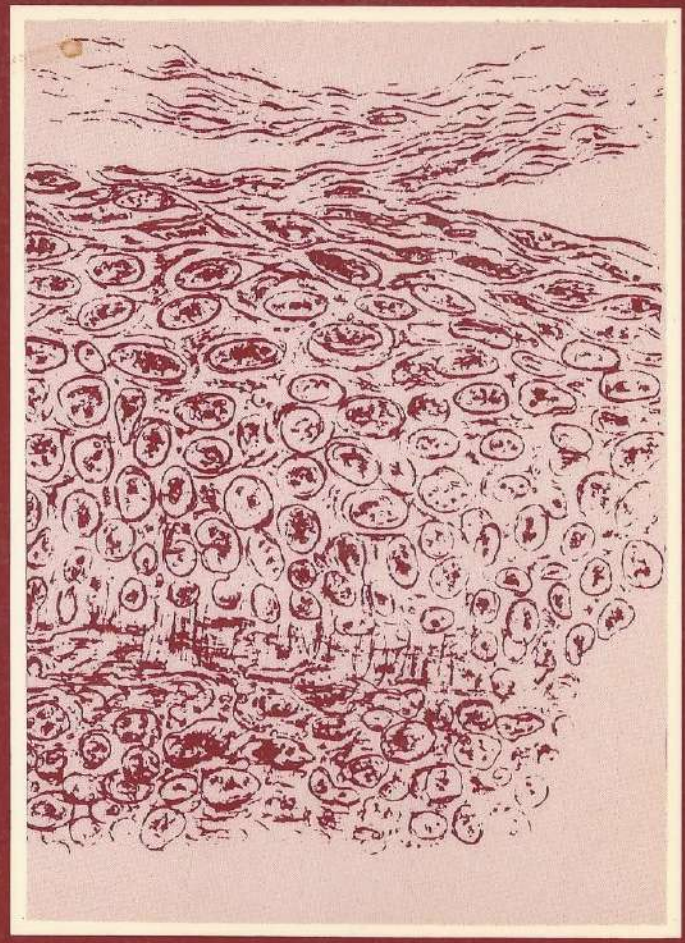
CHOLESTEATOMA PATHOGENESIS

H.W. LUTGERT

CHOLESTEATOMA PATHOGENESIS

AN *IN VITRO* AND *IN VIVO* STUDY ON THE INFLUENCE OF THE GASEOUS ENVIRONMENT ON MIDDLE EAR EPITHELIUM IN RELATION TO CHOLESTEATOMA PATHOGENESIS



H.W. LUTGERT

CHOLESTEATOMA PATHOGENESIS

AN *IN VITRO* AND *IN VIVO* STUDY ON THE INFLUENCE OF THE GASEOUS ENVIRONMENT ON MIDDLE EAR EPITHELIUM IN RELATION TO CHOLESTEATOMA PATHOGENESIS

PROEFSCHRIFT

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden, op gezag
van de Rector Magnificus Dr. J.M.M. Beenakker, hoogleraar
in de faculteit der Wiskunde en Natuurwetenschappen,
volgens besluit van het college van dekanen te verdedigen
op woensdag 16 januari 1991 te klokke 14.15 uur.

door

HENDRIK WIEPKE LUTGERT

geboren te 's-Gravenhage in 1954

Offsetdrukkerij Kanters
Alblasserdam

PROMOTIECOMISSIE

Promoter:	Prof. Dr. J.J. Grote
Co-promoter:	Dr. C.A. van Blitterswijk
Referent:	Dr. M. Ponec-Waelsch
Overige leden:	Prof. Dr. W.Th. Daems
	Prof. Dr. P.H. Schmidt
	Prof. Dr. B.J. Vermeer

Het in dit proefschrift beschreven onderzoek werd verricht in het Laboratorium voor Otobiology en Biocompatibiliteit, verbonden aan de afdeling Keel-, neus-, en oorheelkunde van het Academisch Ziekenhuis te Leiden en het Laboratorium voor Electronenmicroscopie van de Rijksuniversiteit te Leiden (E.M. foto's: L.D.C. Verschragen). Het analisten werk werd grotendeels verricht door S.C. Hesseling verbonden aan het Lab. voor Otobiologie en Biocompatibiliteit. De statistische analyses werden verricht door H.C.M. Franken, afdeling Medische Statistiek.
Foto's: B. van der Lans.
Adviezen en Lay-out: Uitgave Advies Bureau N. Zitman

De drukkosten van dit proefschrift werden gedragen door: H & M Financieel Medisch Consultatie Bureau, Rijnsburgerweg 3, Leiden.

Aan Marlene en onze kinderen

Aan mijn moeder en in herinnering aan mijn vader

CONTENTS

Omslag: Pentekening van cholesteatoom matrix, Lucae 1873.

CHAPTER I

GENERAL INTRODUCTION

Cholesteatoma: What is in a name ?

The name cholesteatoma or Fettgeschwülst (Fatty tumor) was introduced by Müller[192] in 1838. He found a multi-layered pearly tumor of fatty substance deep in the petrous bone, which released an abundance of cholesterol-like crystals, when he dissected it for further investigation. The cholesterol was interspersed between fatty concentric layers. Because he assumed that cholesterol and fat were the major components of the tumor he named it cholesteatoma. This description was not correct, because cholesterol or fat(stearin) are not essential components of this tumor. With improvement of the quality of histological and morphological investigation in time, it became clear that the name cholesteatoma was erroneous. It became evident that the active tissue of this disease resembled the epidermis, consisting of a squamous stratified epithelium with a varying amount of keratin production. Therefore many otologists tried to introduce a more adequate name for this pathological entity. The best-known of these are:

Molluscous tumor (Toynbee[298]) 1841
Perlgeschwülst (Virchow[311]) 1863
Margaritoma (Graigie[148]) 1889
Cholesteatosis (Young[330]) 1950
Epidermosis (Tümarkin[303]) 1958
Squamous cholesteatosis (Birrel[24]) 1958
Squamous epitheliosis (Birrel[24]) 1958
Epidermoid cholesteatoma(Friedmann[90]) 1959
Inactive/Active cholesteatosis (Harris[111]) 1962
Keratoma (Marquet,[171] Broekaert[34])1980,1982
Epidermoid cyst (Kavanagh[145]) 1986

The term molluscous tumor of Toynbee[298] was based on his conviction that he believed that the origin of the process was located in the ceruminal glands of the external ear canal, which secondarily invaded the middle ear. A few years after the name cholesteatoma had been put forward by Müller,[192] Virchow[311] remarked that this description was inadequate, because he had found a strong resemblance to existing epidermis. He advised re-introduction of the term Perlgeschwülst. Graigy[148] proposed a Greek name, calling it a margaritoma, probably hoping that this more scientific name would persist, but no one adopted this term afterwards.

Much confusion was raised by the introduction of terms such as cholesteatosis[330] and squamous cholesteatosis[24] as a relation with cholesterol was suggested. The same holds for the term cholesterol granuloma[214] which concerns a completely different histological entity. Nevertheless many cholesteatoma were called cholesterol granuloma.[171] New names were introduced which tried to indicate the histological resemblance or origin with epidermis, i.e., epidermosis,[304] epidermoid cyst[145] or keratoma[171] However, none of these descriptions managed to replace the misnomer cholesteatoma, although keratoma is still occasionally used by some authors(Broeckaert,Marquet). So the possibility that the name cholesteatoma will be changed after 150 years does not seem very likely.

DEFINITION

An interesting and unresolved problem is to formulate an adequate definition of the entity cholesteatoma, especially since the pathogenesis is still not known. To illustrate the problem of a correct definition, we will present an overview of the discussion about this matter. It is not enough for the otologist to have a histo-pathological definition, since there are clinically divergent cholesteatoma, and the pathogenesis of the process may differ between these varieties. For instance, a residual cholesteatoma develops out of incompletely removed cholesteatoma in the middle ear, whereas a primary or recurrent cholesteatoma, probably arises due to an active ingrowth of epidermis into the middle ear cleft. Furthermore, the presence of a stratified squamous cornifying epithelium in the middle ear cleft can not always be considered pathologic. On the contrary, in a (modified) radical cavity a healthy epidermal lining is even favorable. The ingrowth of epidermis along the borders of an eardrum perforation into the middle ear cannot be called cholesteatoma where there is no local damage, and an adequate keratin clearance into the external ear canal. The first three definitions discussed here, have in common that all assume that the cholesteatoma originates from existing epidermis:

-1- Epidermosis of the tympano-mastoid cavity is a pathological process by which squamous epithelium of epidermal origin grows into that cavity (Tümarkin[304]).

-2- Cholesteatoma is an accumulation of cellular debris and exfoliated keratin in the middle ear, derived from ectopic basal cells of the stratified pavement epithelium (Ars[9]).

-3- A cholesteatoma or keratoma consists of a matrix that originates from the basal cells or malpighian layer of keratinizing squamous epithelium, which envelops an accumulation of exfoliated keratin and dead squamous epithelial debris. This mass of desquamated material, which may become infected, is situated at an ectopic site within the middle ear cleft (Marquet[172]).

The first of these definitions lacks the important information that the squamous epithelium in the middle ear cleft must be cornifying, or that this epithelium will damage the anatomical integrity of the region. Definition two (Ars) focuses attention on the accumulation of debris, whereas the presence of the cornifying epithelium itself is a much more important factor. Furthermore, the term stratified pavement epithelium does not indicate whether this epithelium is cornifying or not, or that this epithelium is located in the middle ear cleft. The Marquet's definition seems to give full information if we consider the origin to be existing epidermis. However, it fails to assign the accumulation of debris to the matrix itself, and does not indicate that the pathological process will become locally destructive in due time. The following two definitions do not refer to the pathogenesis. The authors have tried to give a histo-pathological description.

-4-Cholesteatoma is stratified squamous epithelium trapped and growing in foreign sites within the temporal bone, resulting in the production of a progressively expanding tumor mass, consisting of new growth of epithelium, various stages of degenerating epithelium and abundant keratin and is usually associated with cholesterol and chronic inflammatory cells (Cody[63]).

-5-Aural cholesteatoma is usually a cystic structure produced by keratinizing squamous epithelium; the laminated keratin from its inverted surface accumulates in the cyst-like cavity and may contain an admixture of purulent and necrotic matter. The epidermoid cholesteatoma rests on a fibrous stroma of variable thickness, and the stratified squamous epithelium shows hyperkeratosis (Friedmann[92]).

Cody's definition refers to existing epithelium having become "trapped" in the temporal bone, suggesting that the epithelium itself had no intention to be there. The strong association with cholesterol is risky because this correlation hardly exists. Several studies have revealed only occasional concurrence of cholesteatoma and cholesterol granuloma.[90]

Histologically and morphologically, Friedmann's definition seems quite correct, since he describes a keratinizing squamous epithelium accumulating its keratin in the cyst-like cavity. The statement that cholesteatoma shows hyperkeratosis is too strong, because the rate of keratin production cannot be determined by light microscopy, the mass of keratin is not an indicator. Of course this histological definition has the disadvantage that the location of the disease, aural cholesteatoma, is vague. The definition of Sadé combines clinical and histological characteristics of cholesteatoma :

-6-Cholesteatoma is a middle ear condition (disease) presenting stratified squamous epithelium, which produces macroscopic amounts of keratin, which are not self-cleansing. Histopathologically, all cholesteatoma resemble a classic epidermoid cyst (Sadé[254]).

This definition does not include a possible mode of pathogenesis and all known theories can fit into this concept. The description that it must produce macroscopic amounts of keratin is vague and incorrect. Cholesteatoma can be clinically destructive without a macroscopic accumulation of debris. The addition that all cholesteatoma look like an epidermoid cyst rightly excludes the epithelial lining of a radical cavity, but not all cholesteatoma are cyst-like since they have generally an open communication with the outer environment. The definition given by Gristwood[103] is probably the most practical:

-7-Cholesteatoma is a non-malignant destructive ear disease due to the presence within the middle ear cleft of keratinizing stratified squamous epithelium.

The above mentioned problems associated with the definition of this chronic middle ear disease and the diversity of names used for cholesteatoma, can be considered as a reflection of the basic problem that the pathogenesis of most cholesteatoma is not known. The aim of the studies reported in this thesis was to shed some light on the pathogenesis of cholesteatoma in order to increase our understanding of this destructive middle ear disease.

AIM OF THE STUDY

The earliest concepts concerning the origin of cholesteatoma were published in the middle of the nineteenth century. In those first articles it was assumed that the tumor arose in the petrous or temporal bone itself. At present, the possibility of "de novo synthesis" of cholesteatoma in the middle ear cleft, is not very popular but is still supported by a group of otologists, who believe that middle ear mucosa can change by metaplasia into a cornifying epithelium. A more accepted view of "de novo synthesis" is that embryonic inclusion of ectodermal tissue can lead to cholesteatoma.

At the end of the nineteenth century, the origin of this pathological entity was more and more often ascribed to the existing epidermis of the external ear canal or ear drum. This theory is at present, without any doubt, the most popular one. If we look at the morphological and histological features of the cholesteatoma, we see in general a tissue having all of the characteristics of a stratified squamous cornifying epithelium like the existing epidermis. Furthermore, there is in most cases a direct connection of the cholesteatoma with the epidermal lining of the tympanic membrane or external ear canal. The concept that the middle ear mucosa itself can be transformed into a stratified squamous cornifying epithelium, and will result in a true cholesteatoma, may seem farfetched, especially because clinically its pathogenesis seems to be so obvious.

As mentioned before, most cholesteatoma have a direct connection with the epidermis of the external ear canal, suggesting ingrowth of this epidermis into the middle ear. The mucosal lining of the middle ear belongs morphologically to the respiratory epithelium of the lower and upper respiratory tracts. In the upper part of the lower airways the phenomenon of metaplasia is a well known process which may lead to a complete morphological change of the mucosa into a stratified squamous epithelium. Whether this metaplastic squamous epithelium can differentiate into a cornifying epithelium is still a matter of discussion. Whether this metaplastic shift of the respiratory epithelium might also occur in the middle ear, is discussed in chapter II.

Several *in vitro* and *in vivo* studies have suggested that one of the factors which can influence the histology and morphology of middle ear mucosa, is the extracellular concentrations of carbon dioxide and oxygen. This conclusion is partially based on clinical observations, which will be reviewed in chapter II, indicating a possible relationship between the extracellular gas composition in the middle ear cleft and the morphology of its epithelial lining. The gas composition in the middle ear differs greatly from that of the ambient air. The CO_2 concentration in the middle ear is about 5%, and high compared to the concentration of 0.03% in the open air. The O_2 concentration amounts to about 10%, which is a twofold lower than is the case in the ambient air(21%). If there is a direct connection of the middle ear cavity with the outer environment, a dramatic shift in the physiologic gas composition will occur. This change in the CO_2/O_2 ratio may influence the morphology of the middle ear mucosa, in view of the following clinical observations:

1) In cases of tympanic membrane perforation we occasionally see a cornifying epithelium, particularly on the promontory, which macroscopically has no connection with the epidermis of the tympanic membrane.[249]

2) The morphology of the middle ear epithelium is usually altered after the insertion of ventilation tubes to treat otitis media with effusion.[268,274]

3) In most cases of cholesteatoma there is a defect in the external ear canal or tympanic membrane permitting direct communication with the outer air.

4) Many otologists have reported that patches of cornified epithelium which could not be removed during eradication of a cholesteatoma, had disappeared, by the time of the second look operation. In those cases a healthy middle ear lining was found, usually with an intact tympanic membrane.[247]

These observations suggest that there might be a correlation between a possible change in the environmental gas concentration of the middle ear epithelium, and a related change in the morphology of the middle ear mucosa. For this reason, the main questions posed in this study are:

1) Will the morphology and cell metabolism of the middle ear epithelium be influenced by changes in the extracellular CO_2 and O_2 concentration?

2) Is there a relationship between any influence exerted on the middle ear mucosa by CO_2 and O_2 and cholesteatoma pathogenesis?

We performed *in vitro* and *in vivo* studies to investigate a possible influence of the CO_2/O_2 ratio. In Chapters III, IV, and V the results are reported of the *in vitro* experiments. Middle ear epithelium of the rat, cultured in serial cultivation, was exposed to different concentrations of CO_2 and O_2 during a period of two weeks, to detect any changes in proliferation rate, degree of terminal differentiation, and morphology during this period.

Chapter VI discusses the histology and morphology of two clinically different types of cholesteatoma. The primary acquired and recurrent cholesteatoma considered as one group, and the residual type. These two groups of cholesteatoma are expected to encounter different extracellular gas concentrations. In this study, the residual type represents the outgrowth of incompletely removed cholesteatoma, during an intact canal wall operation, and the outgrowth of the cornifying epithelium occurred in the gaseous environment of the intact middle ear cleft. The primary acquired and recurrent cholesteatoma were, however, in direct communication with the ambient air via defects in the tympanic membrane or bony wall of the external ear canal. Of course the part of the cholesteatoma facing the middle ear cleft may be exposed to the middle ear gas composition, which may, or may not, be altered.

Another method to find out whether an epithelium is basically influenced by environmental factors is to study its cytokeratin pattern. Cytokeratins are water insoluble proteins belonging to the intermediate filaments. All tissues of epithelial origin are characterized by the presence of cytokeratins. In all, 19 cytokeratins, each with its own molecular weight and isoelectric value, are known. In general, it can be said that each type of epithelium has its own set of cytokeratins differentiating, for instance, a cornifying- from a non-cornifying epithelium. The cytokeratin composition is furthermore determined by the type of cell, the stage of histologic differentiation, the growth environment and the disease state of the tissue. Chapter VII deals with cytokeratin expression of middle ear mucosa, cholesteatoma matrix, and epidermis of the auditory canal wall.

It is conceivable that the cytokeratin expression of a cholesteatoma resembles the cytokeratin pattern of its tissue of origin. Furthermore, the results of this investigation were used as a reference for the cytokeratin study reported in Chapter VIII. In this experiment small biopsy specimens of human middle ear mucosa were taken from patients with and without a tympanic membrane perforation. So middle ear mucosa was examined which had been exposed, *in vivo*, either to a gas composition similar to that of the ambient air, or to one occurring normally in the intact middle ear. In this context the environmental gas concentrations in this study may be compared to those of the *in vitro* experiment as mentioned in Chapters III-V. An alteration in the cytokeratin pattern may indicate a shift in the degree of terminal differentiation of the epithelium.

The aim of this experiment was to find out whether a difference in the extracellular CO_2/O_2 ratio would influence the cytokeratin expression of the human middle ear mucosa *in vivo*. Chapter IX discusses the overall results of the experiments and studies in relation to current knowledge. A review of current knowledge is given in Chapter II.

CHAPTER II

REVIEW OF CURRENT LITERATURE

Epidemiology of cholesteatoma

Incidence:

The incidence of cholesteatoma is still a disputed subject: not only are there differences in the percentages within similar groups, but also the incidence is influenced by age, race, ethnic background, and clinical type of the cholesteatoma.

Incidence in general population:

Publications on the overall occurrence in an urban population are sparse; Harker[108] reported an incidence of 0.006%, Ruben[241] estimated that 0.004%, and Tos[295] that 0.015% of a general population suffer from cholesteatoma. Lastly, Podoshin[221] found an incidence of 0.27%. With respect to the percentage given by Ruben and Podoshin, only individuals older than 10 years were investigated. Sadé[246] estimated that about five million people in the world are afflicted with cholesteatoma.

Incidence in children:

Some large-scale studies reported the incidence of cholesteatoma in children before the age of 16. Karma[144] concluded that 0.07% of children would develop cholesteatoma before this age with an annual incidence of 0.0045%. Cohen[66] found the same percentage, 0.07%, as Karma before the age of 16. Harell[107] reported the prevalence in North American children to be 0.5%, 35% of their group was black. Tos[296] reported an incidence of 0.4% in children; however, children treated for chronic OME (adenoidectomy and/or grommets) had a higher risk, 2.0%, of acquiring cholesteatoma. Kokko[147] and McKinnon[165] also showed a possible relation between OME and cholesteatoma, since they found an incidence of 0.7% and 1.0% in the OME group. Karma[144] and van Cauwenberghe[51] found an incidence of 0.2 and 0.5%, respectively, in their treated OME groups.

Age:

There is no general agreement in the literature about the age at which there is a greater incidence of cholesteatoma. Tschopp[301] found the highest incidence at 15 years of age(12%). Harker[108] found three peak occurences i.e., between 10-19 years, 20-29 and 40-49 years. Palva[206] reported that 10% of his cholesteatoma patients were under 16 years of age.

He concluded that cholesteatoma occurs not as frequent in children as in older people. However, Shatz[266] reported 33% of cholesteatoma under 13 years, Cody[64] 22% under 16 years and Sheehy[267] 18% under the age of 15.

Sex distribution:
Several authors have reported that males are more often afflicted by this chronic ear disease, as 60 to 75% of the cholesteatoma patients belong to the male population.[53,63,155,216,242,266,305]

The contra-lateral ear:
Sadé[253] found that the incidence of cholesteatoma in the contra-lateral ear varied between 5.6 and 13.2% and was related to the stage of the cholesteatoma in the other ear. Most publications mention a prevalence of about 10% for the contra-lateral ear.[70,199,305]

Racial and ethnic prevalence:
Harrel[107] reported the prevalence of chronic otitis media and cholesteatoma in black Americans to be statistically significantly lower compared with the caucasian population. Only 12% of the cholesteatoma group were black in a population composed of 38% blacks and 48% whites. The risk of developing cholesteatoma for those contracting a chronic otitis media did not differ between the two racial groups. Similar conclusions were drawn by Griffith.[102] Concerning the ethnic differences all reports discussed in the following section concern juvenile populations. The highest incidence has been found in an Eskimo(Alaskan) population, Beal[17] recorded 19% chronic otits media(COM) and 13% of this group were diagnosed as having cholesteatoma. Tschopp[301] recorded an incidence of 12% in a similar group but 13 years later he found a prevalence of 2.6% in the same age group. According to Tschopp, this was the result of an improved health care system. In contrast Baxter[16] found an incidence of 0.06% in another Eskimo group in Alaska. Johnson[140] diagnosed COM in 14% of Navajo Indian children and cholesteatoma in 6.4% of this group. McCafferty[176] found a high incidence(11.7%) of COM in Aboriginal children, but cholesteatoma occurred very rarely in 0.05%.

Relation with congenital defects:
In this context the cleft palate has been considered an important concomitant congenital defect. Severeid[265] found an incidence of 9.1% in a group of 160 patients treated for cholesteatoma, a relation with a malfunction of the Eustachian tube is suggested[325]. Harker[109] reported a frequency of 9.2% in 190 patients. De La Cruz[69] observed cholesteatoma in ears with congenital atresia of the external ear canal in 14% of cases, usually localized lateral to the atretic plate.

Morphology of cholesteatoma.

Introduction

The first papers concerning the morphology of cholesteatoma were published in the second half of the nineteenth century. The shiny pearly appearance of this tumor attracted much attention, but the histological findings confused the authors.

For example, a pathologist like the famous Virchow[311], wrote an extensive report on cholesteatoma in 1863. He concluded that this pearly tumor originates from elements belonging to connective tissue. It was built up of epidermoid cells, that produced elements identical to those seen in true epidermis. So although he saw an epidermis he did not realize that he was looking at an epidermis. However, most of the cholesteatoma he described were located at the apex of the petrous bone, far away from the existing epidermis, and were probably primary cholesteatoma. A few years later he studied cholesteatoma of the middle ear cleft and concluded that cholesteatoma could be considered normal epidermis. Wendt[317] (1873) too concluded that the tissue assumed characteristics like those of the true epidermis, creating a rete Malpighii with hyperkeratosis. As he said in his own words; "daß Mucosa eine Oberhautartige Beschaffenheit annehmen kann unter Bildung eines Rete Malpighii".

Lucae[163] described in the same year the histological characteristics of the cholesteatoma localized in the middle ear. He mentioned the basal and spinosal cell layers and the production of keratin. He concluded that the tumor had all the features of epidermis and should be considered and treated as normal epidermis. All subsequent papers, concerning the histopathology, up to the present, agree that what we are dealing with, is a true epidermis located in the middle ear cleft.

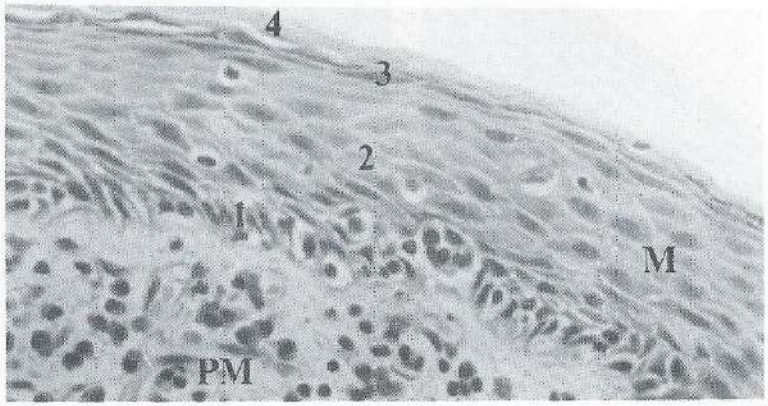


Fig. 1 Cholesteatoma matrix(M) and perimatrix(PM). The four cell layers of the matrix, i.e.; stratum basale(1), stratum spinosum(2), stratum granulosum(3) and stratum corneum(4) can be distinguished.

Light and electron microscopy

Morphologically the tumor may present as a cyst or have a pearl-like appearance.[159,160,247] The wall of the cholesteatoma consists of the epidermis or matrix, and is formed by a typical keratinizing squamous epithelium, in which four cell layers can be recognized: The basal, spinosal(Malpighian), granular, and keratin layers.[32,158,260,322] Thus, the morphology of the matrix is identical to that of the epidermis(Fig. 1).

The pearl-like cholesteatoma, found in the middle ear cavity, often displays a very thin matrix, in which no distinction can be made between the basal and spinosal cell layers(Fig. 2a), however, the granular layer can always be detected, independent of the thickness of the matrix[159](Fig. 2b). The matrix deposits the keratin in concentric layers into the pearl (Fig. 2a). The tissue surrounding the matrix is called the perimatrix (Fig.1) and is generally composed of connective tissue, but in cases with a pearly cholesteatoma it will be only present where the process is attached to the middle ear lining. The remainder of the matrix is covered in these cases with a thin layer of middle ear mucosa.[158] In a non-infected ear the perimatrix will be composed of loose connective tissue containing collagen-fibers and fibrocytes.

The more common cyst-like variant of the cholesteatoma exhibits different characteristics. The matrix is usually well developed and is sometimes composed of many cell layers, the thickness can vary considerably within the same cholesteatoma.[8,159] (Fig.3) The morphology of the basal layers of the matrix, i.e. the spinosal and basal layers, can be altered by such phenomena as basal proliferation or micro-cholesteatoma formation.

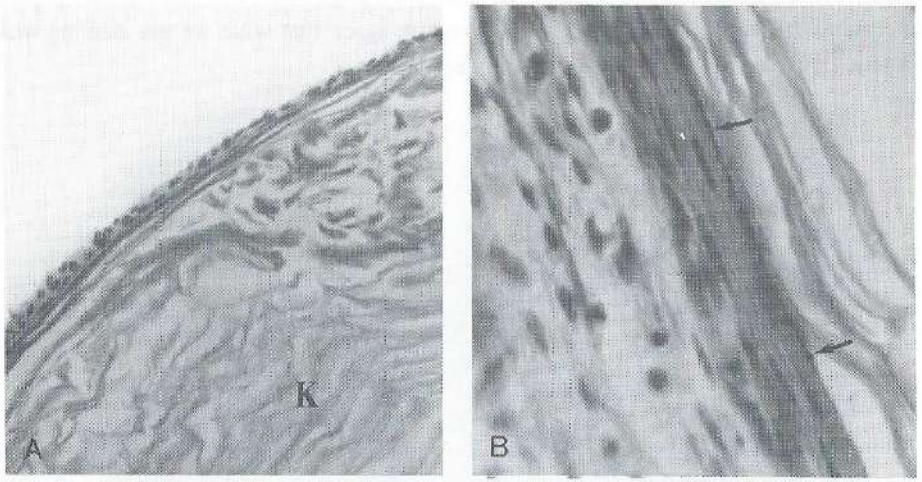
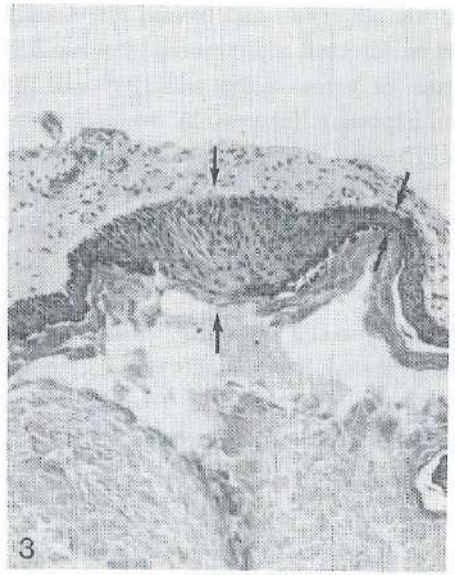


Fig. 2a Very thin matrix of a residual cholesteatoma, the stratum basale and stratum spinosum can not be differentiated, the matrix deposits concentric layers of keratin(K). Fig. 2b Higher magnification of matrix, despite the thin matrix kerato hyalin granules can be detected clearly.



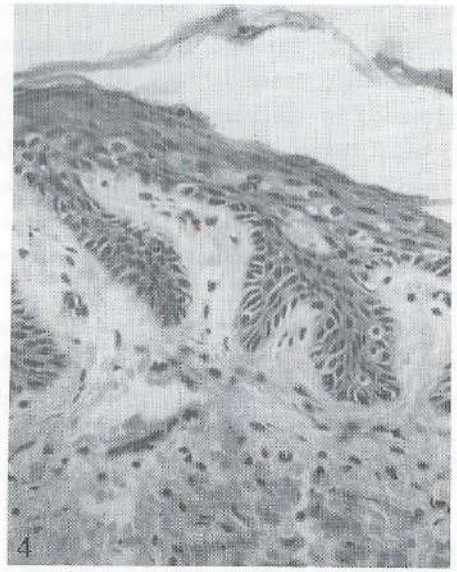


Fig. 3 Abrupt variation in number of cell layers within the same cholesteatoma matrix (arrows).

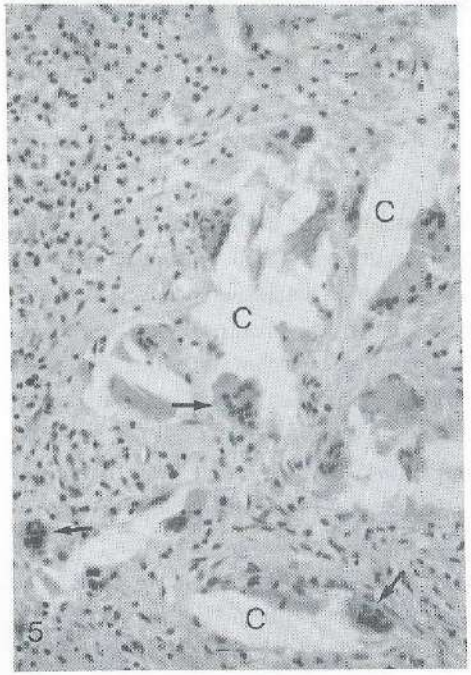
Fig. 4 Papillary projections of the basal layers of the matrix into the perimatrix.

Basal proliferation can lead to papillary projections[114,322] of the matrix into the sub-epidermal tissue(Fig. 4). Within this thickened basal layer small epithelial cysts or 'whorls' may develop.[160] These phenomena will be further discussed in relation to the pathogenesis. The aspect of the perimatrix depends on the presence and degree of inflammation or infection in the middle ear.[159,186] A non-infected cholesteatoma generally displays a perimatrix consisting of a thin layer of connective tissue with fibroblasts and poor vascularization[159]. In general, however, signs of chronic infection are encountered, the perimatrix is thickened and round cell infiltration may be seen, with increased vascularization, and degenerating cells.[8,32,158,260] Occasionally, local hemorrhage is present, and especially in this tissue cholesterol granuloma and giant cells may be found.[214,250,260] (Fig. 5). The ultrastructural studies underscore the fact that the matrix of the cholesteatoma consists of stratified squamous cornifying epithelium. The basal cells rest on a basement membrane, and are attached to it by hemi-desmosomes, which are closely related to bundles of tonofilaments, located in the cytoplasm of the cell. The basement membrane itself is anchored to the perimatrix by fine reticular fibres.[158,162,260]

Depending on the thickness of the matrix and the presence of inflammation, the basal cells may have a squamous, cuboidal or columnar appearance.[157] The spinosal cell layer shows the many intercellular desmosomes, which are related to tonofilaments.[247,260]

In the granular layer kerato-hyalin granules are abundant, and toward the stratum corneum a thickening of the cell membrane can be detected, representing what is called the cornified envelope.[37](Fig. 6) The appearance of kerato-hyalin granules[88] and the formation of the cornified envelope(CE) are important features in the process of terminal differentiation leading to cell death. The cell nucleus and other structures present in the cytoplasm are in a disintegrating state and are lost in the stratum corneum. The cells in this layer are extremely flat and filled with amorphous material. This process of terminal differentiation will be discussed in more detail in the next section.

Langerhans cells are seen frequently throughout the matrix and their incidence seems to be higher compared to that in the epidermis of the tympanic membrane or external ear canal.[32,259] The presence of Merkel cells[32] has also been reported. The implication of the above-mentioned cells and their role in cholesteatoma pathogenesis will be discussed elsewhere.



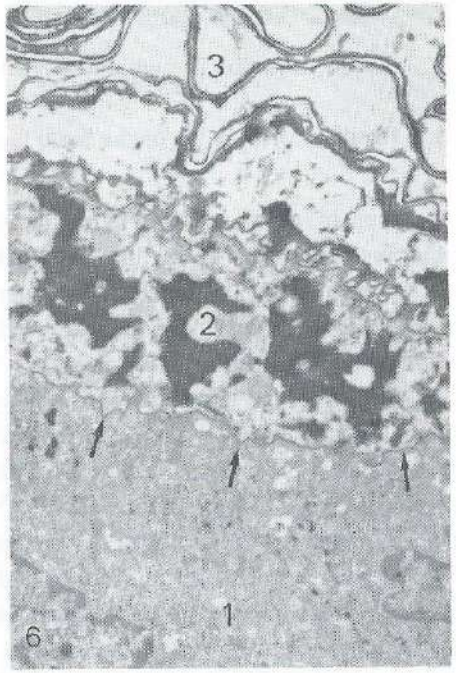


Fig. 5 Cholesterol granuloma with cholesterol clefts(C) and giant cells(arrows).

Fig. 6 Transmission electron microscopic picture of the cholesteatoma matrix, displaying the stratum spinosum(1), stratum granulosum(2), and stratum corneum(3). The thickening of the cell membrane (arrows) represents the cornified envelope.

Terminal differentiation

To describe the process of terminal differentiation we shall discuss the four cell layers of the epidermis, proceeding from the basal cells up to the stratum corneum. In this case the concomitant phenomena will be observed by electron microscopy. The cells of the basal layer are subdivided in two types[314]:

1) *The stem cell*, which by cell division creates a new stem cell, and a transit-amplifying cell. The stem cell itself will never enter the process of terminal differentiation.

2) *The transit-amplifying cell*, however, will after numerous cell divisions, undergo terminal differentiation.[314]

During terminal differentiation of the cell a cytosolic protein, called involucrin[314], can be detected. This water-insoluble protein is the major precursor of the cornified envelope. Involucrin is present in all stratified epithelia with or without a stratum corneum[13], and can be found only above the basal layers.[13,56] Involucrin, becomes more and more concentrated at the cell periphery when it passes from the spinosal cell into the granular cell layer.[234] This protein will undergo cross-linking by the enzyme transglutaminase[47,273] localized in the upper spinosal- and granular layers.[47,185] This cross-linking enzyme only has activity in the presence of intra-cellular calcium ions.[233] Histochemical studies showed that the enzyme activity shifted from the cytoplasm to the cell membrane, during the process of CE formation.[37,185] The cross-linking of involucrine together with membrane-associated proteins will create the insoluble cornified envelope[273], probably located on the inner side of the cell membrane. This structure is seen by transmission electron microscopy as a thickening of the cell membrane. During the terminal differentiation of the cell, increased filling of the cytoplasm, with keratin filaments or tonofilaments can be seen as well. Meanwhile, the nucleus and cytoplasmatic organelles are "digested" in the granular layer. The squames in the keratin layer are packed with these tonofilaments,[99] and all cytoplasmic structures have disappeared. It is assumed that transglutaminase is co-responsible for a stable embedding of the tonofilaments in the cytoplasm.[37]

It is not yet known which stimuli trigger terminal differentiation of cells. The process is associated with increasing cell size[273,233,311,313] and the starting point is probably correlated with insufficient space for the enlarged cell on the substratum.[13] The extracellular calcium concentration can influence the degree of cornified envelope formation in cultured cells.[26] *In vivo* studies showed a calcium gradient throughout the cell layers of the epidermis, the calcium concentration increasing during the process of terminal differentiation.[222]

The process of homeostasis between proliferation and terminal differentiation, is not known yet. The main theory is that during terminal differentiation the cell releases a substance, called chalone, which suppresses proliferation of the basal cells. The loss of differentiated cells by desquamation decreases the concentration of chalones and stimulates proliferation. It is important to keep in mind that tonofilaments, involucrin, and transglutaminase can be found in non-cornifying epithelia, and that cornified envelopes occasionally occur in these tissues.[13,34,37] Tonofilaments are composed of intermediate filaments or cytokeratins, as will be discussed in the following section.

Cholesteatoma and their divergent terminal differentiation

The cholesteatoma matrix show some differences in terminal differentiation, all reflecting a disturbed and incomplete terminal differentiation :

1) Histochemical studies on the localization of transglutaminase in the cells of the cholesteatoma matrix revealed that the shift of this enzyme, from cytoplasm to cell membrane became retarded during terminal differentiation. This can be interpreted as a reduced chemical stabilization of the cornified envelope, because this enzyme, as is the case in epidermis, should transfer its activity to the cell membrane.[36,37]

2) The process of cross-linking of the tonofilaments which mainly fill the dead cells of the stratum corneum is incomplete in cholesteatoma.[34] This finding may explain why, as some authors have reported, the stratum corneum of the cholesteatoma lacks any structural integrity, displaying a disordered mass of corneocytes, as compared to healthy epidermis.[34,185]

3) The events of terminal differentiation lead to a gradual loss of DNA content in the healthy epidermis. In cholesteatoma this process of nuclear degradation evolves more slowly or is postponed.[35]

4) Involucrin was found to occur in larger quantities in cholesteatoma than in the external ear canal skin.[56] This was considered an indication of a more active terminal differentiation of the epithelial cells in cholesteatoma.[56]

5) Cholesteatoma cells can show a very much higher level of dividing activity than is seen in the cells of normal skin.[204]

Cytokeratins

Cytokeratins are intermediate filaments, representing a class of insoluble cytoplasmic proteins. The diameter(10 nm) of these structures lies between those of microtubules(25 nm in diameter) and micro filaments (7 nm), hence their name intermediate filaments.

These filaments occur in all cells and can be subdivided into five classes: 1) *desmin* in myogenic cells, 2) *neuro* filaments in neuronal cells, 3) *glial* filaments in astroglia and astroglia related cells, 4) *vimentin* in mesenchymal cells and 5) the cytokeratins or keratins found in all epithelial tissues.[281] The cytokeratins constitute a group of aqueous insoluble proteins and 19 different subunits have been identified in human epithelial tissues.[281]

They can be divided into acidic and basic groups and each epithelium has at least one doublet of one acidic and one basic cytokeratin. The different cytokeratins are further distinguished by their molecular weight ranging between 40,000 and 70,000 kd.[281] In general, a simple epithelium express cytokeratins small of size, whereas the more complex and differentiated epithelia contain a larger number of different cytokeratins with an overall heavier molecular weight.[318] The different epithelia are not only characterized by their own set of cytokeratins, but these cytokeratins may also be related to a specific place within this epithelium.[135,193,198] They can be visualized, for instance, immunologically with poly- and monoclonal antibodies.[135,194,281,198,287,331]

The cytokeratins are associated with other proteins located in the cytoplasm. In the epidermis the best documented associated protein is filaggrin,[57,281] which may cross-link with the cytokeratins to create a flexible insoluble structure.[280] The cytokeratins are thought to have a more mechanical than dynamic or metabolic role. Microscopical studies have shown that they are peri-nuclear located, radiating to the periphery of the cell and terminate in intercellular junctions like desmosomes or tight-junctions. For this reason they are also called cytoskeletal keratins indicating an involvement with cell shape, nuclear location and intercellular connections.[281] The cytokeratins may aggregate into tonofilaments,[261] which have often been described in reports of fine morphological studies.[146,159] However, cytokeratins have also been identified in cells where no tonofilaments could be visualized by electron microscopy.[261] Because each epithelium has a characteristic set of cytokeratins (CK) a differentiation can be made between (simple) non-stratified epithelia, stratified non-cornifying, and cornifying epithelia.[198,201,287]

For example CK 1,2,10, and CK 11 are only found in a cornifying epithelium, whereas CK 19 is detected in all epithelia except epidermis.[287] CK 14 and CK 5 are expressed in all stratified epithelia thus, the following simplified scheme can be composed[281,287]:

Simple non-stratified epith.	:	CK 19	CK 17	CK 8	CK 6
Stratified non cornifying epith.	:	CK 19	CK 17	CK 8	CK 6
	+	CK 14	CK 5		
Cornifying epithelium	:	CK 14	CK 5		
	+	CK 1	CK 2	CK 10	CK 11

Of course, more keratins can be detected in, for instance, the epidermis. This scheme is thus incomplete, it only serves as an example to show the specificity of the cytokeratins.

The different cytokeratin numbers correlated with the molecular weight in combination with the isoelectric pH value, as can be determined by two-dimensional gel electrophoresis. On this basis cytokeratins have been numbered from 1 to 19, according to Moll.[188] Numbers 1-8 corresponded with basic CK, number 9-19 with the acidic CK. In both groups the lower number represents the "heavier" CK. The cytokeratin composition of the cells is dependent not only on cell type or degree of differentiation, but can also be changed by the growth environment or the diseased state.[37,193,287,318] For instance, non-cornifying epithelia, will express CK 1 and 10 if they become cornified due to a vitamin A deficiency.[97] Cells grown *in vitro* have a different CK composition than those grown *in vivo*.[281] Keratinocytes in culture assumed a cytokeratin expression resembling the epidermis in fetal life, which is completely different than the CK expression of epidermis in adults.[283] Wild et al.[318] noted an inter-individual variation in CK expression in squamous non-cornifying epithelium of the upper respiratory tract. All epithelia showed an identical basic set of CK, as well as an additional composition of varying CK. It was postulated that these differences were due to local environmental factors in the upper respiratory tract, such as hydration, inflammation, and desiccation.

Because we used rat middle ear mucosa in our culture experiments and in the cytokeratin experiments biopsy specimens of human middle ear epithelium, a short review of the morphology of these epithelia is given in the following section.

Morphology of human and rat middle ear mucosa

The morphology of the non-infected middle ear mucosa of man[161] and the rat [5,151] has been extensively studied by both light and electron microscopy. Since no essential differences were found between these two kinds of middle ear mucosa,[5,105,18] no distinction will be made here in the discussion of their morphology. In general, it can be said that the major part of the middle ear cleft and adjacent pneumatized bone is covered by a simple squamous or cuboidal non-ciliated epithelium.[39,75,146]

The meso- and hypotympanum show a more complex epithelium containing ciliated and secretory cells.[5,146] Some studies[39,115,156,243] have dealt with the distribution of the ciliated and non-ciliated cells in the middle ear. The findings indicated that most of the ciliated cells are located in and near the orifice of the Eustachian tube.[161] Furthermore, two or three tracts of such cells lead to the tubal orifice, one starting in the hypotympanum, the other at the border of the meso- and epitympanum, and the third, a minor one, on the promontory itself. The ciliated cells are cuboidal or resemble a pseudo-stratified columnar epithelium, and are intermingled with secretory cells containing varying numbers of granules.[158]

Ultrastructural studies revealed that, basically, three types of cell can be distinguished in mucosal biopts of material from the promontory.[146] The characteristics of these cells will be briefly discussed, because in our studies, biopsy specimens were taken from the human promontory. The three cell types in question are: Ciliated cells, non-ciliated cells, and basal cells.[146] From the apical surface of the ciliated cells, ciliae and microvilli protrude into the middle ear cavity. Above the nucleus which is located in the center of the cytoplasm there are granules varying in size. The ciliated cell has all the characteristics of those located in the respiratory tract.

The non-ciliated cell, whether squamous or cuboidal in appearance, is always covered at the luminal surface by variable number of microvilli, and may occasionally contain a mono-cilium. The cells are in direct contact with adjacent cells via desmosomes.[146] The non-ciliated secretory cell occurs in two or three types, depending on the kind of granules they contain. Lim[158] distinguished three types of secretory cells; the goblet cell, the most active type, containing large lightly stained granules, and the dark granulated cell. The third type is the intermediate cell containing a mixture of both types of granule.

The basal cells are connected by finger like cytoplasmic processes, and are located on the basement membrane. The cytoplasm has a dark appearance due to large numbers of tonofilaments, and the nucleus is more irregular.[146] The subepithelial lining of the middle ear mucosa is formed mainly by a thin layer of connective tissue which contain fibroblasts, collagen fibers, occasionally macrophages, plasma cells, mast cells, and lymphocytes.[5,161,146]

Conclusions

Light and electron microscopical studies showed that the matrix of the cholesteatoma has all of the histological characteristics of a squamous stratified cornifying epithelium. The morphology, however, may vary not only between cholesteatoma but also within one and the same specimen.[260,159] This morphological variation can often be associated with the presence or absence of inflammation or infection in the perimatrix.[160,260]

With respect to terminal differentiation, it seems evident that there are some differences. The observations indicate that the process of terminal differentiation is slowed down and not always completed, at the time when the cells are lost by desquamation. This incomplete cornification[35] may be partially due to the hyperproliferative state of the cholesteatoma matrix.

PATHOGENESIS OF CHOLESTEATOMA

Introduction

The first reports on cholesteatoma located in the temporal bone appeared in the middle of the nineteenth century. In the current literature credit is given to Cruveillier[68] (1829) as the first to describe the pathological features of a "tumeur perleé" or pearly tumor in the human temporal bone. It was probably Müller[192] who called this tumor a cholesteatoma because he found an abundance of cholesterol crystals and fatty tissue while investigating this tumor with a light-microscope. The name, as mentioned before, is a misnomer because neither cholesterol nor fat(stearin) is an essential component of this tumor. Virchow[311] described a cholesteatoma, located in the apex of the petrous bone, which was probably a congenital cholesteatoma or epidermoid cyst. Virchow considered it to be a neoplasm which had developed from elements of fibrous tissue.

The first articles about the pathogenesis of cholesteatoma appeared in the period between 1850 and 1890. Most theories about the pathogenesis discussed at present originated in this period. Furthermore, it is interesting that the lively discussion on this subject occurred mostly in the German journals, because many famous otologists lived in that country. Tröltsch[299] was one of the first who put forward a theory about the origin of this disease. His theory which, "especially due to his outstanding reputation", was accepted by many contemporaries was in vogue for quite a long time. He thought that the central part of the cholesteatoma was the result of a concentrated retention of debris in the middle ear due to chronic infection. The middle ear mucosa identified this debris as a foreign body and, not unlike an oyster, deposited layers of epithelial material on this central clump of debris. This desquamative reaction of surrounding mucosa created the pearly tumor.

The first otologists who cautiously and with great respect for Tröltsch, contradicted this theory were Wendt[317] and Lucae.[163] Both of whom introduced the metaplasia theory in different journals in 1873. A few years, later Habermann[106] and Bezold[19] published their ideas about the pathogenesis, which were in sharp contrast to the metaplasia theory. According to them the origin of the cholesteatoma must be sought in the existing epidermis, and this was the beginning of a long discussion which has not ended yet. All theories published so far can be divided into two main categories :

I- Epidermal Theories

All theories that belong to this group assume that the origin of the cholesteatoma must be sought in the existing epidermal lining of the tympanic membrane or external auditory canal. How this epidermis invades the middle ear cleft is explained by three main concepts(Fig. 7) namely:

1.*The migration Theory* (Habermann[106]),
2.*The retraction theory* (Bezold[20]),
3.*The proliferation theory* (Lange[153]).

II- In Situ Theories

The hypotheses that can be assigned to this group, have in common the view that the site of origin of the cholesteatoma was located within the temporal or petrous bone. The two main concepts are:

4.*The congenital theory* (Virchow[311]),
5.*The metaplasia theory* (Wendt[317]).

The five main theories mentioned above will be discussed separately. It must be kept in mind that there are also other concepts concerning the pathogenesis of cholesteatoma. Some of them are no longer discussed, for instance, the old theories of von Tröltsch or Toynbee.[298] Two other hypotheses, not to be discussed here, are the traumatic and iatrogenic theory,[322] which probably constitute a minority of the cholesteatoma and are only discussed incidentally in the current literature.

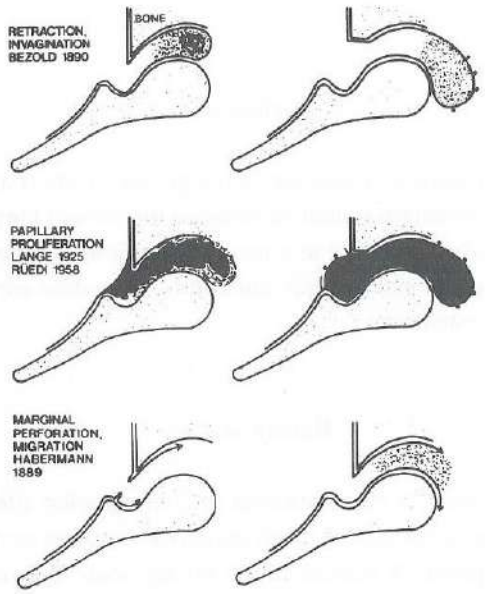


Fig. 7 Schematic illustration of the development of cholesteatoma by the three most often mentioned pathogenic theories: Retraction theory, proliferation theory, and migration theory.

THE EPIDERMAL THEORIES

MIGRATION THEORY

Introduction

The migration theory was introduced in 1888 by Habermann,[106] who described a cholesteatoma located in the middle cerebral fossa which was connected by a strip of skin with the epidermis of the external ear canal. According to Habermann the skin had grown into the middle ear through a defect in the ear drum located postero-superiorly. When this skin was followed, it was found to continue over the promontory and into the epitympanum where it had eroded the bony lining of the tegmen tympani, causing a lethal meningitis. According to Habermann, this phenomenon of ingrowing skin was often seen in cases of middle ear infection and concomitant perforations of the tympanic membrane. Several colleagues at that time confirmed his observations, although not all agreed that this was the main cause of cholesteatoma formation.[20,149,153,163] Necrotizing otitis media occurred quite often in those days,[114,305] and let to large perforations of the tympanic membrane. The subsequent lining of the middle ear cleft with epidermis was considered a normal part of the healing process, as long as the clearance of keratin was not obstructed.[21,163,153]

Definition

A well defined description or definition of the process of cholesteatoma formation by migration of existing epithelium cannot be found in the current literature. The migration theory can be described as referring to a process of migration of existing epithelium of the tympanic membrane or external ear canal into the middle ear cleft, leading to the clinical picture of cholesteatoma.

Human studies

According to Tümarkin,[304] a cholesteatoma can only develop after the integrity of the epidermis covering the ear drum, has been impaired. A defect in the epidermis usually occurs in retraction pockets. A rupture of the pocket leads to invasion or migration of cornifying epithelium induced by the infected middle ear mucosa. Buckingham[42] noted ingrowth of the epidermal layer around the edges of central perforations, leading to accumulation of keratin in the middle ear.

Palva[208] and Karma[143] observed that the larger the perforation was, the more extensive the epidermal invasion into the middle ear. Migration of epidermis up to the medial wall of the middle ear was seen, not only in cases with marginal perforations but, occasionally, also in central perforations. The presence of the cornifying epithelium in the middle ear cleft resulted in 30% of the cases to actual cholesteatoma formation. No correlation could be found between the development of cholesteatoma and the presence of infection or inflammation. Lim[159] and Palva[208,211,213] studied more in detail the junction between the cholesteatoma matrix and the middle ear mucosa, which they called the *advancing front*. In fact, three phenomena were observed :

1. The transition from the epidermis to the mucosa is abrupt without inflammatory signs. The basement membrane of the two epithelia is continuous without a disruption at the border.[159,208]

2. There is a transition zone composed of a metaplastic area. Starting from the edge of the epidermis, we see a gradual disappearance of the 4 characteristic cell layers, to become middle ear mucosa. This "junction metaplasia" will be discussed further in the chapter on pathogenesis. Again, the basement membrane of the epithelia remains intact.

3. The third type is characterized by a disruption of the basement membrane and inflammatory reactions are seen in the immediate vicinity of the advancing front. The basal layers of the matrix undermine the mucosa by invasion into the subepithelial tissues and the mucosa seems to be "pushed away".

This phenomenon was described as early as 1925 by Hellmann[114] who found this third type of transition zone in all of his cholesteatoma. The direction of invasion of matrix seems to be determined by the direction in which the basement membrane is disrupted. Electron microscopy showed that the fine fibrils attached to the basement membrane (anchoring fibers) may not only pull away this structure, but also serve as a guide for the migration of the basal cells.[162]

In sum, the first two transition zones suggest that there is some kind of contact inhibition, without further migration of one of the epithelia. The last type is more easily associated with a clinically active cholesteatoma, and the term *advancing front* must be reserved for this last-described process. Histo-pathologically, the lesions in the basement membrane can be considered (pre) neoplastic[110] because this structure ought to be resistant to infection. However, defects in the basement membrane are no longer specific for carcinogenic states, because similar defects have been observed in benign skin diseases, such as active psoriasis.[67]

Zechner[336] observed that migration of epidermis only occurred in cases of chronic infection of the middle ear followed by alteration of the mucosa into granulation tissue or even necrosis of the middle ear lining. Eigler,[77] however, found no correlation between migratory ingrowth of epidermis and inflammation. Yamashita[324] reported no differences in epidermal ingrowth of ears with central or marginal perforations, migration occurring in both groups. However, no mention was made of the extent of invasion, or of a possible correlation with middle ear infection.

Animal studies

In the late 1950's Friedmann[90] and Fernandez[86,87] published their experiments done in guinea pigs. Friedmann saw migration of epidermis through defects in the ear drum after inoculation of the middle ear of the guinea pig with Pseudomonas pycocyanea, this occurred in 10% of the animals. He infected the middle ear by injection through the tympanic membrane. In human material, obtained during surgical intervention, he also found evidence of ingrowth of epithelium spreading underneath the mucosa of the middle ear. The covering pictures, however, showed only that the perimatrix of the cholesteatoma was covered by mucosa.

Fernandez instilled quinine-HCl into bullae of guinea pigs which caused a heavy inflammation resulting in marginal perforations of the pars tensa of the ear drum. He concluded that the epidermis could grow into the middle ear under favorable conditions such as:

1. presence of a perforation,
2. maintenance of a hyperplastic epidermis by inflammation and,
3. necrosis of middle ear mucosa or transformation of the epithelium into granulation tissue.

Similar studies were done with chinchillas by introducing propylene glycol into the middle ear, which gave perforations with subsequent migration of epidermis and cholesteatoma formation.[141,321] Hörmann[130] saw ingrowth of epidermis after cauterization of the Eustachian tube in guinea pigs resulting in chronic purulent otorrhoea. Abramson[1,2] introduced skin into the bullae of guinea pigs and migration, proliferation and subsequent development of cholesteatoma were enhanced if a concomitant inflammatory reaction was induced.

In vitro studies

Recent studies on factors that might induce migration, revealed the presence of fibronectin.[55] This is a glyco-protein, produced during inflammatory reactions by basal cells of the epidermis, or fibroblasts, endothelial cells, and macrophages, located in the sub-epithelial tissues. This fibronectin was found in the granulation tissue of infected middle ears and it was suggested that it might be one of the inductive forces guiding epidermis into the middle ear.[3] In vitro studies[286] showed the enhanced migratory and proliferative activity of cultured keratinocytes (from newborn rats) after exposure to fibronectin.

RETRACTION THEORY

Introduction

The retraction theory was published shortly after the appearance of Habermann's[106] article on the migration theory. It was Bezold[20] who in 1890, focused attention on the Shrapnell membrane, or pars flaccida. He concluded that there was a correlation between tubal dysfunction and pathology in the pars flaccida. Dysfunction of the Eustachian tube would lead to retraction of the tympanic membrane, especially in the pars flaccida, because this structure has no organized lamina propria. The combination of infection and retraction in this area could lead to either a defect with subsequent migration of epidermis into the epitympanum, or to inadequate keratin clearance of the pocket, both leading to cholesteatoma. Bezold associated cholesteatoma formation with chronic middle ear infection, tubal dysfunction and subsequent retraction pocket development. This theory is still supported by many otologists today.

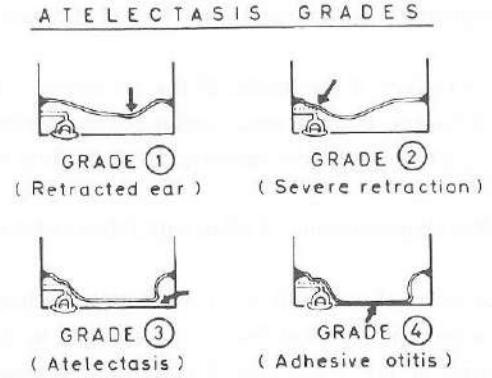


Fig. 8 Different grades of atelectasis of the middle ear according to Sadé.

Definition

Schuknecht[264]: A retraction pocket is an invagination of a replacement membrane into the middle ear space, a cutaneous layer of the tympanic membrane in the wrong place. According to Schuknecht, the retraction pocket could only develop after damage of the tympanic membrane was healed by a less resistent "replacement" membrane. Paparella[215]: The retraction pocket is an inward displacement of the tympanic membrane from the normal position.

Classification

The retraction pocket(RP) can be located in the pars tensa or the pars flaccida. The pars tensa RP is often associated with atelectasis of the middle ear cleft. Retraction of the pars tensa can occur in cases with disintegration of the normally well organized lamina propria, i.e. atrophic changes have taken place. This disintegration is thought to be caused by permanent negative pressure[9] or inflammation.[87,196] Sadé[251] subdivided the pars tensa RP into two groups. Those which retracted toward the promontory, resulting in atelectasis of the middle ear, and those which retracted toward or into the attic region, resulting in retraction pockets. The first group leading to atelectasis was divided into five types (Fig. 8):

Type 1. slight retraction not touching the ossicular chain.
Type 2. touching the incus and or stapes.
Type 3. touching the promontory(atelectasis)
Type 4. tympanic membrane adhesive to promontory (adhesive otitis media)
Type 5. atelectatic tympanic membrane combined with a perforation.

The second group comprising "true" retraction pockets of the pars tensa:

Type A. small retraction pocket- if the fundus of the retraction is easily seen
Type B. large retraction pocket- into the attic, fundus not easily seen but can be "sucked out". If the retraction pocket is not self-cleansing but its debris can be removed it is called an infected RP
Type C. retraction pocket cholesteatoma- if filled with infected keratin which can not be sucked out.

It is conceivable that many of us would call Type B already cholesteatoma. It can be difficult to distinguish a retraction pocket from cholesteatoma by determine whether its contents can be removed or not. Sadé also distinguished between central retraction pockets and those situated postero-superiorly or marginally.

Sadé[251] subdivided the RP of the pars flaccida into three types (small,medium and large), but the classification given by Tos[293] is more practical and requires less subjective interpretation:

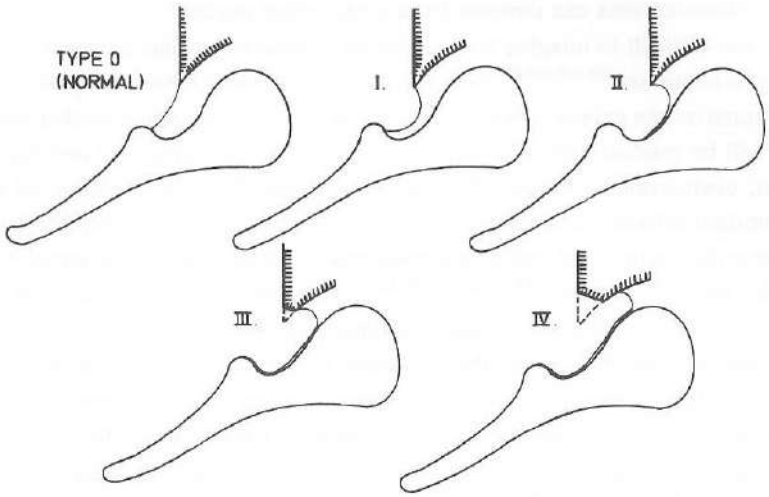


Fig. 9 Schematic illustration of different types of retraction pockets of the pars flaccida, according to Tos.[293,297]

Type 0. Normal Shrapnell membrane.
Type 1. RP towards the neck of the malleus but not touching it.
Type 2. RP makes contact with the neck of the malleus
Type 3. RP beyond the bony annulus, but the fundus can be seen.
Type 4. RP extends toward the head of the malleus. The fundus can be visualized due to some resorption of the bony annulus.
Type 5. Attic cholesteatoma, deep retraction pocket whose fundus cannot be seen.

Paparella et al.[215,328] proposed a classification which encompassed all types of retraction pocket. Classification was based on the size of the external aperture of the RP, extension of the pocket into the middle ear, and on histo-pathological criteria, which meant that the RP could only be classified after it had been removed, which is not very practical in the clinical situation.

Lastly, Tümarkin[304] proposed that the name, retraction pocket, be changed to pre-epidermosis, and Lacher[152] to pre-cholesteatoma to underscore the potential of retraction pockets to become cholesteatoma.

Human studies

The main question in relation to the retraction theory is: Is there substantial evidence that a cholesteatoma can develop from a retraction pocket?

It is not difficult to imagine how a retraction pocket can change into a cholesteatoma, and several authors[2,81,168,169,225] have published their ideas about this postulated process. To summarize the general opinion: The transition from retraction pocket into cholesteatoma will be gradual, one of the main causes being that during the development of the pocket, obstruction of keratin clearance will occur. The accumulation of debris, with concomitant inflammation within the pocket or in the adjacent subepithelial tissues will further enhance the tendency of the epidermis to invade the middle ear cleft. In the last decade, several articles[2,40,43,189,220,237,251,328] have discussed this process in the clinical situation, often sustained by photographic evidence. Because many reports have confirmed the hypothesis that the retraction pocket is the precursor of cholesteatoma, it is interesting to mention which factors could induce retraction pocket formation.

Tos et al.[293,294,297] have done comprehensive follow-up studies to establish the pathological changes occurring in the tympanic membrane following otitis media with effusion (OME). Retraction pockets of the pars flaccida and pars tensa were seen frequently in 34.5% and 26%, respectively. In 12% these two types of retraction pocket occurred simultaneously. Surprisingly, the incidence of severe retraction pockets, type IV, was the same as in a control group of otherwise healthy children (±4%). An important difference was that only the OME group showed pars flaccida and pars tensa cholesteatoma (1.7% and 0.3%, respectively).

Several authors[15,144,200,283,328] found a correlation between the persistence of OME and the incidence of retraction pockets. In most of the studies however, no control group was used. In general, more retraction pockets were seen in the pars flaccida, although there was a marked variation of the incidence, from 0 to 36%.[15,144,154,283,316] Edelstein[74] observed a higher incidence of retraction pockets in the pars tensa. Retractions of the pars tensa in children treated for OME ranged between 1.8 and 15%, this part of the tympanic membrane exhibited other pathological features such as tympanosclerosis and atrophy.[15,70,154,189,199,200,283]

Most authors of follow up studies have reported also the incidence of cholesteatoma in children treated for OME, i.e. ranging between 0.07 and 1%. Most of the cholesteatoma were located in the pars flaccida.[4,181,187,200,297] According to Magnan[166], advanced retraction of the pars tensa (adhesive otitis media) can lead in almost 25% of the cases to cholesteatoma formation. This study was based on histological examination of temporal bones, a definition of what was considered a cholesteatoma was not given.

In general, it was not possible to compare the results of the various studies, because of differences in the age of target groups, the duration of (untreated) OME, the duration of follow up, criteria for retraction pockets, and for cholesteatoma. As already mentioned almost none of the authors discussed his findings in relation to a control group. In sum, it was difficult to obtain a clear picture of a possible correlation between otitis media with effusion and retraction pocket development. A negative middle ear pressure due to tubal dysfunction is thought to be one of the main etiological factors in retraction pocket formation. Holmquist[123] found a negative middle ear pressure of at least -500 mmH_2O in the majority of his patients with retraction pockets. Shinkawa[272] recorded in ears with retraction of the tympanic membrane, values of at least -160 mmH_2O. Sadé[247] and Buckingham[41] recorded mild negative middle ear pressures(1.5-5 mmH_2O) in ears with retraction pockets. They rejected negative middle ear pressure as an important factor.

Lange[153] in 1925 remarked that the initial factor in retraction pocket formation must be the negative middle ear pressure. To maintain this condition, however, inflammatory reactions in the subepithelial tissues were obligatory. More recently, Abramson[3] investigated the presence of myo-fibroblasts in the sub-epithelial tissues. These myo-fibroblasts might play a role in the retraction of the pocket by active contraction.

Eustachian tube function was studied in relation to the presence of retraction pockets and cholesteatoma. Generally, an active tubal dysfunction was observed in a higher percentage than in control groups with normal ears, although the interpretation of the kind of dysfunction differed considerably.[27,45,82,83,93,112,124,125,129,168] Various tests were used, and most of them were performed under non- physiological conditions. Adequate interpretation of the clinical relevance of these experiments is difficult. Especially because values found in normal ET function vary between authors too. Furthermore, in children a drastically poorer Eustachian tube function will be found compared to that in adults.[54,78]

The aeration of the epitympanum is considered to be mainly dependent on the tympanic isthmus.[4,187,215,292] Mal-aeration of the epitympanum, due to a blocked tympanic isthmus, can occur despite the presence of a normally functioning of the Eustachian tube.[218] For instance, Paparella[215] found in his temporal bone study a correlation between the patency of the tympanic isthmus and the stage of retraction pocket formation in the pars flaccida. Obstruction of the tympanic isthmus can be the result of swollen mucosal folds or granulation tissue, however, also retraction of the postero-superior area of the pars tensa can block the tympanic isthmus.[4]

Retraction of the pars flaccida will occur in this case, after the pars tensa retraction pocket has obstructed the tympanic isthmus, with subsequent absorption of gasses in the epitympanum. The simultaneous presence of retraction of the pars tensa and flaccida can be explained this way.[328]

Animal studies

Retraction pocket formation has been incidentally mentioned in reports of animal experiments. Chole[59] induced inward displacement of the tympanic membrane, by ligating the external ear canal in mongolian gerbils. The formation of his "retraction pocket" was due to the accumulation of keratin in the external ear canal and the retraction pocket resembled cholesteatoma.

Fernandez[87] introduced quinine-HCL or quinine-sulphate into the bullae of guinea pigs. This caused inflammatory reactions with perforations and necrosis. A cicatricial reaction of the healing sub-epithelial tissues caused an inward displacement of the ear drum. He concluded that negative middle ear pressure is not always necessary for retraction pocket formation.

Other animal studies, done by Honda[127] and Huang[134], also concerned the use of chemical irritants, introduced into the middle ear, leading to inflammation and than to subsequent retraction pocket formation.

Obstruction of the Eustachian tube by ligation or cauterization has been done in several animal experiments, where it lead to the development of otitis media with effusion and negative middle ear pressures.[86,277,278,320] This procedure only occasionally led to the formation of retraction pockets,[128,282] except in the study done by Wolfman.[320] In that study blind cauterization of the Eustachian tube was done in mongolian gerbils and caused a high percentage of retraction pockets and cholesteatoma formation. The transformation of the retraction pocket into a cholesteatoma is, not mentioned in other reports of animal experiments, this report being an exception.

PROLIFERATION THEORY

Introduction

This theory introduced by Lange[153] and Manasse[170] was based on histological and clinical observations of the epidermis and subepithelial tissue, located at the junction of the external ear canal and tympanic membrane. In particular, the region of Shrapnell's membrane and the poster-superior region of the pars tensa. The basal and spinosal layers of the epidermis showed an increased number of cells, indicating hyper-proliferation. Long rete pegs invaded the underlying connective tissue. Re-organization of these basal cones led to the formation of micro-cholesteatoma deep in the subepithelium, without interruption of the integrity of the overlying epidermis.

The skin in the above-mentioned region was thought to have special proliferating properties, which some authors explained by preceding embryological events. The existence of such a special biological behavior has been investigated in several studies. Various chemical irritants, applied to the ear drum or middle ear, induced phenomena such as invasion and micro-cholesteatoma in animal experiments. Thus the presence of a subepithelial inflammatory reaction, may play an essential part.

Within this concept, immunologic reactions have also been considered to play an inductive role in the invasive behavior of the epidermis. The proliferation theory can be applied to any site where epidermis can invade the sub epithelial tissues, un-obstructed by such barriers as the lamina propria. The proliferation theory is often mentioned in combination with the migration and/or retraction theory. In the next section the above-mentioned items will be discussed in more detail.

The Definition

No definition comprising the concept belonging to the proliferation theory was found in the current literature. The process of cholesteatoma formation, according to the proliferation theory, may be defined as an invasion of the basal layers of the epidermis into the sub-epithelial tissues, leading to cholesteatoma.

Human studies

The first article dealing with invasion, and proliferation of the basal layers of the pars flaccida date from 1917, and was written by Manasse.[170] In 1925, Lange[153] published a histological and clinical article, describing the ingrowth of the basal and especially the spinosal cell layers into the subepithelial connective tissues. According to Lange, this invasion occurred in the pars flaccida, due to a chronic middle ear infection. The deep invading cones of basal cells developed small epidermoid cysts which were expected to lead to clinical cholesteatoma. This micro-cholesteatoma formation in human material was also reported by others.[143,211,238,263,337] It is not certain whether the lamina basilaris of the epidermis had been disrupted before the invasion. Damage to this structure, was seen incidentally with the electron microscope in human material.[61,211,229,247] Rauchfuss[229] studied the temporal bones of children, and concluded that persistence of mesenchymatous tissue in the attic enhanced the ingrowth of epithelium. If infection was present, this tissue readily changed into granulation tissue. The presence of this mesenchymatous tissue can persist until the end of the first year or even longer.[228,256]

In cases with a perforation of the pars tensa the same process of basal invasion and cholesteatoma formation, was seen at the border between migrating epidermis and middle ear mucosa, i.e., in the area called the "advancing front".[209,211] The epidermis invaded the subepithelial tissues whilst undermining and "pushing away" the middle ear epithelium.[89,114] Proliferation and invasion through an intact lamina propria of the pars tensa has never been documented. Most authors assume that invasion in the pars tensa cannot occur if the lamina propria is intact. In this context it is important to refer to the papers by Boedts[29], Lim[157], Marquet[174], and Ars,[9] who found in normal human tympanic membranes defects in the lamina propria, without a history of perforation or infection. These physiologic locus minoris resistentiae, might facilitate invasion of basal cells.

Almost all cholesteatoma occur in the region of the pars flaccida or the postero-superior area of the pars tensa. It is not surprising that some authors have tried to explain this phenomenon by ascribing special biological properties to this part of the epidermis.

Histological studies on human temporal bones of healthy ears, showed acanthosis and hyperkeratosis at the junction of the external ear canal and tympanic membrane.[143,220,238] Ingrowth of basal cones into the underlying tissue was not observed, but in cases with infection, papillary proliferation occurred.[143] Eigler[76] found at the rim of marginal perforations ingrowth of basal layers and subsequent cholesteatoma formation, even in the absence of inflammation. The specific biological activity of the epidermis was explained by its embryological origin. The deep skin of the external auditory canal represents the remnant of ingrowing ectoderm of the first epithelial groove, this invasive behavior would still be present in postnatal life.[76]

The epithelial-mesenchymal interaction[179] has also been put forward to explain the differences in proliferative activity of the epidermis in this area.[3,6,77,182] This concerned especially the pars flaccida because the morphology of the subepithelial tissue differed so strongly from that of the pars tensa, by lacking a well-organized layer of collagen fibers.[62,157] The lamina propria and adjacent middle ear mucosa of the pars flaccida could easily change into granulation tissue under conditions of infection or inflammation. This granulation tissue could induce invasion of the epidermis into the middle ear cleft. Within the context of the epithelial-mesenchymal interaction, it is also of interest to refer to articles dealing with a possible immunologic reaction.

The literature on immunologic reactions at the interface of invading epidermis and subepithelial tissue has gained greatly interest in the last decade. The Langerhans cell (LC) detected in the matrix and perimatrix of cholesteatoma might play an essential part in this concept. The Langerhans cell originates from the bone marrow and this cell is present in all stratified epithelia and underlying connective tissues.[212,308,327]

It is assumed that the LC has an antigen presenting function in the cellular defence system, because this cell also occurs in the lymph follicles in close relation to T-lymphocytes.[96,186,219,306,309,327] In biopsy specimens of cholesteatoma, the LC was found throughout the matrix but especially in the suprabasal layer (spinosal cells). T lymphocytes were clustered in the perimatrix beneath aggregations of LC in the matrix, indicating a possible relationship between them.[95] The T-lymphocyte population had equal numbers of T- helper and T-suppressor cells, indicating a "healthy" immunological reaction. Some authors report higher concentrations of LC in cases with recurrent or residual cholesteatoma, suggesting a sensitized cellular response.[219,308]

The development of cholesteatoma has even been considered to be the result of immunologic reactions in chronic otitis media.[306] The induced epidermal spread would be caused by Langerhans cells ,macrophages, lymphocytes, and plasmocytes.[306]. Others, however, do not believe in some kind of systemic disease, considering the LC to be a normal histiocyte that ought to be present in this tissue.[175,212]

In sum, there are indications that an immunologic reaction takes place in the vicinity of the cholesteatoma matrix. If this reaction is triggered by an antigen, e.g. keratin, is not known. The presence of Langerhans cells, macrophages, T-lymphocytes but also plasma cells, and mast cells does indicate that an immunological process can be involved. Whether this process can induce cholesteatoma does not seem likely, but the invasive behavior of cholesteatoma might be partially explained this way.

Animal studies

Cholesteatoma have been induced in animals by applying chemical irritants to the external ear canal or the tympanic membrane. For example; tar, benz-pyrene, croton, or olive oil, in all these cases cholesteatoma developed by migration of epithelium through perforations of the tympanic membrane.[262] Ruedi[238] was the first to observe cholesteatoma formation behind an intact ear drum in his animal experiments. He applied a mixture of talcum and fibrin to the inner surface of the tympanic membrane of guinea pigs.

This led to active invasion of basal cells, into the underlying granulation tissue. In these cases the lamina propria of the tympanic membrane changed into granulation tissue without concomitant perforation. The invading columns divided into branches and the cells showed keratinization, and typical cholesteatomatous cysts were formed within the columns. Identical experiments with such irritants as propylene glycol, benz-pyrene, olive oil, talcum powder, quinine, ethyl alcohol were used in various animal species such as the rat, chinchillas, hamsters, and rabbits.[29,87,128,173,277,321]

In most animals the irritants were applied through the bulla into the middle ear. In some cases cholesteatoma developed due to the above-described ingrowth of epidermal basal cells. The same results were obtained by applying irritants to the lateral surface of the tympanic membrane or adjacent skin of the external ear canal.[94,277,282]

Steinbach[277,279] applied various irritants to the lateral surface of the tympanic membrane of rabbits, e.g. gelfoam, histoacryl, NaOH, and HCl. This resulted in hyperkeratosis, acanthosis, and cholesteatoma if applied in the region of the pars flaccida. Application of the irritants to other areas of the tympanic membrane did not led to cholesteatoma formation.

Grote[105], inoculated rat middle ears with Staphylococcus aureus. Inflammatory reactions were most prominent in the area of the pars flaccida, although cholesteatoma formation was not observed.

A common feature in all animal experiments was, that damage occurred in the lamina propria of the pars tensa or flaccida due to inflammatory reactions. Whether the integrity of the lamina basilaris must also be disturbed before invasion of the basal cells can occur is uncertain, but some electron microscopical studies suggested defects in this structure.[61,94,211,239]

In Vitro Studies

Within the context of the epithelial-mesenchymal interaction[33,127,128] *in vitro* studies were performed, to establish the influence of such substances as prostaglandin (PGE2),[142,332] leukotriens,[142] and endotoxins,[202,284,285] which are especially detectable during infection in granulation tissue. Prostaglandin and endotoxin can influence the proliferation of keratinocytes, as was demonstrated *in vitro* experiments.[285] Granulation tissue-conditioned medium decreased protein synthesis and increased the production of cornified envelopes of cultured keratinocytes[133], indicating that the substances in granulation tissue can influence the process of terminal differentiation.

Conclusions

The proliferation theory is often related to the retraction and migration theories, and many otologists believe that all three concepts play a role in cholesteatoma formation.[127,174,196,333] Migration of epidermis into the middle ear cleft was observed frequently in the pre-antibiotic era. Then, middle ear infections led more often to large perforations of the tympanic membrane as well as to necrosis of middle ear mucosa.[114,305]

As long as the keratin clearance of ingrowing epidermis was not obstructed, this process could be considered a normal part of wound healing. In fact, a middle ear could be completely lined by epidermis without the development of cholesteatoma.[208] It was suggested that concomitant infection could induce in these cases the clinical picture of cholesteatoma.[319]

The migration occurred mainly where marginal perforations were present but has also been observed around the edges of central perforations. In a non-infected ear, migration through these defects may occur, but normally will not result in cholesteatoma.[29,201] At present, necrotizing otitis media does not seem to play an important role within cholesteatoma pathogenesis, probably due to the better control of middle ear infections provided by antibiotics.[43]

A retraction pocket may develop without infection, due to negative middle ear pressure. The epidermis of this dry pocket consists of a thin layer without signs of hyperproliferation or enhanced keratosis.[328,335] Chronic infection or inflammation in the retraction pocket, with obstruction of the keratin clearance, or subepithelial inflammation, may lead to increased basal proliferation and keratinization. These processes can lead to advancement of the pocket into the middle ear cleft[328] or rupture of the retraction pocket [303] leading to migration of epidermis. The basal proliferation which can result in cholesteatoma, as described in the preceding chapter dealing with the proliferation theory, results in micro-cholesteatoma occurring beneath the intact epidermal layer.

The link between the three theories is disruption of or damage to the lamina propria of the tympanic membrane, which will usually occur in combination with a chronic middle ear infection, changing the healthy middle ear mucosa and lamina propria into granulation tissue.[174] The biological properties of the epidermis (wound-healing), and the inductive forces of the granulation tissue may lead to the invasion of stratified squamous epithelium into the middle ear cleft, leading to the formation of cholesteatoma.

IN SITU THEORY

CONGENITAL ORIGIN

Introduction

A congenital origin of cholesteatoma was postulated as early as the end of the last century. Kuhn[148] and Küster[150] mentioned what was called the primary or true cholesteatoma, meaning that infection of the middle ear had played no role in the

pathogenesis, and that a congenital inclusion of epidermis in the temporal bone was probably responsible for its formation. Küster saw concomitant congenital defects of the branchial grooves in the head and neck region.

McKenzie[178] and Holmes[122] thought that most cholesteatoma were originally congenital and had a cystic structure, perforation of the tympanic membrane or middle ear infection being secondary effects, due to rupture of the epidermoid cyst.
Derlacki[72] and Cawthorne[52] published with regularity about case reports of congenital cholesteatoma behind an intact ear drum.

Definition

Derlacki introduced three conditions before a cholesteatoma could be considered congenital:

1 - development behind an intact tympanic membrane
2 - no history of aural infection
3 - originating from embryonic inclusion of squamous epithelium in the temporal bone, or from undifferentiated tissue that changes into squamous epithelium.

The Derlacki's criteria were applied by most authors who wrote on this subject. Of course, the last two conditions of Derlacki can never be verified completely, but reports of the presence of embryonic ectodermal tissue are known, as will be discussed below.

Human studies

One possible source of epidermal cells found in the middle ear cleft and which should transform into a non-keratinizing epithelium during fetal life was first put forward by Teed.[290] The epidermal structure was "located at the dorso-lateral pole just medial of the neck of the malleus". No other similar observations were made until 50 years later, when Michaels[184] reported on the so-called "epidermoid formation".

This epidermoid structure was seen in ca. 65% of fetal temporal bones from 10 to 33 weeks of gestation. It was found in the "anterior superior middle ear mucosa , adjacent to the posterior edge of the anterior limb of the bony tympanic ring. It demarcated two different middle ear mucosae, one a ciliary and the other a non-ciliary epithelium".[155,184] The origin of this formation was thought to be ectodermal tissue of the first branchial groove, and the structure could show a cap of keratin in fetal life.

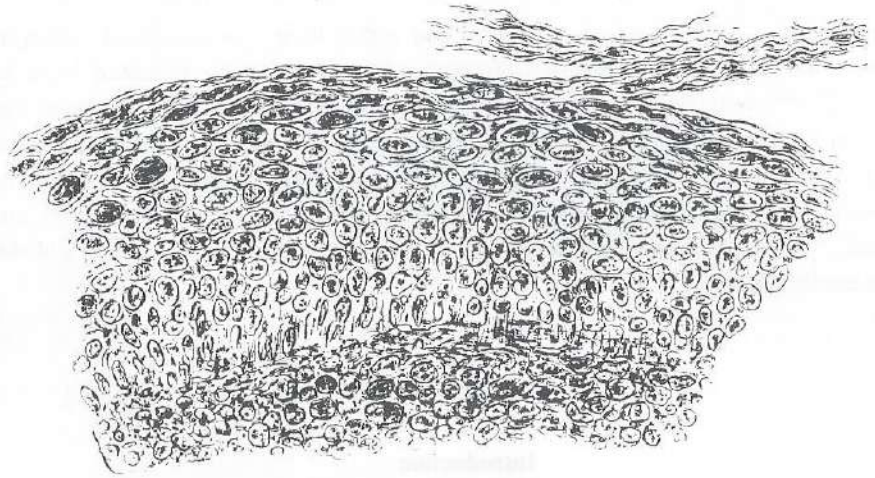


Fig. 10 The first drawing of a cholesteatoma matrix, as was published by Lucae in 1873.

Many congenital cholesteatoma are, according to some papers, indeed located in the antero-superior quadrant of the middle ear.[65,74,155,216] Michael did not adhere to Derlacki's criteria completely, because he did not consider a history of middle ear infection to be an exclusion criterium, provided, that no perforation or otorrhoea had occurred.

In contrast to Michael's hypothesis stands the theory of Aimi,[4] who claimed that the place of preference for congenital cholesteatoma was located in the postero-superior region, i.e. the tympanic isthmus. The tympanic isthmus was postulated to represent the end bud of the first branchial groove. Aimi found that small epidermoid cysts occurred in this region. The cysts might remain inactive during life if no infection or inflammation developed in the subepithelial tissue of the cornifying epithelium. For this reason, the small congenital cholesteatoma might remain undetected during a life time. His observations were also confirmed by others.[227,257,258,331] Aimi's theory supports Michael's opinion that only in cases with a combination of a middle ear infection and perforation, the diagnosis congenital must be abandoned. Especially since infection can activate the dormant congenital cholesteatoma. According to Derlacki,[72] however, we cannot speak of a congenital cholesteatoma if a middle ear infection has taken place.

Sanna[257] stated that at least some of the cholesteatoma with a postero-superior perforation could have been congenital with secondary perforation. Estimations of the incidence of congenital cholesteatoma lie between 1 and 5% of the total incidence of cholesteatoma.[63,131,155,217,226,242,253,257]. One exception is made by Parisier,[216] who reported an incidence of 10%, possibly due to the fact that it was based on a retrospective study, and the criteria did not include a history free of aural infection.

Cholesteatoma behind an intact eardrum and with a history of middle ear infection may have developed out of invasive proliferating basal cells, as suggested by some authors.[138,217,284] Apart from the above-mentioned authors, it is the general opinion that congenital cholesteatoma can and will occur, but that the incidence is so low that this concept plays a minor role within the range of cholesteatoma pathogenesis. A possible increase in congenital cholesteatoma in children was observed by Parisier[216] and Edelstein,[74] which may have been the result of the "doctor's greater awareness", of this type of cholesteatoma.

THE METAPLASIA THEORY

Introduction

Current literature relates the metaplasia theory to Wendt,[317] as the first otologist to advocate and introduce this theory. However, slightly before Wendt, and at least in the same year, 1873, Lucae[163] published an article about the metaplastic origin of cholesteatoma. He saw a correlation between the chronic middle ear infection and cholesteatoma formation. The chronic infection forced the middle ear mucosa to become granulation tissue, which underwent metaplastic changes leading to a cornifying epithelium(fig.10). Wendt was more precise: he called this proces a "desquamative-like change of the mucosa exhibiting a rete Malpighii(prickle cell layer) and hyperkeratosis".

Except for a paper by Wingrave[319] in 1910, who believed that desiccation of the middle ear mucosa induced formation of a cornifying epithelium, no other papers on the metaplasia theory appeared, until the middle of the present century. Review of the literature showed clearly that many contradictory reports have been presented.

Definition

A clear definition or exact description of what must be considered as the metaplastic process leading to cholesteatoma cannot be found in the literature. Within this context, it is more relevant to pose the following question;

Can mucosa of endodermal origin change into the cornifying epithelium, normally seen in ectoderm-derived tissue?

A definite answer to this question cannot be given yet, especially because few authors specify clearly what they mean by a cornifying epithelium.

Normally, as we have already mentioned, we can distinguish four types of cell layer: the stratum basale, stratum spinosum, stratum granulosum, and the stratum corneum. The third cell layer, the stratum granulosum, which shows kerato-hyalin granules represents an important stage in what is called "terminal differentiation". This process of terminal differentiation characterized by cornified-envelope formation and subsequent cell death creates a true cornifying epithelium(see page 13). In most of the papers this important granular cell layer is not mentioned when the metaplastic cornifying epithelium is discussed.

Classification

The mucosal lining of the middle ear is considered to be an extension of the respiratory epithelium of the upper and lower respiratory tracts, Lim.[161] Metaplasia of respiratory epithelium, especially in the lower respiratory tract, is a well-documented phenomenon.[183,300] The influence of various exogenic factors on this epithelium has been studied.[90,110,183]

According to Auerbach,[10] three phases in the process of metaplasia of the bronchial epithelium can be distinguished:

-*Phase 1*: The first change is characterized by proliferation of the basal layers, the uppermost cells retaining their original features.

-*Phase 2*: In the second phase stratification and flattening of cuboidal or columnar cells occur.

-*Phase 3*: Finally, above the basal cells a typical spinosal cell layer develops, but a granular cell layer or keratinization does not occur.

Auerbach could not find indications pointing to further differentiation into a true cornifying epithelium. However, if we consider transformation into an epidermis as a possibility, than a *phase 4* representing the final stage in metaplasia should be added. This *phase 4* epithelium would have a stratum granulosum and stratum corneum.

Human studies

The papers on this subject can be subdivided into two groups. The authors of the first group believe, that metaplasia cannot differentiate any further, than according to Auerbach type 3 metaplasia. Thus keratinization or cholesteatoma formation are not in order.

The second group of authors believe that squamous metaplasia with keratinization can occur. It is not certain that in all cases true cornification was in order. Keratin-like squames can also be the result of increased exfoliation of squamous epithelium. The two groups will be reviewed separately;

Squamous metaplasia without keratinization

Friedmann,[90] who examined an impressive number of human mucosal biopsy specimens obtained during surgery, found metaplasia up to the prickle cell layer. Metaplasia, however, could result not only in a squamous epithelium but also in an active mucus-producing epithelium, as seen in otitis media with effusion, a conclusion which has been confirmed by others.[91,101,116,143,151,161,244] Other papers on squamous metaplasia without keratinization, in human middle ear mucosa, were published by, Rüedi,[240] Birrel,[24] Karma,[143] Zechner,[334] Tümarkin,[302] Lim.[160]

Tümarkin[302] assigned special properties to the squamous epithelium(pavement epithelium) of the attic. In cases with infection this epithelium reacted by proliferation and throwing off of paper-like squames. This enhanced exfoliation without the formation of a prickle cell layer could lead to cholesteatoma formation according to Tümarkin. Another type of metaplasia was introduced by Palva,[208,210,211] who observed two types of metaplasia, the independent metaplasia and junction metaplasia, the latter representing a transitional zone between the invading epidermis and the normal middle ear mucosa. In this area the keratin and granular cell layer gradually disappeared, and the mucosa resembled the type 3 metaplasia of Auerbach.[10] The independent type had no connection with existing epithelium, but exhibited the same features.

Squamous metaplasia with keratinization

Bernstein[22] found stratified squamous epithelium with keratin production in biopsy specimens of middle ear mucosa of patients with otitis media with effusion, however, the presence of a granular cell layer was not documented. The same can be said about the article by Bendek,[19] and the many articles by Sadé.[245,247,249,252] Sadé advocated reintroducing of the possibility of a metaplastic origin of cholesteatoma. The basic concept in Sadé's[247] view is that all cells have the same genetic information, stored in the chromosomes, which enables them to differentiate into other types of cell. According to Sadé, the stem cell of the human middle ear mucosa can differentiate into four cell types: 1) another stem cell, 2) an intermediate cell, 3) a mucus-producing cell, or 4) a keratin-producing cell.

Local environmental conditions were thought to determine the cell type into which the cell would differentiate. Sadé presented a substantial amount of histological material, showing stratified squamous epithelium with keratin production in human middle ears. Most of the biopts were taken from ears treated for a chronic (cholesteatoma) infection or otitis media with effusion. The biopts were taken from places clinically devoid of cholesteatoma. However, because the clinical and histological diagnoses may differ,[205] it remains uncertain whether this squamous epithelium had no connection with the cholesteatoma matrix or the meatal epidermis.

The same can be said about the articles by Bernstein[22] and Bendek.[19] Sade[244] found squamous metaplasia in an incidence of up to 60% in the mucosal biopts. The histological features were studied by light microscopy but a detailed description of the individual cell layers of the cornifying epithelium was not given. Lim[160] studied mucosal biopsy specimens taken from the promontory of 180 ears with OME, and in 3 cases observed a squamous cornifying epithelium. The biopts were studied electron microscopically and the granular and corneal cell layers were described. These ears had no history of perforation or operative procedures. Ten cases showed a non-cornifying metaplastic epithelium without a granular cell layer, whether this concerned a distinct class of epithelium or a transitional form to become a cornifying epithelium could not be determined.

Bodelet and Wayoff[28] studied human mucosa from ears which had been treated surgically for cholesteatoma in the past. They found a classic squamous cornifying epithelium, well-documented by TEM pictures, since a granular cell layer with keratohyalin granules was described. The number of patients in this study was not mentioned. Palva[205] took mucosal biopts during operations on ears that were clinically free of cholesteatoma, but suffered from a chronic infection. In 56% he found a cornifying epithelium the cause of which he did not exclude to have been due to metaplasia, because most of the ears had marginal perforations he concluded that a migratory origin was more likely. This article, however, has been cited by many to support the metaplasia theory. Palva concluded that cornifying epithelium which clinically resembled middle ear mucosa, was probably replaced by normal mucosa if chronic infection was healed. Palva held the opinion that this mild cornifying epithelium would probably not led to cholesteatoma after closure of the tympanic membrane. In later publications, Palva[211] withdrew this last conclusion by stressing the importance of removing all cornifying epithelium.

Many questioned whether a metaplastic cornifying epithelium could indeed result in cholesteatoma. Sadé[247] subdivided the metaplastic cornifying epithelium into simple stratified squamous epithelium and cholesteatomatous stratified epithelium. The former as frequently encountered in OME, would not lead to cholesteatoma, the second type encountered in chronic otitis media would lead to actual cholesteatoma formation.

Animal studies

In reports on animal experiments,[90,321] the formation of non-cornifying squamous metaplasia has been ascribed to infection or chemical irritation of the middle ear. Wright[321] is one of the few authors who stated that he did not see a granular cell layer, to confirm that this epithelium was not cornifying. Squamous metaplasia with keratin production has also been observed in the animal model. It was induced by cauterizing the Eustachian tube in mongolian gerbils,[320] introducing virulent micro-organisms into the middle ear of the rat,[310] or antibiotic eardrops in chinchillas.[307] In all these studies mention is made of proliferation of the basal cells ,development of a spinosal cell layer and keratinization, a granular cell layer is not specifically mentioned.

Greenberg[101] instilled crude tobacco tar into the middle ear of mongrel cats, which gave rise to a cornifying epithelium with a documented granular cell layer. As explicitly mentioned, there were no breaks in the tympanic membranes. A vitamin A deficient diet led to squamous metaplasia of the hamster tracheal epithelium.[110] Transmission electron microscopy revealed kerato-hyaline granules and a thick cornifying layer. Chole[58,60] fed rats a diet free of vitamin A, which led to a cornifying epithelium in the middle ear cleft, but Chole did not describe a granular cell layer.

Banoczy[14] changed the local environment of the buccal mucosa of rabbits by exposing the latter to the outer environment. Due to desiccation and lack of saliva, the mucosa showed increased keratinization without the appearance of a granular cell layer. The authors believed that the granular cell layer is not essential for true keratinization, and mentioned other sub-microscopic granules as responsible for this process.

Squamous metaplasia was induced by influencing the epithelium- mesenchyme interaction. Sweeny[288] implanted tracheal respiratory epithelium of the mongrel dog on the denuded cartilage of the dog's ear, that underwent a complete transformation into normal epidermis. Re-implantation of this cornifying epithelium in the trachea led again to a normal respiratory epithelium. Change of epithelium by inward migration was excluded, because no signs of migration were noted at the junction sites, transformation of the epithelium was observed first in the central part of the graft. McLoughlin[179] reported that embryonic chicken epidermis differentiated into a mucus- or keratin-producing epithelium depending on the type of underlying mesenchymal layer.

In Vitro Experiments

In Vitro experiments[13,100] revealed that when a cell suspension of trachea derived respiratory epithelium was cultured, all cells became rich in keratins and could create cornified envelopes.

The same observations were made by van Blitterswijk,[25] who cultured middle ear epithelium. Bijlsma[23] cultured tubotympanic epithelium of the rat in a medium lacking vitamin A and saw complete keratinization within 14 days. The epidermis of the external auditory canal cultured in a medium enriched with vitamin A showed loss of kerato-hyaline granules and acquired characteristics of a secretory epithelium.

Conclusions

With respect to congenital cholesteatoma, it must be kept in mind that the diagnosis is made on the basis of three criteria which often cannot be verified. Concerning a possible metaplastic origin, it can be stated that stratified squamous metaplasia with keratinization in the human middle ear was found mainly in cases with actual cholesteatoma or with a history of chronic otitis media and cholesteatoma. Those articles did not make clear whether this epithelium could have been in continuity with the existing epidermis. The most interesting findings concerned human middle ears with a cornifying epithelium in combination with otitis media with effusion, but without a history of perforation or surgical treatment.
As mentioned before, many articles lacked a well-documented description of the cornifying epithelium. However, some observations made in human biopsy specimens as well as observations made during animal experiments, have indicated that a middle ear mucosa can differentiate into stratified squamous cornifying epithelium, whether this will result in the clinical picture of cholesteatoma remains uncertain.

GAS COMPOSITION AND GAS DIFFUSION IN THE MIDDLE EAR

Human studies

One of the first to measure the gas composition in the human middle ear was Matsumura,[174] he found no CO_2 and a moderately decreased O_2 level(14 - 17%) when compared with the ambient air. Probably pollution with ambient air influenced his results, because more recent studies,[49,85,104,180,236,247] on the composition of the gas in the human middle ear resulted in different values. The O_2 concentration ranged between 5 and 9.5% and for CO_2, the concentrations lay between 2.6 and 6.5%.

Riu[236] found no difference in gas composition between healthy ears and otitis media with effusion. Within this context, Ingelstedt[137] found elevated CO_2 concentrations compared to values found in healthy middle ears.

Elner[80] concluded that there is probably no real ventilation of the middle ear, in the sense of a temporary change in the middle ear gas composition, due to an opening of the Eustachian tube. He estimated that the absorption from the middle ear by surrounding tissues was between 0.7 and 1.1 ml in 24 hours, these values were confirmed by others.[79,137,236] Taking these low values into account, Elner considered the Eustachian tube more as a pressure regulator not influencing the actual gas composition itself. Tubal dysfunction would not influence the gas composition, which explains the minimal differences between aerated ears and those afflicted by otitis media with effusion.

Grontved[104] observed variations in O_2 concentrations from 3 to 17%, between different subjects. In contrast to Elner,[80] he concluded that high O_2 concentrations were due to recent openings of the Eustachian tube introducing relative O_2-rich air, which was slowly absorbed. Grontved proposed that the Eustachian tube could have an essential influence on the composition of the gas in the middle ear. According to the values mentioned above, we can conclude that the CO_2 concentration in the middle ear($\pm$5%) is much higher than that of the ambient air(0.03%). The O_2 concentration in the middle ear($\pm$ 10%) is at least twice as low as that of the outer environment (21%). The CO_2 transport between the blood-tissue compartment and the cavity is 25 times faster relative to that of O_2, due to the high solubility of CO_2 in the blood.[49] The CO_2 tension in the middle ear is therefore considered to be in equilibrium with the surrounding tissues.[137]

O_2 and N_2 are absorbed slowly[49,84,85,104,137] due to the higher total gas pressure in the middle ear compared with the surrounding tissues.[49,80,137] Bylander[48] performed tympanometric studies in sleeping children, on the assumption that the Eustachian tube does not function during sleep. He observed initially an increase in pressure but after one hour there was a steady decrease of the pressure. After a few hours a steady state of a moderately negative middle ear pressure was reached and persisted during the remainder of the sleep period.

This observation contradicts the *ex vacuo theory*, where it is supposed that a continuous absorption of gas will occur if no tubal opening takes place, leading to high negative middle ear pressure. Various articles, on human and animal studies, are opposing the *ex vacuo theory* or are in agreement with Bylanders' observations, as will be discussed next. Sadé[255] and Luntz[164] observed a spontaneous re-insufflation of atelectatic middle ears during sleep, suggesting an active gas diffusion into the middle ear cleft.

Shinkawa[270] and Magnuson[168] reported positive pressures in healthy ears after a nights' sleep and before the onset of active functioning of the Eustachian tube. The pressure decreased immediately after swallowing and became close to the ambient pressure. Hypo-ventilation caused an increase of the pressure. The same observations were made by Hergils,[117-119] who furthermore reported that hyper-ventilation resulted in a decrease of middle ear pressure.

As the pCO_2 in blood normally increases during sleep,[119] the authors attributed the recorded pressure variations to diffusion of CO_2, entering and leaving the middle ear by diffusion.

Hergils[120] demonstrated that the healthy middle ear could reduce an experimentally applied negative middle ear pressure in the absence of a tubal opening. Buckingham[46] visualized the development of positive intra-tympanic pressure in 37% of cases after myringotomy in healthy ears, however, in 13% a negative pressure developed and in 9% positive and then a negative pressure. Buckingham[46] and Sadé[247] found only mildly negative middle ear pressures, of 6-8mmH_2O and 1.7-5mmH_2O, respectively, in otitis media with effusion. This is in contrast to most authors who reported negative middle ear pressures of 300mmH_2O and more in case of OME.[177,295] Pollitzerization of the human middle ear with air,[180,323] or O_2 at various concentrations[174,180] led to a marked pressure drop, probably due to absorption of O_2.

Animal studies

The gas composition of the middle ear found in animal studies,[190,203,271] did not differ drastically from human values, reported concentrations lying for O_2 between 11.7 and 12.1%, and for CO_2 between 4.7 and 7.5%. Cantekin[50] found that the physiologic gas pressure in the middle ear of the Rhesus monkey are almost in equilibrium with the mucosal blood-tissue gas pressures. The middle ear pressure in cases with a non-functioning Eustachian tube, decreased slowly in the first hour, after which a steadily mildly negative middle ear pressure of -60mm H_2O persisted. Pollitzerization of the middle ear with air and especially with O_2 led to a marked pressure drop. Cantekin concluded that a negative middle ear pressure of more than 60 mm Hg is not the result of a blocked Eustachian tube and subsequent absorption of gasses. In his opinion high negative middle ear pressure occurred, if the eustachian tube over ventilated the middle ear, changing the physiologic gas composition, and inducing increased resorption of O_2, as was observed in his experiment.

Shinkawa[271] found that an increase of the middle ear pressure in chinchillas during hypo-ventilation was due to a diffusion of CO_2 into the middle ear. He substantiated this observation by taking gas samples at the moment of increased pressure, which showed an increased CO_2 concentration. Ostfeld[203] induced changes in the middle ear gas composition by changing the arterial and venous pCO_2 and pO_2 pressures, in healthy dogs. Yee[326] found no relation between middle ear pressure and the O_2 pressure in the blood. Buckingham[44] found a direct relation between pCO_2 in the blood and middle ear pressure, suggesting diffusion or absorption of CO_2 into the middle ear, no evidence for O_2 absorption was found within the duration of the experiment(2 hr).

At last, Yee[326] found a steady state of middle ear pressure in anaesthetized rhesus monkeys breathing room air. In some cases even an increase in pressure was found.

Conclusions

The cited studies indicate that the "*ex vacuo theory*" cannot be considered the only mechanism regulating middle ear pressure and aeration. According to this theory, there is a constant absorption of O_2 and N_2, resulting in negative middle ear pressure, which must be compensated for by regular openings of the Eustachian tube(ET).

In many experiments the ET was regarded as non-functioning, if the person was recumbent, and neither swallowing nor yawning occurred. If this presumption is correct then there must be at least an other mechanism influencing middle ear pressures, since a steady state of mild negative or even positive middle ear pressures was found in these studies. Gas samples taken from normal human middle ears and during hypo- and hyperventilation in animals suggest that the middle ear gas composition and pressure is related to the gas pressures in the blood-tissue compartment. As Sadé already proposed, there is probably a complicated co-operation between gaseous pathways to and from the middle ear and Eustachian tube functioning.

THE INFLUENCE OF CO_2 AND O_2 ON CELLS *IN VIVO* AND *IN VITRO*

Culture studies

The influence of O_2 and CO_2 on cultured cells has rarely been studied by research workers in our field. In the culture studies[25,133,207,223,224,285,286] on cells or explants published in E.N.T. journals, use was made of the standard gas conditions of 5% CO_2 mixed with humidified air. These studies dealt mainly with keratinocytes. The first studies concerning the possible influence of environmental gas concentrations on cells in culture were performed by co-workers of Sadé e.g. Weismann[315] and Drucker.[73] Sadé was inspired by the classic study done by Moscona,[191] and the clinical observations of Hilding[121] and Young.[329]

Moscona demonstrated that the differentiation of an embryonic tissue can be changed by extra-cellular CO_2 and O_2. Young and Hilding observed alteration of the morphology of nasal mucosa due to closing of the nares in patients with ozaena. Weismann and Drucker exposed explants of mucosae from the rabbit trachea, human adenoid, and middle ear, to gas of various concentrations of CO_2 and O_2 as well as different pH values.

The animal experiments of Weismann[315] with explants of rabbit trachea revealed that the ratio of goblet and ciliary cells could be influenced by the environmental CO_2 concentration and pH. A high concentration of CO_2 in combination with an elevation of the pH led to an increase of mucus-producing cells. At lower CO_2 concentrations the ciliated cells pre-dominated numerically.

Drucker[73] cultured human adenoid tissue in air only, or air enriched with 5% CO_2. The number of mucus-producing cells was not altered, but at 5% CO_2 a 50% increase of ciliary cells occurred in the outgrowth of the explants. Explants of middle ear mucosa were also cultured, but there is only a short note reporting that the behavior of this tissue was similar to that of epithelium covering the adenoid and that in 70%, epithelial growth occurred. The possible influence of gas concentrations was not referred to.

According to Sadé, the results of these studies must be viewed with reserve, as it was very difficult to control the environmental conditions, e.g. maintaining of a constant pH. Differences in atmospheric CO_2 concentrations can influence pH of the medium, which is difficult to neutralize with bicarbonate used as a buffer in these experiments. Van Blitterswijk[25] brought middle ear epithelium of the rat into serial cultivation, at standard gas concentrations(5% CO_2 with air). The culture results were consistent and reproducible, and provided a good basis for further investigation of middle ear mucosa. Use was made of the experience accumulated in those studies in the culture experiments reported in the following chapters.

Articles on the effect of the O_2 tension on the growth and metabolism of various cell types are found in the journals dealing with basic cell research and cell physiology. These articles showed that cultured epidermal cells, i.e. keratinocytes[126,276,289] required a higher O_2 level for optimal growth than fibroblasts,[31,38] amniotic fluid cells,[28,113,291] or the WI-38 cells.[11,12] The last three cell types showed enhanced colony formation and growth upon lowering of the O_2 concentration from ambient($\pm$21%) values to percentages ranging from 2.5 and 10%. Optimal culture conditions for the keratinocyte included an O_2 concentration of ca. 18%. A marked decrease in proliferation rate was noted below 5% or above 30% O_2. At very low or high O_2 concentrations, enlargement of the keratinocytes was noted, as well as a more granular cytoplasm.[289,291]

Hardly anything has been written about the influence of different CO_2 concentrations on cells in culture. One of the main problems encountered in studies on the influence of this gas is the maintenance of a constant pH of the growth medium.[248,291]

Balin[11] concluded that raising of the CO_2 concentration from 5 to 10% during cultivation of human endothelial cells led to inhibition of proliferation. He attributed this phenomenon partially to the drop of pH, which could not be completely compensated for by a proper choice of buffer combinations in the culture medium.

Tissue studies

Moscona[191] exposed chorionic epithelium of the chicken embryo to air by removing the egg shell. This mucosa normally has a respiratory function but differentiated into keratinizing epithelium when exposed to air. High O_2 concentrations induced the same phenomenon "in ovo", but keratinization was always stopped with the addition of 5% CO_2 to the gas mixture. In this embryonic tissue the presence of CO_2 determined whether the cell differentiated into a mucus- or keratin-producing cell. Reimer[230,231] studied the effect of O_2 on ciliary cells of human antral mucosa and epithelium of the rabbit trachea *in vitro*. These epithelia required ambient O_2 concentrations(20%) to display normal ciliary function.

Rabbit sinus mucosa showed immediate reduction of ciliary activity when exposed to an atmosphere containing 5.2% O_2 and 8.6% CO_2. This gas composition was chosen, because it correlated with the gas concentrations Ingelstedt[137] had found in otitis media with effusion. The ciliary activity further decreased by increasing the CO2 concentration to 10 and 15%. Sinus mucosa of the rat *in vivo* was not affected by low O_2 concentrations, but a CO_2 concentration of 5% decreased the ciliary activity. The main conclusion drawn by Reimer was that the function of a ciliated epithelium lining a non-ventilated cavity will improve under proper ventilation of that cavity.

Conclusions

As the articles reviewed above indicate, changes in the levels of O_2 and CO_2, can influence cell metabolism or the morphology of cultured cells or tissue explants *in vitro* and existing epithelia *in vivo*. The epithelia investigated within the field of otorhinolaryngology concerned those of the trachea, adenoids, and sinus mucosa. No studies on explants of middle ear epithelium were found except, a short note by Drucker.[73] With refined culture techniques cell suspensions of middle ear mucosa of the rat can be cultured serially at present. This means that the influence of O_2 and CO_2 on this epithelium can now be studied.

REFERENCES

1 Abramson MA, Asarch RG, Litton WB. Experimental aural cholesteatoma causing bone resorption. Ann Otol Rhinol Laryngol 1975;84:425-432

2 Abramson M, et al. Cholesteatoma pathogenesis; Evidence for the migration theory. In: McGabe BF,Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co,1977;176-187

3 Abramson M et al. Epidermal-mesenchymal interactions in the middle ear. In: Tos M, Thomsen J, Peitersen E,eds. Cholesteatoma and Mastoid surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ,1989;9-12

4 Aimi K. The tympanic isthmus: its anatomy and clinical significance. Laryngoscope 1978;7:1067-1081

5 Albiin N, Hellstrom S, Stenfors LE et al. Middle ear mucosa in rats and humans. Ann Otol Rhinol Laryngol 1986; suppl 125:2-15

6 Albrecht W. Zur Pathogenese des Mittelohr Cholesteatoms. Acta Otolaryngol (Stockh) 1931;15:375-379

7 Andreasson L, Ingelstedt S, Ivarsson A et al. Pressure dependent variation in volume of mucosal lining of the middle ear. Acta Otolaryngol(Stockh) 1976;81:442-449

8 Anniko M, Mendel L. Cholesteatoma. Acta Otolaryngol(Stockh) 1981;91:275-283

9 Ars B. The lamina propria and cholesteatoma. Clin Otolaryng 1989;14:471-475

10 Auerbach O, Petrick TG, Stout AP et al. The anatomical approach to the study of smoking and bronchogenic carcinoma. Cancer 1956;9:76

11 Balin AK, Goodman DB, Rasmussen H et al. Atmospheric stability in cell culture vessels. In Vitro 1976;12:687-692

12 Balin AK, Goodman DBP, Rasmussen H et al. The effect of oxygen on the growth and metabolism of WI-38 cells. J Cell Physiol 1976;89:235-250

13 Banks-Schlegel S, Green H. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J Cell Biolog 1981;90:732-737

14 Banoczy J. Keratinization in explanted oral mucosal flaps of rabbits. Arch Oral Biol 1971;16:1289-1294

15 Barfoed C, Sorensen KM, Rosborg J. Secretory otitis-Cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;357-359

16 Baxter JD, Ling D. Ear disease and hearing loss among the Eskimo population of the Baffin Zone. Canad L Otolaryngol 1974;3:110.

17 Beal DD. Prevention of otitis media in the Alaskan native. In: Glorig A, Gerwin KS eds. Otitis Media: Proc Nat Conf, Callier Hearing and Speech Center Dallas. Springfield, Thomas CC. 1972:158

18 Van der Bek JMH. Experimental obstruction of the Eustachian tube. Thesis, 1984, Nijmegen.

19 Bendek A. Histopathology of transudatory-secretory otitis media. Arch Otolaryng;78:33-38

20 Bezold F. Cholesteatoma perforation der membrana flaccida ShrapnelliZeitschr Ohrenheilk 1890;20:5-23

21 Bezold F. Uber das Cholesteatoms des Mittelohres.Zeitschr Ohrenheilk 1891;21:252-264

22 Bernstein JM, Hayes ER. Middle ear mucosa in health and disease. Arch Otolaryng 1971;94:30-35

23 Bijlsma PJ, Liauw L, Bakaletz et al. Effect of vitamin A on the growth and differentiation of rat tubotympanic and auditory canal epithelium in organ culture. Pers Communic.

24 Birrel JF. Cholesteatosis of the attic. J Laryngol 1958;72:620- 625

25 Blitterswijk van CA, Ponec M, v Muijen GNP et al. Culture and characterization of rat middle ear epithelium. Acta Otolaryngol(Stockh) 1986;101:453-466
26 Blitterswijk van CA, Hesseling SC, Grote JJ. The effect of calcium on terminal differentiation, proliferation and morphology of cholesteatoma matrix, meatal epidermis and middle ear epithelium. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;77-81
27 Bluestone CD, Casselbrant ML, Cantekin AI. Functional obstruction of the Eustachian tube in the pathogenesis of aural cholesteatoma in children. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;211-224
28 Bodelet B. Wayoff M. Métaplasie et Cholesteatome. Ann Otolaryngol 1972;89:411-41
29 Boedts D. The tympanic epithelium in normal and pathological condition. Acta Oto Rhino Laryngol(Belgica) 1978;32:299-419
30 Brackertz M, Kubbies M, Feige A et al. Decreased oxygen supply enhances growth in culture of human mid-trimester amniotic fluid cells. Hum Gent 1983;64:334-338.
31 Bradley TR, Hodgson GS, Rosendaal M. The effect of oxygen on haemopoietic and fibroblast cell proliferation in vitro. J Cell Physiol 1978;97:517-522
32 Bremond G, Magnan J. Evidence for the migration theory: Ultrastructural cell types in the epithelial layer of cholesteatoma. In:McGabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977:187-193
33 Bretlau P, Sorensen CH, Jorgensen B. Experimental studies of cholesteatomas. Acta Otolaryngol(Stockh) 1981;91:575-584
34 Broekaert D, Cooreman K, Coucke P et al. Keratinization of aural cholesteatoma (Keratoma) In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;161-173
35 Broekaert D, v Oostveldt P, Goucke P. Differentiation and keratinization in middle ear cholesteatoma. Acta Otolaryngol(Stockh) 1987;105:90-99
36 Broekaert D et al. Keratinization of middle ear cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma an Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;85-92
37 Broekaert D, Cornille A, Eto H et al. A comparative immunohistochemical study of cytokeratin and vimentin expression in middle ear mucosa and cholesteatoma, and in epidermis. Virch Arch Pathol Anat 1988;413:39-51
38 Brosemer RW, Rutter WJ. The effect of oxygen tension on the growth and metabolism of a mammalian cell. Experim Cell Res 1961;25:101-113
39 Buch Nh, Jorgensen MB. Eustachian tube and middle ear embryology and pathology. Arch Otolaryngol 1964;79:472-480
40 Buckingham RA, Ferrer JL. Etiology of middle ear cholesteatoma. Ann Otol Rhinol Laryngol 1968;77:1054-1057
41 Buckingham RA, Ferrer JL. Middle ear pressure in Eustachian tube malfunction: manometric studies. Laryngoscope 1973;83:1585-1593
42 Buckingham RA. Cholesteatoma and chronic otitis media following middle ear intubation. Laryngoscope 1981;91:1450-1456

43 Buckingham RA. The clinical appearance and natural history of cholesteatoma. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;13-21
44 Buckingham RA, Stuart DR, Geick MR et al. Experimental evidence against middle ear oxygen absorption. Laryngoscope 1985;95:437-442
45 Buckingham RA. Patent Eustachian tube in the underaerated middle ear: a paradox. Ann Otol Rhinol Laryngol 1988;97:219-221
46 Buckingham RA, Ferrer JL. Observations of middle ear pressures. Ann Otol Rhinol Laryngol 1980; 89 suppl 68:56-61
47 Buxmann MM. Keratin cross-linking and epidermal transglutaminase. J Invest Dermat 1985;65:107-112
48 Bylander AK, Ivarsson A, Tjernstrom O et al. Middle ear pressure variations during 24 hours in children. Ann Otol Rhinol Laryngol 1985;94 suppl120:33-35
49 Cantekin EI, Bluestone CD. Membrane ventilating tube for the middle ear. Ann Otolo Rhinol Laryngol 1976;85 suppl25:270-276
50 Cantekin EI, Doyle WJ, Phillips DC et al. Gas absorption in the middle ear. Ann Otol Rhinol laryngol 1980;89:suppl 68:71-75
51 Van Cauwenberghe PB et al. Relationship between secretory otitis media and cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma an Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;319-324
52 Cawthorne T. Congenital cholesteatoma. Arch Otolaryngol 1963;78:248-252.
53 Chalton RA, Stearns MP. The incidence of bilateral chronic otitis media. J Laryngol Otolog 1984;98:337-340
54 Chan KH, et al. Comparison of eustachian tube function tests between children with cholesteatoma/retraction pockets and those with chronic otitis media with efffusion. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma an Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;485-487
55 Chao WY, Sugita T, Huang CC. Fibronectin in middle ear cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;129-134
56 Chao WY, Huang CC. Expression of involucrin in human middle ear cholesteatoma. Am J Otolog 1989;10:385-389
57 Chao WY, Huang CC. Localization of fillagrin in human middle ear cholesteatoma. Acta Otolaryngol(Stockh) 1989;107:249-253
58 Chole RA. Squamous metaplasia of the middle ear mucosa during vitamin A deprivation. Otolaryngol Head Neck Surg 1979;87:837-844
59 Chole RA, Henry KR, McGinn MD. The gerbilline cholesteatoma: an experimental model. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam Kugler Publ, 1982;291-296
60 Chole RA, Frush DP. Quantitative studies of Eustachian tube epithelium during experimental vitamin A deprivation and reversal. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;321-327
61 Chole RA, Tonling SP. Basal lamina breaks in the histogenesis of cholesteatoma. Laryngoscope 1985;95:270-275
62 Chole RA, Kodama K. Comparative histology of the tympanic membrane and its relation to cholesteatoma. Ann Otol Rhinol Laryngol 1989;98: 761-766

63 Cody DTR. The definition of cholesteatoma. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;6-9
64 Cody TR, Taylor WF. Mastoidectomy for acquired cholesteatoma. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma.Birmingham, Alabama: Aesculapius Publishung Co, 1977;337-351
65 Cohen D. Locations of primary cholesteatoma. Am J Otolog 1987;8:61-65
66 Cohen D, Tamir D. The prevalence of middle ear pathologies in Jerusalem school children. Am J Otolog 1989;10:456-459
67 Cox A. The dermal-epidermal junction in psoriasis. J Invest Dermat 1969;53:428-435
68 Cruveiller J. Anatomie pathologique du corps humain. Paris 1829;1:341
69 Cruz De La, Linthicum FH, Luxford WM. Congenital atresia of the external auditory canal. Laryngoscope 1985;95:421-427
70 Deguine C, Deguine O. The contralateral ear in cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;393-398
71 Derlacki EL. Congenital cholesteatoma of the middle ear and mastoid. Arch Otolaryngol 1973;97:177-182.
72 Derlacki EL. Etiological aspects of cholesteatoma. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;208-211
73 Drucker I, Weismann Z, Sadé J. Tissue culture of human adult adenoids and of middle ear mucosa. Ann Otol Rhinol Laryngol 1976;85:327-333
74 Edelstein DR, Parisier SC, Ahuja GS et al. Cholesteatoma in the pediatric age group. Ann Otol Rhinol Laryngol 1988;97:23-29
75 Eggston AA, Wolfe D. Histopathology of the Ear, Nose and Throat. Williams and Wilkins Co. Baltimore, 1947.
76 Eigler G. Das Mittelohr Cholesteatom als Ontogenetisch Problem. Arch Ohr Nase Kehlkopf Heilk 1950;156:557-559
77 Eigler G. Das Verhalten der hinteren oberen Gehörgangs Epidermis beim randstandigen Perforationen Hals Nase Ohr Wegweiser Berlin 1951;2:281-283
78 Elner A, Ingelstedt S, Ivarsson A. The normal functioning of the Eustachian tube. Acta Otolaryngol(Stockh) 1971;72:320-328
79 Elner A, Ingelstedt S, Ivarsson A. A method for studies of the middle ear mechanics. Acta Otolaryngol(Stockh) 1971;72;191
80 Elner A.Normal gas exchange in the human middle ear. Ann Otol Rhinol Laryngol 1976;85 suppl 25:161-164
81 Estancona NG, Muniz L. Pathogenic factors in cholesteatoma In: Tos m, Thomsen J, Peitersen,eds. Cholesteatoma and Mastoid surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;19-23
82 Falk B. Tubal dysfunction in patients with cleft palate. In: Sadé J, ed. Cholesteatoma and Mastoïd surgery. A'dam : Kugler Publ, 1982; 229-233
83 Falk B, Magnuson B. Eustachian tube closing failure. Occurrence in retraction type cholesteatoma. In:Tos M, Thomson J,Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;477-480

84 Felding JU, Lildholdt T. Direct measurement of middle ear gas composition. In:Lim DJ, Bluestone CD, Klein JO, Nelson eds.Recent advances in otitis media. Proceedings of the fourth international symposium. Decker ,Toronto 1988 abstr.30
85 Felding JU, Rasmussen JB, Lildholt T. Gas composition of the normal and the ventilated middle ear cavity. Scand J Clin Lab Invest 1987;47 suppl 186:31-41
86 Fernandez F. Some observations on the pathogenesis of middle ear cholesteatoma. Arch Otolaryng 1959;69:537-542
87 Fernandez C. Aural cholesteatoma experimental observations. Laryngoscope 1960;1959 69;537-547
88 Freedberg IM. Epidermal differentiation and keratinization. In: McGabe BF,Sadé J, Abramson M, eds. Cholesteatoma. Birmingham,Alabama: Aecsulapius Publishing Co,1977;38-44
89 Friedmann I. The comparative pathology of otitis media experimental and human. J Laryngol Otolog 1955;69:588-601
90 Friedmann I. Epidermoid cholesteatoma and cholesterol granuloma. Ann Otol Rhinol Laryng 1959;68:57-59
91 Friedmann I. The pathology of secretory otits media. Proc Roy Soc Med 1963;56:695-699
92 Friedmann I. The pathology of epidermoid cholesteatoma. In: McGabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;10-23
93 Fujita A, et al. The etiological role of the retraction pocket in cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 421-424.
94 Galic M, Neumann H et al. The formation of ultrastructural epithelial and subepithelial connective tissue changes in experimentally induced cholesteatomas in guinea pigs. Arch Oto Rhino Laryngol 1985;241:267-270
95 Gantz BJ. Epidermal Langerhans Cells in Cholesteatoma. Ann Otol Rhinol Laryngol 1984;93:150-156
96 Gantz BJ. Immunobiologic aspects of acquired cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;171-174
97 Gigi-Leitner O. Cytokeratin expression in squamous metaplasia of the human uterine cervix. Diff 1986;31:191-205
98 Gardner E, Gray DJ, O'Rahilly. Anatomy. Philadelphia, Toronto, London, Saunders WB Co, 1969.
99 Green H. Terminal differentiation of cultured human epidermal cells. Cell 1977;11:405-416
100 Green H. The keratinocyte as differentiated cell type. Harvey Lect Series 1979;74:101-138
101 Greenberg SD, Dickey JR, Neely JG. Effects of tobacco on the ear. Ann Otol Rhinol Laryngol 1973;82:311-317
102 Griffith TE. Epidemiology of otitis media: an interracial study. Laryngoscope 1979;89:22-30
103 Gristwood RE. Review; Chronic otitis media with epidermoid cholesteatoma. Clin Otolaryngol 1976;1:337-342
104 Grontved A, Moller A, Jorgensen L. Studies on gas tension in the normal middle ear. Acta Otolaryngol(Stockh) 1990;109:271-277

105 Grote JJ, van Blitterswijk, Bakker DJ. Tympanic membrane structure during a Staph. Aureus infection of the middle ear. Acta Otolaryngol(Stockh) 1989;107:225-234
106 Habermann. Zur Entstehung Cholesteatoms des Mittelohrs. Arch Ohrenheilk 1888;27:42-50
107 Harell M, Pennington FR, Morrison WV. Prevalence of cholesteatoma in black americains. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam: Kugler Publ, 1982;97-99
108 Harker LA. Cholesteatoma an incidence study. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;308-309
109 Harker LA, Severeid LR. Cholesteatoma in the cleft palate patient. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;37-40
110 Harris CC, Sporn MB, Kaufman DG et al. Histogenesis of squamous metaplasia in the hamster tracheal epithelium caused by vitamin A deficiency or benzo[a]pyrene ferric oxide. J Nat Canc Inst 1972;48:743-747
111 Harris J. Cholesteatosis and chronic otitis media Laryngoscope. 1962;72:954
112 Hauser R, Münker G. Tubal closing failure and its possible significance in the development of cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;465-469
113 Held KR, Sonnichsen S. The effect of oxygen tension on colony formation and cell proliferation of amniotic fluid cells in vitro. Prenat Diag 1984;4:171-179
114 Hellmann K. Studien über das sekundare Cholesteatom des Felsenbeins. Z Hals Nase Ohrenheilk 1925;11:406-421
115 Hentzer E. Ultrastructure of the normal mucosa in the human middle ear, mastoid cavities and Eustachian tube. Ann Otol Rhinol Laryngol 1970;79:1143-1157
116 Hentzer E. Ultrastructure of the middle ear mucosa in secretory otitis media. Acta Otolaryngol(Stockh) 1972 73;394-401
117 Hergils L, Magnuson B. Morning pressure in the middle ear. Arch Otolaryngol 1985;111:86-89
118 Hergils L, Magnuson B. On middle ear pressure regulation. Proc Int Conf on acute and secretory otitis media. A' dam Kugler public 1985;93-98
119 Hergils L. Middle ear pressure under basal conditions. Arch Otolaryngol Head Neck Surg 1987;113:829-832
120 Hergils L, Magnuson B. Regulation of negative middle ear pressure without tubal opening. Arch Otolaryngol Head Neck Surg 1988;114:1442-1444
121 Hilding AC. Experimental surgery of the nose and sinus change in the morphology of the epithelium following variations in ventilation. Arch Otolaryngol 1932;16:9
122 Holmes E. A review of three hundred and three cases of cholesteatoma. Ann Otol Rhinol Laryng 1938;47:136-143
123 Holmquist J, Lindeman P. Tympanometric studies in ears with cholesteatoma and retraction pockets. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;225-228
124 Holmquist J, Lindeman P. Eustachian tube function in ears with cholesteatoma. Am J Otolog 1988;9:363-365

125 Holmquist J. Eustachian tube function in cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;459-463
126 Horikoshi T, Bakin AK, Carter M. Effect of oxygen on the growth of human epidermal keratinocytes. J Invest Dermat 1986;86:424-427
127 Honda Y. Relation between middle ear cholesteatoma and secretory otitis media. In: Tos M, Thomsen J, Peitersen,eds. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;3-7
128 Honda Y et al. Experimental cholesteatoma. In:Tos M, Thomsen J, Peitersen E, eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 33-36
129 Honjo I, et al. Eustachian tube function in patients with cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;455-458
130 Hörmann K. Cholesteatoma and eustachian tube dysfunction. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;515-519
131 House JW. Cholesteatoma with intact tympanic membrane; a case report of 41 cases. Laryngoscope 1980;90:70-76
132 House HP. An apparent primary cholesteatoma; case report. Laryngoscope 1953;63:712-713
133 Huang CC, Yi ZX, Chao WY. Effects of granulation tissue conditioned medium on the in vitro differentiation of keratinocytes. Arch Otol Rhinol Laryngol 1988;245:325-329
134 Huang CC, Yi ZX. Cholesteatoma and granulation tissue. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;215-222
135 Huszar M, Gigi-Leitner O, Mol R et al. Monoclonal antibodies to various acidic(type1) cytokeratins of stratified epithelia.Diff 1986;31:141-153
136 Ingelstedt S, Jonson B. Mechanisms of the gas exchange in the normal human middle ear. Acta Otolaryngol(Stockh) 1966 suppl 224:452-461
137 Ingelstedt S, Jonson B, Rundcrantz H. Gas tension and pH in middle ear effusion. Ann Otol 1975;84:198-201
138 Jahnke V. Clinical, pathological and therapeutic aspects of cholesteatoma in children. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;25-27
139 Johnson A, Hawke M. Cell shape in the migratory epidermis of the external auditory canal. J Otolaryngol 1985;14:273-281
140 Johnson RL. Chronic otitis media in school age Navajo indians. Laryngoscope 1967;77:1990-1995
141 Jove MA. Vassali L, Raslan W et al. The effect of isoretinoin on propylene glycol-induced cholesteatoma in chinchilla middle ears. Am J Otolaryngol 1990;11:5-9.
142 Jung TTK et al. Arachidonic acid metabolites in human and experimental cholesteatoma. In:Tos M, Thomsen J, Peitersen E, eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley Milano: Kugler&Ghedini Publ, 1989; 53-58
143 Karma P Middle ear epithelium and chronic ear disease. Acta Otolaryng (Stockh) 1972 suppl 307.

144 Karma PH et al. Occurrence of cholesteatoma and secretory otitis media in children. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma an Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 335-338.
145 Kavanagh KT, Giltman LI, Babin RW. Erosive mucosal cysts of the temporal bone. Am J Otol 1986;7:270-274
146 Kawabata I, Paparella MM. Ultrastructure of normal human middle ear mucosa. Ann Otol Rhinol Laryngol 1969;78:125-130
147 Kokko E. Chronic secretory otitis media in children. Acta Otolaryngol(Stockh) 1974;suppl 327:7
148 Kuhn. Zum Cholesteatomfrage. Arch Ohrenheilk 1888;26:63-76
149 Küpper. Arch Ohrenheilk 1876;11:18-19
150 Küster A. Discussion. Berliner Klinischenwochenschr. 1889;10:213-214
151 Kuypers W, vd Beek JMH, Willart ECT. The effect of experimental tubal obstruction on the middle ear. Acta Otolaryngol(Stockh) 1979;87:345-352.
152 Lacher G. Aspects of pre-cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;413-417
153 Lange W. Uber die Entstehung der Mittelohr Cholesteatoma. Zeitschr Hals Nase Ohernheilk 1925;11:250-271
154 Larsen PL, Tos M. Tympanic membrane sequelae after recurrent acute otitis media. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 365-371
155 Levenson MJ, Michaels L, Parisier SC et al. Congenital cholesteatoma in children: an embryologic correlation. Laryngoscope 1988;98:949-955
156 Lim DJ, Hussl B. Human middle ear epithelium. An ultrastructural and cytochemical study. Arch Otolaryngol 1969;89:835-849
157 Lim D. Human tympanic membrane. Acta Otolaryngol(Stockh) 1970; 70:176-186
158 Lim DJ, Shimada T. Secretory activity of normal middle ear epithelium. Scanning and transmission electron microscopic observations. Ann Otol Rhinol Laryngol 1970;80:319-329
159 Lim DJ, Saunders WH. Acquired Cholesteatoma. Ann Otol Rhinol Laryngol 1972;81:2-11
160 Lim DJ, Birck HG, Saunders WJ. Aural cholesteatoma and epidermization: a fine morphological study. In:McGabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;233-252
161 Lim DJ. Normal and pathological mucosa of the middle ear and Eustachian tube. Clin Otolaryngol 1979;4:213-234.
162 Lim DJ. Epithelial invasion, contact guidance, and contact inhibition. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;141-152.
163 Lucae A. Beitrage zur Kenntniss de Perlgeschwülst des Felsenbeins. Arch Ohrenheilk 1873;7:255-279
164 Luntz M, Eisman S, Sadé J Diurnal middle ear pressure fluctuations in atelectatic ears. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;507-510
165 MacKinnon DM. The sequel to myringotomy for exudative otitis media. J Laryngol 1971;85:773.
166 Magnan J, Bremond GA. Adhesive otitis and cholesteatoma. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982; 67-69

167 Magnuson B. Tubal closing failure in retraction type cholesteatoma and adhesive middle ear lesions Acta Otolaryngol(Stockh) 1978;86:408-417
168 Magnuson B, Hergils L. Presssure regulation in healthy and diseased ears. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;471-475
169 Makino M, Amatsu M. Epithelial migration in retraction pocket and cholesteatoma. In: Tos m, Thomsen J, Peitersen,eds. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989; 313-316
170 Manasse P. Das Cholesteatom des Mittelohres, bzw des Felsenbeines. In; Handbuch der pathologischen Anatomie des menslichen Ohres. Bergmann JF, Wiesbaden, 1917;64
171 Marquet J. Cholesteatoma or Keratoma. Acta Oto Rhino Laryngol(Belgica) 1980;34:5-11
172 Marquet J. My current cholesteatoma techniques. Am J Otolog 1989;10:124-130
173 Masaki M et al. Longitudinal stduy of the tympanic membrane in experimental cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 65-69
174 Matsumura H. Studies on the gas composition of air in the tympanic cavity. Arch Otolaryngol 1955;61:220-222
175 Mayot D. et al. Immunological characteristics of human cholesteatoma matrix. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;181-182
176 McCafferty GJ, Coman WB. Cholesteatoma in Australian Aboriginal children.In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977; 293-301
177 McCandless GA, Thomas GK. Impedance audiometry as a screening procedure for middle ear disease. Trans Am Acad Ophtalmol Otolaryngol 1974;78:98-102
178 McKenzie D. The pathogeny of aural cholesteatoma. J Laryng Rhinol Otol 1931;46:163-190
179 McLoughlin SB. Interaction of epidermis with various types of foreign mesenchym. In: Fleischmayer CR, Billingham RE eds. Epithelial Mesenchymal Interactions. Baltimore:Williams and Wilkins, 1968;244-251
180 Melvill Jones G. Pressure changes in the middle ear after altering the composition of contained gas. Acta Otolaryngol(Stockh) 1961;53:1-11
181 Mertens J, Schwenk B, Rudert H. The value of secretory otitis media treatment for diagnosis and prevention of cholesteatoma.In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;339-341
182 Meyer M. Uber Konstitution und Mittelohr Schleimhaut. Zeitschr Hals Nase Ohrenh 1931;29:106-113
183 Michaels L. The metaplastic process in epithelial strata. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982; 299-303
184 Michaels L. An epidermoid formation in the developing middle ear: possible source of cholesteatoma. J Otolaryngol 1986;15:169-174
185 Michel S, Schmidt R, Shroot B et al. Morphological and biochemical characterization of the cornified envelopes from human epidermal keratinocytes of different origin. J Invest Dermatol 1988;91:11-15

186 Mogi G, Ichimiya I, Kawauchi H. Immunology of cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;1653-155
187 Mohri M, Makino K, Amatsu M. Relationship between cholesteatoma and upper respiratory tract infection. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;343-348
188 Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors, and cultured cells. Cell 1982;31:11-24
189 Moller P et al. Tympanic membrane changes and retraction pockets after secretory otitis media. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;351-355
190 Morgenstern C. Oxygen supply of middle ear mucosa under normal conditions and after Eustachian tube occlusion. Ann Otol Rhinol Laryngol 1980;89 suppl68:76-77
191 Moscona AA. In: Montagna, Lobitz WC eds. The Epidermis. Acad Press. 1964:83-96
192 Müller J. Uber den feinen Bau und die Formen der Krankhaften Geschwülste. Berlin Wochen Zeitschr 1838;1:50
193 Muijen van GNP, Warnaar SE, Ponec M. Differentiation related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn and adult epidermis. Exp Cell Res 1987;171:331-345
194 Van Muijen GNP, Ruiter DJ, Franke WW et al. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. Exp Cell Res 1986;162:97-113
195 Nager FR. The cholesteatoma of the middle ear: its etiology, pathogenesis, diagnosis and therapy. Ann Otol Rhinol Laryngol 1925;34:34
196 Nager GT. Cholesteatoma of the middle ear: Pathogenesis and surgical indication. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;193-204.
197 Nager GT. Cholesteatoma of the middle ear: Pathogenesis and surgical indication. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;193-204.
198 Nagle RB, Moll R, Weidauer H et al. Different patterns of cytokeratin expression in the normal epithelia of the upper respiratory tract. Different 1985;30:130-140
199 Nakamura H, et al. Cholesteatoma in view of the contralateral ear. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;389-392
200 Oates J, Bennet RJ. The persistent sequelae of otitis media with effusion. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 316-364.
201 O'Donoghue GM. The migration theory of cholesteatoma formation. J Laryngol Otolog 1986;100:395-398
202 Okazaki N, DeMaria TF, Briggs BR et al. Experimental otitis media with effusion induced by nonviable Hemophilus influenzae. Am J Otolaryngol 1984;5:80-92
203 Ostfeld E. Middle ear gas composition. Ann Otol Rhinol Laryngol 1985;94 suppl120:32-33

204 Ottaviani F. et al. Middle ear cholesteatoma. A cytodiagnostic study of cellular proliferative activity. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;119-124
205 Palva T, Palva A, Dammaert K. Middle ear mucosa and chronic ear disease. Arch Otolaryng 1968;87:21-29.
206 Palva T, Karma P, Karja J. Cholesteatoma in children. 1977 Arch Otolaryngol 1977;103:74-77
207 Palva t, Thesleff I, Saxen L. Organ cultures on human skin and cholesteatoma epithelium. Acta Otolaryngol(Stockh) 1978;85:307-312
208 Palva T, Karma P, Makinen J. The invasion theory. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;249-264
209 Palvat T, Makinen J. Why does middle ear cholesteatoma recurr. Arch Otolaryngol 1983;109:513-518
210 Palva T, Johnson LG. Findings in a pair of temporal bones from a patient with secretory otitis media and chronic middle ear infection. Acta Otolaryngol(Stockh) 1984;98:208-220
211 Palva T, Lehto VP, Virtanen I et al. Junctions of squamous epithelium with middle ear mucosa. Acta Otolaryngol (Stockh) 1985;99:297-304
212 Palva T, Taskinen E. Inflammatory cell subpopulations in chronic otitis media. Arch Otolaryngol Head Neck Surg 1987;113:149-154
213 Palva T. Surgery related to the histopathology in chronic inflammatory middle ear disease. J Laryngol Otolog 1988;102:851-856
214 Paparella MM, Meyerhoff WL. Clinical significance of granulation tissue in chronic otitis media. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam Kugler Publ,1982;387-395
215 Paparella MM, Yoon TH, Schachern PA. Pathology and pathogenesis of retraction pockets and cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;401-411
216 Parisier Sc, Levenson MJ, Edelstein DR et al. Management of congenital pediatric cholesteatoma. Am J Otolog 1989;10:.
217 Parker Cross J. Otomastoid cholesteatomas with intact eardrum. Laryngoscope 1981;91:1145-1149.
218 Parker DA, Reid AP, Patel PJ et al. Localized middle ear effusions: three unusual cases in adults. J laryngol Otolog 1989;103:83-85
219 Passali D, Belussi L. The importance of B and T lymphocytes and langerhans cells in relapsing and non-relapsing cholesteatomas. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;165-170
220 Pfaltz CR. Pathogenesis of cholesteatoma in children. In: Tos M, Thomsen J, Peitersen,des. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;635-637
221 Podoshin L, Fradis M, Ben-David Y et al. Cholesteatoma: an epidemiologic study among members of kibbutzim in northern Israel. Ann Otol Rhinol Laryngol 1986;95:365-368
222 Malmquist KG, Carlsson et al. Proton and electron microprobe analysis of human skin. Nucl Instr Meth Physic Res 1984;B3:611-617

223 Proops DW, Parkinson EK. Tissue culture of human cholesteatomatous keratinocytes. Clin Otolaryngol 1983;8:165-170
224 Proops DW, Hawke M, Blight A. The morphology of keratinocyte colonies derived from normal skin, migratory skin and cholesteatoma. In: Tos M, Thomsen J, Peitersen,des. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;289-292
225 Proud GO. Chronic otitis media and cholesteatoma. Ann Otol Rhinol Laryngol 1980 suppl 74:47-49
226 Radulovic R, Miroslav M. Some remarks on the pathogenesis of cholesteatoma. In:Tos M, Thomsen J, Peitersen, eds. Cholesteatoma and Mastoid surgery. A'dam, Berkeley, Milano: Kugler& Ghedini Publ, 1989;25-29
227 Ralli G, Magliulo G. The cholesteatoma behind an intact tympanic membrane. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 557-560
228 Rauchfuss A. Ultrastructural characteristics of mesenchyme in the middle ear. Arch Otorhinolaryngol 1986;243:162-166
229 Rauchfuss A, Hartwein J. Pathohistology of cholesteatoma and surgical consequences in childhood. In: Tos M, Thomsen J, Peitersen,des. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;639-642
230 Reimer A, Huberman K, Klementsson K et al. The mucociliary activity of the respiratory tract. Acta Otolaryngol(Stockh) 1981;91:139-148
231 Reimer A. The effect of carbon dioxide on the activity of cilia. Acta Otolaryngol(Stockh) 1987;103:156-160
232 Rhodin JA. Histology. London, Oxford University Press 1974;618
233 Rice RH, Green H. Relation of protein synthesis and transglutaminase activity to formation of the cross-linked envelope during terminal differentation of the cultured human epidermal keratinocyte. J Cell Biolog 1978;76:705-711
234 Rice RH, Green H. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. Cell 1979;18:681-694
235 Rigner P, Renvall U, Tjellstrom A. The incidence of cholesteatoma in children over a ten year period. In: Tos M, Thomsen J, Peitersen,des. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;623-625
236 Riu R, Flottes J, Bouche J, LeDeu R. La physiologie de la trompe d'Eustache. Paris:Librairie Arnette, 1966:153-156
237 Rosen G. Posterior superior retraction pockets *vs* prussacks retraction pockets as a cause of cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;425-437
238 Rüedi L. Acquired Cholesteatoma. Arch Otolaryng 1963;78:44-261
239 Rüedi L. Cholesteatoma formation in the middle ear in animal experiments. Acta Otolaryngol(Stockh)1059;50:233-242
240 Rüedi L. Pathogenesis and surgical treatment of the middle ear cholesteatoma Acta Otolaryngol(Stockh) 1979 Suppl 361
241 Ruben RJ. The disease in society: evaluation of chronic otitis media in general and cholesteatoma in particular. In: Sadé J,ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;111-116

242 Ruben RJ, et al. Efficacy of early diagnosis of primary cholesteatoma in infants. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989; 545-548.
243 Sadé J. Middle ear mucosa. Arch Otolaryngol 1966;84:137-143
244 Sadé J, Weinberger J.Mucus production in the chronically infected ear: A histological and histochemical study. Ann Otol Rhinol Laryngol 1969;87:148-155
245 Sadé J. Cellular differentiation of the middle ear lining. Ann Otol Rhinol Laryngol 1970;80:376-383
246 Sadé J. Prologue. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;1-3
247 Sadé J. Pathogenesis of attic cholesteatoma: The metaplasia theory. In: McGabe,BF, Sadé J, Abramson M, eds. Cholesteatoma.Birmingham, Alabama: Aesculapius Publishing Co, 1977;212-223
248 Sade J, Weisman Z. The phenotypic expression of middle ear mucosa. In: McGabe,BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;52-61
249 Sadé J. Retraction pockets and attic cholesteatoma. Acta Oto Rhino Laryngol (Belgica) 1980;34:62-84
250 Sadé J, Teitz A. Cholesterol in cholesteatoma and in the otitis media syndrome. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;125-132
251 Sadé J, Avraham S, Brown M. Dynamics of atelectasis and retraction pockets. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;267-281
252 Sadé J, Babyatzki A, Pinkus G. The metaplastic and congenital origin of cholesteatoma. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;305-319.
253 Sadé J, et al. Data concerning aural cholesteatoma and retraction pockets. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;7-11
254 Sade J. Treatment of cholesteatoma. Am J Otolog 1987;8:524-533
255 Sade J, Luntz M. Modified radical mastoidectomies as gas pockets. Acta Otolaryngol(Stockh) 1989;107:456-459
256 Sancha Fernandez JM, Yarnoz Y. Study on the mesenchymal clearance factor and its importance in the middle ear pneumatization process in rat and in man.Acta Otolaryngol(Stockh) 1981;91:557-565
257 Sanna M, Zini C. Congenital cholesteatoma of the middle ear. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;29-36
258 Sanna M, Zinni C. Congenital cholesteatoma of the middle ear. Am J Otolog 1984;5:368-373.
259 Schaper J, vd Heyning J. Cholesteatoma of the middle ear in human patients. Arch Otolaryngol 1976;102:663-668
260 Schaper J, vd Heyning J. Cholesteatoma of the middle ear. An ultrastructural study. Acta Oto Rhino Laryng(Belgica) 1980;34:12-15
261 Schlegel R, Banks S, Pinkus GS. Immunohistochemical localization of keratin in normal human tissues. Lab Invest 1980;42:91-96
262 Schroer R. Entwicklungsgeschichte und experimentelle Untersuchungen zur Genese des Mittelohrcholesteatoms. Arch Ohren Nasen Halsheilk 1957;170:265..

263 Schwarz M. Die Entwicklung des Cholesteatoms im Mittelohr ab statu nascendi. Hals Nase Ohrenheilk 1985;33:180-182
264 Schuknecht HF. Pathology of the ear. Harvard University Press, Cambridge, Massachusets. 1974
265 Severeid LR. Development of cholesteatoma in children with cleft palate: a longitudinal study. In: McGabe,BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;287-289
266 Shatz A, Sadé J. Cholesteatoma in children. In: Tos M, Thomsen J, Peitersen,eds. Cholesteatoma and Mastoid surgery.A'dam, Berkeley, Milano:Kugler&Ghedini Publ, 1989;627-633
267 Sheehy JL, Brackmann DE, Graham MD. Cholesteatoma surgery: Recurrent and residual disease. Ann Otol Rhinol Laryngol 1977;86:451-463
268 Shiboru M, Mamoru S, Hozawa K, Takasaka T et al. Ultrastructural pathology of the middle ear mucosa in OME. Acta Otolaryngol(Stockh) 1987; suppl 435:90-99
269 Shimada T, Lim DJ. Distribution of ciliated cells in the human middle ear. Electron and light microscopic observations. Ann Otol Rhinol Laryngol 1972;81:203-211
270 Shinkawa H, Okitsu T, Yusa T et al. Positive intratympanic pressure in the morning and its etiology. Acta Otolaryngol(Stockh) 1987;suppl435:107-111
271 Shinkawa H, Sasaki Y, Inamura N et al. Middle ear pressure and gas diffusion. In:Lim DJ, Bluestone CD, Klein JO, Nelson eds. Recent advances in otitis media. proceedings of the fourth international symposium. Decker ,Toronto 1988 abstr.29
272 Shinkawa H, Ishidoya M, Okitsu T. Changes in tympanograms after middle ear inflation. Eur Arch Otorhinlaryngol 1990;247:125-128
273 Simon M, Green H. Participation of membrane associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell 36:827-834
274 Smyth GDL, Hall S. Aetiology and treatment of persistent middle ear effusion. J Laryngol Otolog 1983;97:1085-1089
275 Sobol S, Reichert TJ, Faw KD et al. Intramembranous and mesotympanic cholesteatoma associated with an intact tympanic membrane Ann Otol Rhinol Laryngol 1980;89:312-317.
276 Staiano-Coico L, Higgins PJ, Darzynkiewicz Z et al. Keratinocyte culture. J Clin Invest 1986;77:396-404
277 Steinbach E. Experimental production of cholesteatoma in rabbits by using non-irritants(skin tolerants). J Laryngol Otolog 1980;94:269-279
278 Steinbach E. Die Pathogenese des Cholesteatoms. Arch Otorhinolaryngol 1981;231:574-582
279 Steinbach E. Experimental studies on cholesteatoma formation. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;333-336
280 Steinert PM, Cantieri JS, Teller DC et al. Characterization of a class of cationic proteins that specifically interact with intermediate filaments. Proc Natl Acad Sci 1981;78:4097-4101
281 Steinert PM. Intermediate Filaments. Cell Biolog 1984;99:22-27
282 Stenfors LE et al. Experimental epitympanic retraction pockets. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;287-290

283 Stewart IA, et al. Attic and posterior marginal retractions in a random sample of 794 children. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;419-420
284 Sugita T, Huang CC, Abramson M. Effect of endotoxin on keratin production of keratinocytes in vitro. Am J Otolaryngol 1986;7:42-46
285 Sugita T, Huang CC, Abramson M. The effect of endotoxin and prostaglandin E_2 on the proliferation of keratinocytes. Arch Otorhinolaryngol 1986 243;211-214
286 Sugita T, Huang CC, Abramson M. The effect of fibronectin on the migration of keratinocytes. Acta Otolaryngol(Stockh) 1987;104:512-525
287 Sun TT, Eichner R, Nelson WG et al. Keratin Classes; Molecular markers for different types of epithelial differentiation. J Investig Dermat 1983;81:109-115
288 Sweeny PR, Farkas LG, Former AW. Metaplasia of adult oral mucous membrane. J Surg Res 1975;19:303-308
289 Taylor WG, Camalier RF. Modulation of epithelial cell proliferation in culture by dissolved oxygen. J Cellular Physiol 1982;111:21-27
290 Teed RW. Cholesteatoma verum Tympani. Arch Otolaryngol 1936;24:455-462
291 Thilo-Korner DGS, Heinrich D, Knorpp K. The influence of different gas atmospheres on the proliferation of human endothelial cells in the 12-multiwell culture dish. Drug Res 1984;34;740-741
292 Thorburn IB. The pathogenesis of chronic otitis media- a clinical study. Proc Roy Soc Med 1968;61:385-399
293 Tos M, Poulsen G. Attic retractions following secretory otitis. Acta Otolaryngol(Stockh) 1980;89:479-486
294 Tos M. Upon the relationship between secretory otitis in childhood and chronic otitis and its sequelae in adults. J Laryngol Otolog 1981;95:1011-1022
295 Tos M. Epidemiology and natural history of secretory otitis Am J Otol 1984;5:459-462
296 Tos M. Incidence, Etiology and pathogenesis of cholesteatoma in children. Adv Oto Rhino Laryngol 1988;40:110-117
297 Tos M et al. The relationship between secretory otitis and cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;325-330
298 Toynbee J. Pathological and surgical observations on the diseases of the ear. Medico-Chirurgical transactions, London, 1841;34:190-211
299 Tröltsch von A. Die anatomie des Ohres in ihrer anwendung auf die Praxis. Monatschr Ohrenheilk Laryngorhinol 1861;100:19
300 Trump BF, McDowell EM, Glavin F et al. The respiratory epithelium III. Histogenesis of epidermoid metaplasia and carcinoma in situ in the human. J Nat Canc Inst 1978;61;563-575
301 Tschopp CF. Chronic otitis media and cholesteatoma in Alaskan native children. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishung Co, 1977;290-292.
302 Tümarkin A. Middle ear suppuration and cholesteatoma. J Laryngol Otolog 1938;53:685-772
303 Tümarkin A. Attic Cholesteatosis. J Laryngol Otolog 1958;68:610
304 Tümarkin A. Pre Epidermosis. J otol Rhinol Laryng 1961;75:
305 Ulrich K. Zwei Beitrage zur genese des Mittelohr Cholesteatoms. Z Ohrenheilk 1917;75:159-165

306 Vandevelde L, Blondiau, Dourov N. Our opinion on the granulation tissue in cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;227-230
307 Vassali L. Am J Otolaryng 1988;8:1-5
308 Veldman JE. Immunology of cholesteatoma. Am J Otolog 1985;6:22-25
309 Veldman JE. The langerhans T-cell repertoire in cholesteatoma. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;157-163
310 Vennix et al. The cytokeratin expression in the normal rat middle ear and during induced OME. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;111-115
311 Virchow R. Ueber Perlgeschwülste. Virchow Archiv 1863;24:317-418
312 Watt FM, Green H. Stratification and terminal differentiation of cultured epidermal cells. Nature 1982;295:434-436
313 Watt FM, Jordan PW, O'Neill CH. Cell shape controls terminal differentiation of human epidermal keratinocytes. Cell Biolog 1988;85: 5576-5580.
314 Watt FM. Keratinocyte cultures: an experimental model for studying how proliferation and terminal differentiation are co-ordinated in epidermis. J Cell Science 1988;90:525-529
315 Weismann Z, Sadé J. Effect of environmental CO_2, O_2 and pH on the growth of respiratory epithelium in vitro. Ann Otol Rhinol Laryngol 1979;88:21-30
316 Wells MD, Michaels L. Role of retraction pockets in cholesteatoma formation. Clin Otolaryngol 1983;8:39-45
317 Wendt H. Desquamative Entzündung des Mittelohrs. Arch Heilk 1873;14:428-446
318 Wildt GA, Mischke D. Variation and frequency of cytokeratin polypeptide patterns in human squamous non-keratinizing epithelium. Exp Cell Res 1986;162:114-126
319 Wingrave VHW. Notes on the pathogeny of cholesteatoma. Proc R Soc Med 1918;3:63-70
320 Wolfman DE, Chole RA. Experimental retraction pocket cholesteatoma. Ann Otol Rhinol Laryngol 1986;95:639-644
321 Wright CG, Meyerhoff WL, Burns DK. Middle ear cholesteatoma: an animal model. Am J Otolog 1985;6:327-341
322 Wullstein HL, Wullstein S. Cholesteatoma, Etiology, Nosology and Tympanoplasty. Otol Rhinol Laryngol 1980;42:313-335
323 Yamashita T, Maeda N, Honda K et al. New findings of middle ear ventilation using xenon gas. In:Lim DJ, Bluestone CD, Klein JO, Nelson eds. Recent advances in otitis media. Proceedings of the fourth international symposium. Decker,Toronto 1988 abstr.32
324 Yamashita T. Histology of the tympanic membrane perforation and the replacement membrane. Acta otolaryngol(Stockh) 1985;100:66-71
325 Yamashita K. Tubal malfunction related to maxillo-palatal abnormalities. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;499-503.
326 Yee A, Cantekin EI. Effect of changes in systemic oxygen tension on middle ear gas exchange. Ann Otol Rhinol Laryngol 1986;95:369-372
327 Yong Chuan W, Buck RC. Langerhans cells in epidermoid metaplasia. J Invest Dermat 1971;56:10-17

328 Yoon Th, Paparella MM, Aeppli DM. Pathology and pathogenesis of tympanic membrane retraction. AM J Otolaryngol 1990;11:10-17
329 Young A. Closure of the nostrils in atrophic rhinitis. J Laryngol Otolog 1967;81:515..
330 Young G. Aural Cholesteatoma or cholesteatosis. A review. Proc R Soc Med 1950;43:75-85
331 Young R, Kwok P, Hawke M. Epidermal cyst attached to the chorda tympani nerve. J Otolaryngol 1988;17:211-213
332 Yung TTK, Juhn SK. Prostaglandins in human cholesteatoma and granulation tissue. Am J Otolog 1988;9:197-200
333 Zechner G. Histomorphologische und histologische untersuchungen an der Paukenhöhlenschleimhaut bei otitis media chronica adhesiva. Mntschr Ohrenh Laryng Rhinol 1965;99:53-74
334 Zechner G. Zur Pathofysiology der Mittelohr Schleimhaut. Mntschr Ohrenh Laryng Rhinol 1966;100:19-50
335 Zechner G. Stratified squamous epithelium in retraction pockets and attic cholesteatoma. In: Sadé J, ed. Cholesteatoma and Mastoid surgery. A'dam : Kugler Publ, 1982;119-124
336 Zechner G. Zur Entstehung de Erworbenes Mittelohr Cholesteatoms. 1985 Laryngol Rhinol Otol 1985;64:67-72
337 Zeroni. Ueber cholesteatom in einem Ohrpolypen. 1899 Arch Ohrenheilk XLII;188-206

CHAPTER III

THE EFFECT OF EXTERNAL STIMULI ON RAT MIDDLE EAR EPITHELIUM IN CULTURE

-EXTRACELLULAR CARBON DIOXIDE CONCENTRATION-

HW Lutgert, CA van Blitterswijk, JJ Grote.

E. N. T. Department, University Hospital Leiden, The Netherlands.

SUMMARY

Serially cultivated rat middle ear epithelium was studied under exposure to various concentrations of carbon dioxide. Three experiments were performed exposing the epithelium to a gaseous environment containing 5 or 10% CO_2 or to an atmosphere almost devoid of CO_2(0.03%). Oxygen was maintained constant at 20% and nitrogen was additionally supplied. Cells exposed at 0.03% CO_2 showed a remarkably different morphology compared with the cultures at 5 and 10% CO_2. Cells could become extremely large at 0.03% CO_2 also showing a great variation in shape and cell size within the culture. Proliferation was significantly impaired at 0.03% CO_2 *vis-a-vis* the 5 and 10% experiments. Terminal differentiation however was not influenced by the different CO_2 concentrations, indicating that *in vitro*, variation in the CO_2 concentration is of no importance regarding a possible differentiation of middle ear epithelium into a cornifying epithelium.

INTRODUCTION

In this paper we present the results of our experiments concerning the influence of various concentrations of CO_2 on proliferation and differentiation of middle ear epithelium in the rat, grown in serial cultivation[1]. The reasons for studying the possible influence of CO_2 on middle ear mucosa were the following observations:

1) In the case of a tympanic membrane perforation one can occasionally see a cornifying epithelium, located particularly on the promontory, which does not seem to be directly connected with the skin of the tympanic membrane or external ear canal.[2,3]

2) In chronic otitis media (cholesteatoma) defects in the external ear canal or tympanic membrane are often present, making a direct connection of the middle ear with the outer air possible.

3) The morphology of the middle ear epithelium is usually altered by inserting a ventilation tube in cases of otitis media with effusion.[4]

4) Many otologists have reported that patches of cornifying epithelium, which could not be removed during eradication of cholesteatoma, had disappeared at the time of the second look operation.[2]

These clinical observations have in common a possible relation between changes in the gaseous environment of the middle ear mucosa and a subsequent change in its morphology. The gas composition of the middle ear has been studied by several authors(Table I), who have reported a relatively high CO_2 concentration between 2.6-8.0% and a low O_2 between 9.5-12.1%. Compared with the ambient air, the difference in CO_2 gas concentration is especially evident. Laboratory animal and human studies concerning the gas composition in the middle ear generally report the same values for CO_2 and O_2.[5,13]

Moscowa reported that the differentiation of an embryonal epithelium could be influenced by altering the concentrations of O_2 and CO_2.[14] The middle ear epithelium is, of course, not an embryonal tissue and has already differentiated into a non-cornifying epithelium.

The literature, however, suggests that several factors, such as tobacco tar,[15] vitamin A,[16] infection,[17] tobacco smoke,[18] and endotoxines[19] can affect the morphology and differentiation of the middle ear epithelium. A difference in the gaseous environment could also be such a factor, and for this reason we cultured middle ear epithelium of the rat at various CO_2 concentrations. The possible influence of this gas on the proliferation and terminal differentiation of middle ear epithelium was the aim of our study, maintaining the other culture conditions as constant as possible.

Table I. *Gas composition of the middle ear*

		O_2 %	CO_2 %
Matsumura, '55 (5)	Human	9.15	
Melville Jones, '61 (6)	Human	9.5	7
Riuet al, '66 (7)	Human	9.5	5.5
Ingelstedt et al, '75 (8)	Human	5.5	8.0
Shinkawa et al, '75 (9)	Chinchilla	11.9	6.0
Cantekin et al, '76 (10)	Human	9.5	6.5
Sade et al, '77 (2)	Human		2.6
Morgenstern, '80 (11)	Guinea pig	10.9	
Ostfeld et al, '80 (12)	Dog	12.1	4.7
Ostfeld, '85 (13)	Guinea pig	11.7	7.5

MATERIALS AND METHODS

Culture conditions: For this study we used serially cultured rat middle ear epithelium (4th passage) originally obtained from the outgrowths of explants from the middle ear of male Wistar rats(body weight approx. 200g) which were stored in liquid N_2.

The cells were plated at a density of $2x10^4$ per cm^2 in combination with lethally irradiated 3T3 cells($1.2x10^4$ per cm^2). In our first experiments the culture medium consisted of a mixture of Dulbecco's Vogt and Ham's F12 medium(3:1) supplemented with fetal calf serum(5%), hydrocortisone(0.4ug/ml), isoproterenol(10-6M). penicillin(100U/ml), and streptomycin(100ug/ml). Epidermal growth factor(10ng/ml) was added on the 4th day. The culture medium was renewed twice a week. In a second experiment we added HEPES(20mM or 4.77g/l) in addition to $NaHCO_3$(0.85g/l) for a better buffer capacity. Cells were cultured in Petriperm® Hydrophil dishes (diameter 7 centimeter) which have a gas permeable bottom. The dishes were placed in Heraeus® incubators in which CO_2 concentrations were 0.03, 5 and 10%; O_2 was kept at a constant 21% and nitrogen additionally supplied. Culture time was 2 weeks and cells were studied on days 1, 4, 6, 10 and 14. For each day three dishes were investigated per gas combination.

Cell counting: Cultures were rinsed in sterile PBS, and after trypsinization(0.5 ml trypsin,time 20-30 minutes),soya trypsin inhibitor was added(1.0 ml). Cells were counted conventionally with the light microscope, in a Bürker counting chamber.

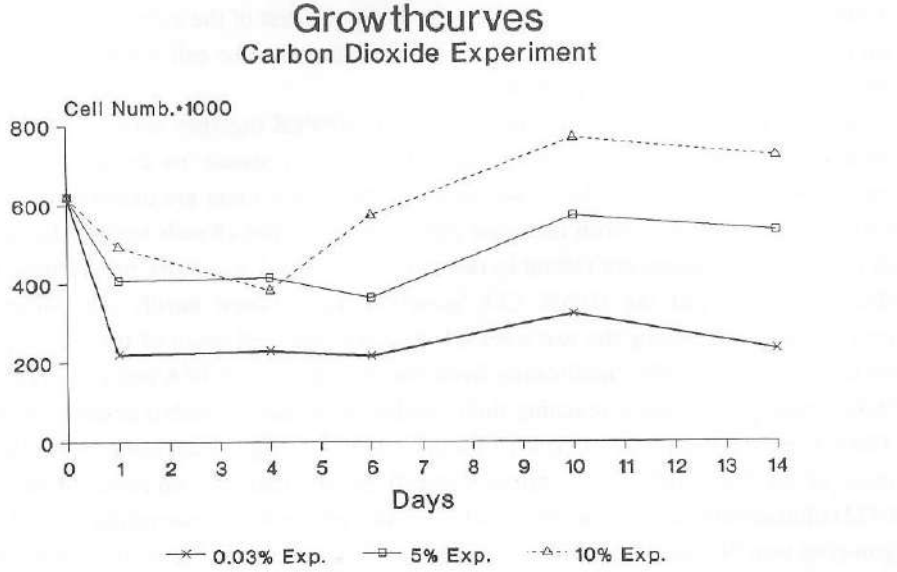


Fig. 1: Growth curves of rat middle ear epithelium cultured at 0.03, 5, and 10% CO_2 with addition of HEPES. The 0.03% CO_2 culture showed a statistically significantly reduced proliferation rate compared with the 5 and 10% cultures.

Scanning electron microscopy: Samples intended for scanning electron microscopy were fixed in glutaraldehyde, the adhering cells on the dish surface were dehydrated, critical point dried, sputtered with gold and studied with a Cambridge S 180 scanning electron microscope.

Promotion of cornified envelope formation: Cornified envelope formation was promoted and scored *ad modum* Rice and Green,[19] using Ionophore X537A.

Statistical evaluation: The results of this experiment were statistically evaluated with the ANOVA and the Scheffé test (modified Student's t test), the level of significance being kept at $p<0.05$.

RESULTS

Growth curves: Middle ear epithelium did not grow at 0.03% CO_2 when using a medium lacking HEPES as a buffer. At 5 and 10% CO_2, however, cultivation of cells was possible in the standard medium. Both curves showed an increase in cell number on days 4 and 6, after which the cell count remained constant for the rest of the culture period. Statistical evaluation revealed no differences in proliferation rate or cell number between the two cultures (ANOVA test $p<0.5479$).

In the second experiment in which we added HEPES together with $NaHCO_3$ to the medium to ensure a better buffer capacity, it was possible to culture middle ear epithelium at 0.03% CO_2. The growth curves of this experiment are plotted in Fig. 1. All cultures started on day 0 with the same cell number, but not all cells survive the moment of plating. The consequent fallout in cell number on day 1 was more pronounced in the 0.03% culture, and the 0.03% CO_2 growth curve showed hardly any increase in proliferation rate during the two weeks. In fact the final cell count of the 0.03% culture on day 14 did not differ significantly from that for day 1 (ANOVA test $p<0.9129$). All three growth curves were reaching their maximum on day 10, with a decline on day 14. The low cell numbers of the 0.03% CO_2 experiment were significant less ($p<0.0001$) than those of the 5 and 10% CO_2 cultures. Table II showed that the end result of the 0.03% CO2 cultured differed significantly from the 5 and 10% cultures, according to the Scheffé grouping test. No statistical differences were seen between the 5 and 10% experiments.

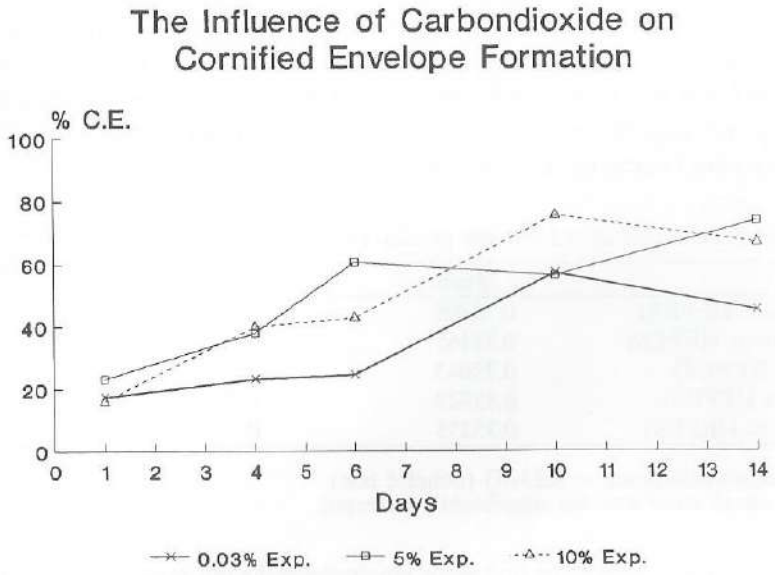


Fig. 2: Cornified envelope formation(C.E.) of rat middle ear epithelium cultured at 0.03, 5, and 10% CO_2 with addition of HEPES. Statistical evaluation revealed no significant differences.

Cornified envelope formation: Promotion of cornified envelope formation was measured in both experiments, with and without the addition of HEPES. The first experiments, with only $NaHCO_3$ as a buffer, showed no differences in cornified envelope formation between the 5 and 10% cultures.

In both experiments, after 14 days of culture, 65% of the cells could create a cornified envelope after the addition of Ionophore X537 A. The end results on day 14 for the 5 and 10% CO_2 experiments were not statistically different(ANOVA test $p<0.8469$). Fig. 2 shows the second experiment with the addition of HEPES and $NaHCO_3$. Cornified envelope formation in the 5 and 10% CO_2 experiment is again not statistically different($p<0.9392$). The 0.03% CO_2 culture, however, exhibited lower values during cultivation time, though the end results of the three curves was not statistically different,according to the Scheffé grouping test(Table III).

Light- and scanning electron microscopy:The morphological findings discussed here are from the cultures of the second experiment with HEPES as a buffer. Differences in morphology could be seen between the cultures grown with 0.03% CO_2 on the one hand, and the cultures grown with 5% and 10% CO_2 on the other hand. The latter were considered as one group, as no apparent differences were found between these cultures. Cultures grown at 5 and 10% CO_2 showed, already from the first day onward, large cell colonies in which cells densely covered with microvilli were recognized. Cells with and without microvilli were intermingled without any apparent pattern.

Table II. *Statistical evaluation of cell number on day 14*

	Mean	
5% CO_2 (without HEPES)	0.78308	A
10% CO_2 (without HEPES)	0.81165	A
5% CO_2 (with HEPES)	0.72043	A
10% CO_2 (with HEPES)	0.85929	A
0.03% CO_2 (with HEPES)	0.35255	B

Minimum significant difference = 0.23163 (Scheffé test)
Means with the same letter are not significantly different

Cell borders were clearly seen in the first week, emphasizing the polygonal appearance of those cells. In contrast to this group, cells cultured at 0.03% CO_2 showed only small islands of cells during the first days, and it was not until the second week that cells with microvilli were detected.

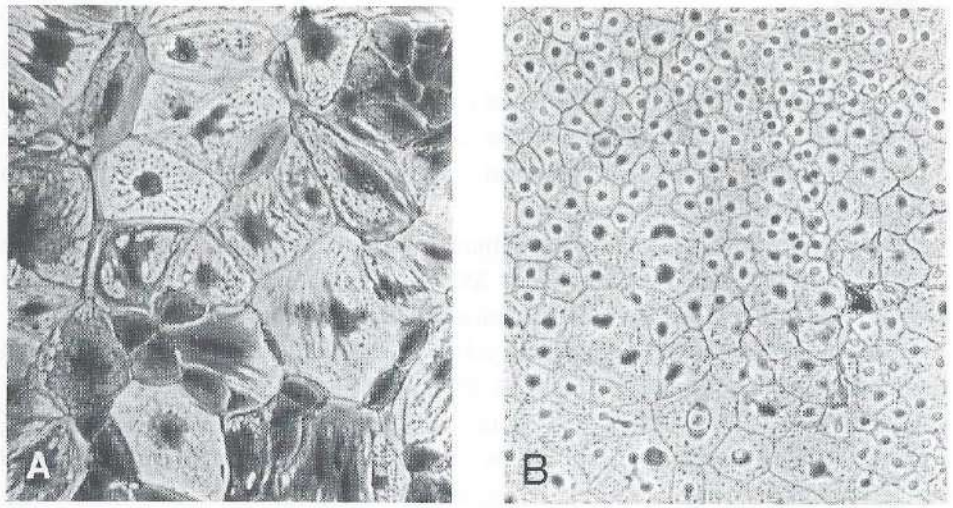


Fig. 3: Light microscopic pictures of rat middle ear epithelium grown at 0.03% CO_2 (A) and 10% CO_2 (B). Both pictures taken on day 10 with the same magnification, displaying the difference in cell size between the two cultures.

These cells were also polygonal in appearance, but their surface area could become extremely large (Fig. 3a,b). Confluence of the culture dishes was evident from day 6 in the 5 and 10% CO_2 groups, though even on day 14 the 0.03% CO_2 cultures showed areas on the dish surface which were unoccupied. Spontaneous cornified envelope formation was found in all cultures. The surface of those structures was highly irregular due to a dense layer of microvilli as has been described by others.[1]

CONCLUSIONS AND DISCUSSION

In this study we investigated the effects of different CO_2 concentrations on morphology, proliferation and terminal differentiation of rat middle ear epithelium in serial cultivation. One of the main problems was to maintain a steady pH in the medium, at different CO_2 concentrations. In the first experiment the culture medium used solely $NaHCO_3$ as a buffer, which dissociated whilst releasing CO_2 to the atmosphere if the gaseous environment has a low CO_2 pressure, resulting in a too high pH at 0.03% CO_2 conditions and leading to cell-death.

In the second experiment the culture medium was enriched with HEPES buffer. The pH of the culture media was measured during this experiment, showing a range from 6.8 to 7.9 . A parallel study was performed in our laboratory to measure the effect of changes in pH ranging from 6.0 to 9.0 in rat middle ear epithelium at 10% CO_2 concentrations. This study showed that pH values as measured in our study did not affect morphology or proliferation in a significant way. It became evident that culturing middle ear epithelium under ambient air conditions (meaning 0.03% CO_2 and 21 % O_2, and 79% N_2) clearly changed the morphology and the proliferation rate of cells. The 0.03% CO_2 growth curve showed significantly lower values compared with the growth curves of the 5 and 10% CO_2 cultures, indicating that conditions are not optimal at 0.03% CO_2.

Table III. *Statistical evaluation of cornified envelope formation on day 14.*

	Mean	
5% CO_2 (without HEPES)	1.80207	A
10% CO_2 (without HEPES)	1.80614	A
5% CO_2 (with HEPES)	1.84185	A
10% CO_2 (with HEPES)	1.81867	A
0.03% CO_2 (with HEPES)	1.65051	A

Minimum significant difference = 0.33358 (Scheffé test)
Means with the same letter are not significant

The degree of terminal differentiation of cells was measured by determine their ability to create cornified envelopes if Ionophore X537A was added to the suspended culture.[19]

In a cornifying epithelium the cornified envelope constitutes an important phase probably resulting in cell death and the formation of the stratum corneum. No significant differences in cornified envelope formation could be found between the different cultures, indicating that a low CO_2 concentration (near zero) does not influence the terminal differentiation of the middle ear epithelium in cultivation, during the period of 14 days.

Morphology was studied by light- and scanning electron microscopy. The enlargement of cell area in the culture at 0.03% CO_2 was a striking feature. The polygonal appearance of those cells was lost, in contrast to the cells in 5 and 10% CO_2 cultures, and confluence on the dish surface was not reached at 0.03% CO_2 after 2 weeks. If the above-mentioned results can be translated to the clinical situation then the following conclusions are feasible. *First*, a low CO_2 concentration of 0.03% (ambient air) may affect the morphology and proliferation of the middle ear epithelium, a situation which may occur in the case of a tympanic membrane perforation or when ventilation tubes are patent. *Secondly*, a low CO_2 concentration alone plays no important role in the degree of terminal differentiation of middle ear epithelium *in vitro*, which is why an effect of CO_2 on the possible transformation of middle ear mucosa into a true cornifying epithelium does not seem likely.

Admittedly, our observation time was short, whereas in the clinical situation the influence of a changed atmosphere can be much longer. In the various experiments N_2 was additionally supplied, resulting in different concentrations of this gas. We have assumed, however, that N_2 does not influence cell metabolism. Furthermore, it is possible that, in contrast to our *in vitro* experiment, the proliferation and differentiation of middle ear epithelium *in vivo* can be influenced by several other factors in its near environment, such as the underlying submucosa, or the presence of endotoxines[20] in case of infection.

REFERENCES

1. Van Blitterswijk CA, Ponec M, van Muijen GNP, Wijsman MC, Koerten HK and Grote JJ. Culture and characterization of rat middle ear epithelium. Acta Otolaryngol(Stockh) 1986; 101:453-466.

2. Sadé J, Weisman Z. The Phenotypic expression of middle ear mucosa. In: McCabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977; 52-61.

3. Sadé J. Cellular differentiation of the middle ear lining. Ann Otol Rhinol Laryngol 1970; 8:376-383.

4. Smyth GDL, Hall S. Aetiology and treatment of persistent middle ear effusion. J Laryngol Otol 1983; 97:1085-1089.

5. Matsumura H. Studies on the composition of air in the tympanic cavity. Arch Otolaryngol 1955; 61:220-222.

6. Melvill Jones G. Pressure changes in the middle ear after altering the composition of contained gas. Acta Otolaryngol 1961; 53:1-11.

7. Riu R, Flottes J, Bouche J, LeDeu R. La physiologie de la trompe d'Eustache. Paris: Arnette, 1966

8. Ingelstedt S, Jonson B, Rundcrantz H. Gas tension and pH in middle ear effusion. Ann Otol Rhinol Laryngolog 1975; 84:198-202.

9. Shinkawa H, Sasaki Y, Inamura N, Okitsu T, Takasaka T. Eustachian tube physiology. 1975:32 Kugler Publications.

10. Cantekin EI, Bluestone CD. Membrane ventilating tube for the middle ear. Ann Otol Rhinol Laryngol 1976; suppl 25:270-276.

11. Morgenstern C. Oxygen supply of the middle ear mucosa under normal conditions and after Eustachian tube occlusion. Ann Otol Rhinol Laryngol 1980; Suppl 68:76-77.

12. Ostfeld E, Blonder J, Crispin M, Szeinsberg A. The middle ear gas composition in air ventilated dogs. Acta Otolaryngol (Stockh) 1980; 89:105-108.

13. Ostfeld E. Middle ear gas composition. Ann Otol Rhinol Laryngol 1985; Suppl 120:33-34.

14. Moscona AA. Studies on stability of phenotypic traits in embryonic integmuntal tissues and cells. In: Montagna W, Lobitz WC, eds. The Epidermis. New York: Acad Press, 1964:83-96.

15. Greenberg DS, Dickey JR, Neely JG. Effects of tobacco on the ear. Ann Otol Rhinol Laryngol 1973; 82:311-317.

16. Fell H. The effect of excess vitamin A on cultures of embryonic chicken skin implanted at different stages of differentiation. Proc R Soc Med 1957; 146:242-248.

17. Vennix P, Kuypers W, Peters TA, Tonnaer ELGM, Ramaekers FCS. The cytokeratin expression in rat middle ear mucosa during induced sterile and infected effusions. Third Int Conf on Cholesteatoma and mastoid surgery. Copenhagen: Abstracts, 1988:101.

18. Auerbach O, Cuyler Hammond E, Garfinkel L. Histologic changes in the larynx in relatin to smoking habits. Cancer 1970; 25:92-40.

19. Rice RH, Green H. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: Activation of the cross linking by calcium ions. Cell 1979; 18:681-694.

20. Okazaki N, DeMaria TF, Briggs BR, Lim DJ. Experimental otitis media with effusion induced by nonviable hemophilus influenzae: Cytologic and histologic study. Am J Otolaryngol 1984; 5:80-92.

CHAPTER IV

THE EFFECT OF EXTERNAL STIMULI ON RAT MIDDLE EAR EPITHELIUM IN CULTURE

- EXTRACELLULAR OXYGEN CONCENTRATION-

HW Lutgert, PJ Bijlsma, CA van Blitterswijk, JJ Grote.

E.N.T. Department, University Hospital Leiden, The Netherlands

SUMMARY

Three experiments were performed in which rat middle ear epithelium was exposed to 5, 10, and 21% O_2, while the CO_2 concentration was held at 5%. Nitrogen was additionally supplied. No differences in morphology could be detected by light and scanning electron microscopy between the results of the 10 and 21% O_2 experiments. At the end of the culture period cells in the 5% O_2 culture showed a significantly larger average cell area compared with the 10 and 21% O_2 cultures. All cultures showed predominantly flat polygonal cells, occasionally covered with microvilli. Neither proliferation nor terminal differentiation of the epithelia was influenced by any of the three O_2 concentrations. Since a 10% O_2 is probably present in the intact middle ear, and a concentration of 21% is expected to occur after an ear drum perforation it may be concluded that the results of this *in vitro* study suggest that physiologic changes in atmospheric O_2 concentrations do not affect the morphology, proliferation, or terminal differentiation of rat middle ear epithelium.

INTRODUCTION

The influence of the O_2 concentration on the proliferation and morphology of cells of various types has been extensively studied *in vitro*. Several authors observed enhanced colony formation and proliferation rate of cultured cells if the O_2 concentration was lower than the level in the open air, mainly between 5 and 10%.[1-4] Brosemer et al.[5] showed that cells originating from human cervical carcinoma, could be cultured in the presence of sub-detectable concentrations of O_2, although optimum growth was obtained between 10 and 35 %. In most of these experiments the CO_2 concentration was kept at a level of 5%, and nitrogen was supplied additionally. These studies showed that cell metabolism can be influenced *in vitro* by changing the oxygen concentration. Optimum results were generally reached if the O_2 concentration approximated values occurring *in vivo*, where the extracellular oxygen concentration is normally between 5 and 7% O_2.[6] In the experiments discussed here we investigated the influence of three oxygen concentrations on serially cultured rat middle ear epithelium. The oxygen concentrations used were 5, 10, and 21 %.

In the first experiment we used 10% O_2 in combination with 5% CO_2 and 85% N_2, which is approximately the same gas composition as that in the intact middle ear.[7-10] In the second experiment the epithelium was cultured at 21% O_2, 5% CO_2, and 74% nitrogen, which represents the gaseous environment normally used for culture experiments in our laboratory.

A concentration of 21% O_2 resembles the oxygen concentration in the middle ear in case of a drum membrane perforation or if a patent ventilation tube is present; however, the carbon dioxide concentration would then have been 0.03%. In the third experiment a concentration of 5% O_2 was introduced because in many of the reported culture studies mentioned above, enhanced proliferation was observed at this concentration. Our aim was to find out whether the morphology, proliferation, and terminal differentiation of cultured rat middle ear epithelium would be influenced by these O_2 concentrations, which may occur in certain clinical situations.

MATERIALS AND METHODS

Culture conditions: For this study we used serially cultured rat middle ear epithelium from male Wistar rats (body weight ca. 200 g). The cultured middle ear epithelium was of the fourth passage.

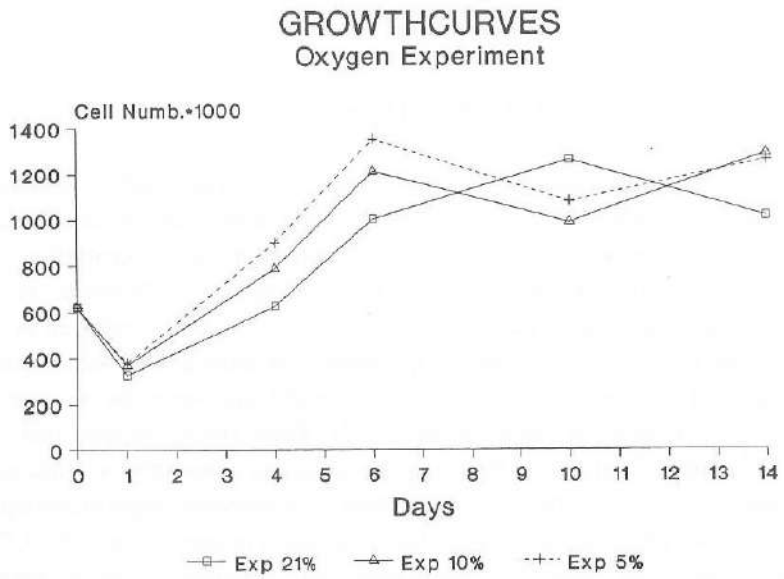


Fig. 1: Growth curves of rat middle ear epithelium cultured at 21, 10, and 5% oxygen. Statistical analysis revealed no significant differences.

The thawed cells were initially plated on dishes (diameter 14 cm) with standard medium and irradiated 3T3- feeder cells. The standard medium consisted of Dulbecco's Vogt and Ham's F12 medium(3:1) supplemented with fetal calf serum(5%), hydrocortisone(0.4ug/ml), isoproterenol(10-6M), penicillin(100U/ml) and streptomycin(100ug/ml). After confluence was reached, trypsin(0.5 ml) was added to the culture during 20-30 minutes, and the trypsinization process was stopped with soya trypsin inhibitor(1.0 ml). The suspended cells were plated on Petriperm Hydrophil® dishes with a diameter of 6 cm, which have a gas permeable bottom.

Middle ear epithelium was plated at a density of $2x10^4/cm^2$ and 3T3 feeder cells at a density of $1.2x10^4/cm^2$. The dishes were placed in Heraeus® incubators in which the oxygen concentration was 5, 10, or 21%. The carbon dioxide concentration was 5% except in exp.6, where 10% CO_2 was used. The culture time was two weeks and cells were studied on days 1, 4, 6, 10, and 14. For each day three dishes were investigated for each gas combination.

Cell counting: For cell counting cultures were rinsed in sterile phosphate-buffered saline(PBS), and after trypsinization (0.5 ml trypsin, exposure time 20-30 min.), soya trypsin inhibitor was added(1.0 ml). The suspended cells were counted in a Bürker counting chamber.

Promotion of cornified envelope formation: Cornified envelope formation of the suspended cells was promoted and scored according to Rice and Green,[11] using ionophore X537A. The percentage of cells capable of creating cornified envelopes was determined on days 1,4, 6, 10, and 14 three cultures being used for each measurement.

Scanning electron microscopy: The material intended for scanning electron microscopy was fixed in glutaraldehyde, the adhering cells on the dish surface were dehydrated, critical point dried, sputtered with gold and studied in a Cambridge S 180 scanning electron microscope.

Cytomorphometric study: For each culture dish 9 photographs were taken with the light microscope at predetermined locations. On each photograph the morphology of 20 cells, also at predetermined locations, was objectivated with the aid of a digitizing X/Y tablet connected to an MOB computer, for calculation of the average cell size.

Statistical evaluation: The results of this experiment were statistically evaluated with the ANOVA test and Scheffé test (modified student t test), the level of significance was taken at $p<0.05$.

RESULTS

Growth curves and cornified envelopes

The rate of cell proliferation at an atmosphere containing 5, 10, or 21% O_2 is expressed by the growth curves in Fig. 1. The cultures were started with $6.25x10^5$ cells on day 0, after which all curves showed a dip on day 1, because not all cells will survive the moment of plating. The cultures grown at 5 and 10% O_2 showed a steady increase in cell number, with a maximum on day 14. The growth curve of cells grown at 21% O_2 reached its optimum on day 10 with a slight backfall on day 14. Statistical evaluation of the three curves (Anova test) revealed no significant differences concerning the overall results ($p < 0.3064$), the course of the curves in time($p < 0.6889$) or the end values on day 14($p < 0.5360$). Fig. 2 shows the percentage of cultured cells, grown at 5, 10, and 21% O_2, which formed cornified envelopes after Ionophore X537A was added to the medium. All curves reached an optimum on day 6, when 70% of cells were able to create cornified envelopes. After day 6 we saw a decline in terminal differentiation and on day 14 only 40% cornified envelope formation was recorded. Statistical evaluation of the results revealed no significant differences between the three experiments.

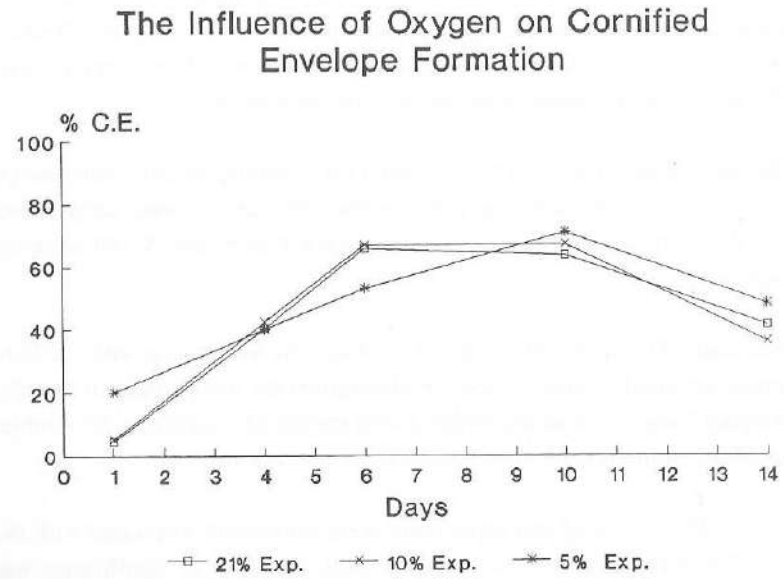


Fig. 2: Cornified envelope (C.E.) formation of rat middle ear epithelium cultured at 21, 10, and 5% oxygen. Statistical analysis revealed no significant differences.

As well as the end values on day 14, as the course of cornified envelope formation in time, was not statistically different($P < 0.9329$). The values on day 14 were statistically significantly lower than those on day 6 and 10 ($p < 0.0001$).

Light and scanning electron microscopy

Confluence on the dish surface was reached in the 10 and 21% culture on day 6, and in the 5% material on day 10. All cultures showed monolayers of predominantly flat polygonal cells. The size of the cultured cells varied considerably, fields of very small cells were intermingled with groups of larger cells(Fig. 3). Especially after day 10 this difference in cell size within the same culture became pronounced. Cornified envelope-like structures could be seen above the plane of cultured cells, and were generally located in the vicinity of cells with a relatively larger cell area (Fig. 3); they were found in all of the cell cultures. Scanning electron microscopy showed the extremely flat appearance of the cultured cells and their polygonal size, especially after confluence was reached (Fig. 4). Cells with and without microvilli were seen, the latter seemed to be clustered between larger cell areas without this feature. The presence of cornified envelopes was easily detected with the scanning electron microscope and they were found in all cultures (Fig. 5).

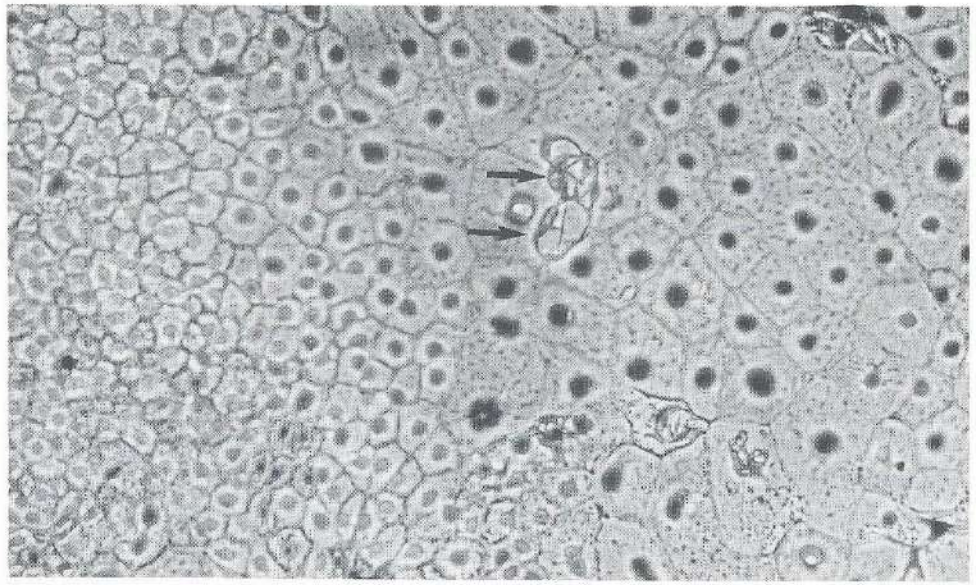
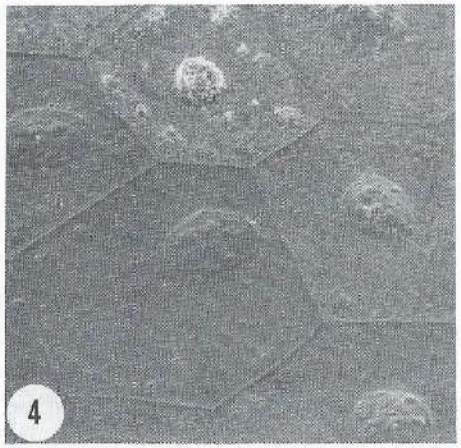


Fig. 3: Light-microscopic picture of cultured rat middle ear epithelium at 10% O_2, displaying the variation in cell size within the same culture. Vesicle like structures (arrows) are seen probably representing cornified envelopes.



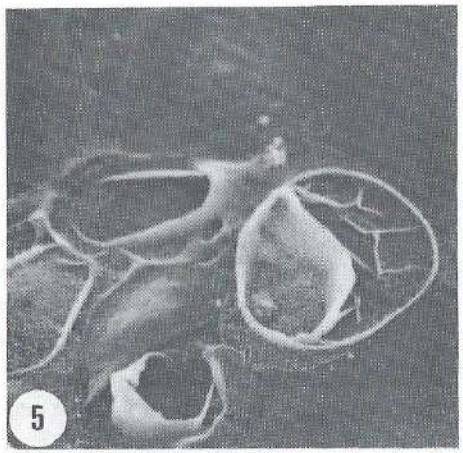


Fig. 4: Scanning electron microscopic picture of middle ear epithelium underscoring the flat appearance and polygonal shape.
Fig. 5: An example of spontaneously formed cornified envelopes.

Cytomorphometric results

In Fig. 6 the average cell-areas of the three culture experiments are plotted. The course of the 10 and 21% curves is almost the same, i.e. in both experiments an increase in cell area was seen on day 4, followed by a decrease in cell area continuing until day 10. The 5% culture to the contrary, displayed a constant increase in cell area with time. Statistical analysis revealed that the average cell size on day 14, in the 5% culture was significantly larger than that in the 10% ($p < 0.0042$) and 21% ($p < 0.0001$) cultures. The morphometric results of the 10 and 21% cultures showed no significant differences ($p < 0.2860$).

DISCUSSION

Some differences in morphology were found between the 5% culture on the one hand and the 10 and 21% cultures, considered as one group, on the other. In the latter group confluence was reached on day 6, whereas this phenomenon was observed in the 5% culture on day 10. Furthermore, the average size of cells grown at 5% on day 14, was statistically significantly larger than the average cell size of the 10 and 21% experiments on that day. Apart from these differences, all cultures showed cells predominantly polygonal in shape, with considerable variation in cell size. These morphological characteristics did not change on days 10 and 14.

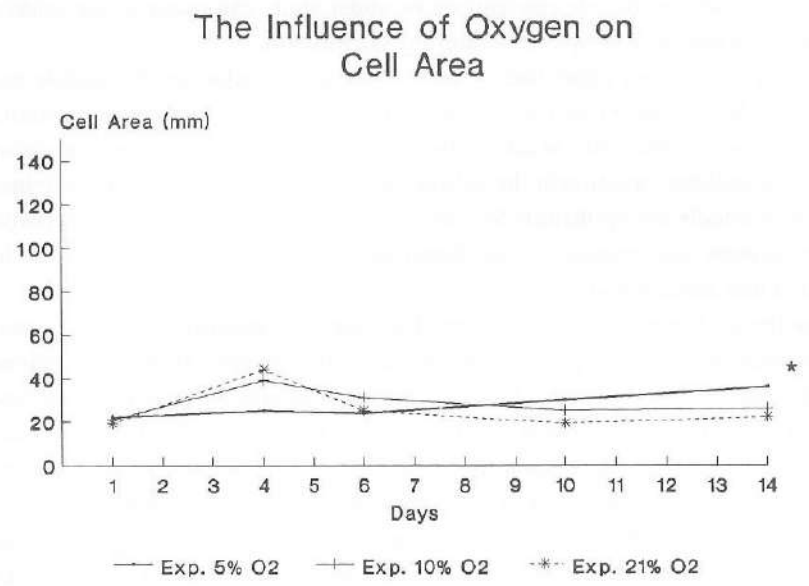


Fig. 6: *) Average cell area on day 14 of the 5% O_2 culture is statistically significantly larger compared to those in the 10 and 21% O_2 cultures.

Scanning electron microscopy underscored the extremely flat appearance of the cells, and revealed the presence of microvilli. Cornified envelopes were found, indicating that *in vitro* the middle ear epithelium can display spontaneous terminal differentiation. These cornified envelopes have been described by others, and were found in all three experiments.[12]

The degree of terminal differentiation was not altered in cultures grown at the oxygen concentrations of 5, 10, or 21%. It is not clear why a backfall in cornified envelope formation occurred in all experiments on day 14. Van Blitterswijk et al., who performed similar studies, saw a maximum degree of terminal differentiation of 100% after 10 days, which remained constant even after 28 days.[12] This might be explained by the addition of HEPES to the growth medium in our experiment, to increase the buffer capacity. HEPES can exhibit cell toxicity at concentrations higher than 40mM, we used a concentration of 20mM in our medium but its potential toxicity must be taken into consideration.

A study similar to this O_2 experiment was performed in our laboratory with changes in the CO_2 concentrations, the percentages used were 0.03, 5, and 10%.[13] This study did not reveal a significant influence of CO_2 on the terminal differentiation of cultured middle ear epithelium. In sum, no correlation was found between changes in the O_2 or CO_2 concentration and the degree of terminal differentiation of the epithelium.

The oxygen and carbon dioxide concentrations under study can occur in the middle ear, for instance in case of a tympanic membrane perforation.

There are of course, many other factors *in vivo* which can influence the middle ear mucosa in cases with a defect of the tympanic membrane, like a changed aeration, temperature or lowered humidity of air in the middle ear. In contrast to extracellular CO_2 and O_2, extracellular variation in the calcium concentration may effect the terminal differentiation of middle ear epithelium *in vitro*, as was demonstrated by van Blitterswijk et al.[14] The calcium concentration may fluctuate in case of otitis media due to osteoresorption and osteodeposition.

Concerning the proliferation rate it became clear that no statistical differences could be found between the cultures grown at 5, 10, and 21% oxygen. Many publications reported that a variety of cells will display an increased proliferation rate at relative low oxygen concentrations ranging between 2.5 and 7.5%.[1-5] However, at 5% O_2 we did not observe an influence on the proliferation rate if compared with the 10 and 21% cultures.

The role of CO_2 may be more important, because our previously mentioned CO_2 experiment showed that proliferation rate was statistically significantly reduced ($p < 0.0001$) at an atmosphere containing 0.03% CO_2 and 21% O_2. Also the morphology of the cultured middle ear epithelium was different if grown at 0.03% CO_2, compared to the 5 and 10% CO_2 cultures. At 0.03% CO_2 many cells did not display a polygonal shape, and the average cell size was significantly enlarged ($p < 0.0001$) compared to the other cultures.

Thus it may be concluded that the oxygen concentrations used in this study i.e. 5, 10, and 21%, did not influence the proliferation, morphology, or terminal differentiation of cultured rat middle ear epithelium significantly. In this context the influence of carbon dioxide seemed to be much more important, especially with respect to the morphology and proliferation rate of middle ear epithelium *in vitro*.

REFERENCES

1. Held KR, Sonnichsen S. The effect of oxygen on colony formation and cell proliferation of amniotic fluid cells in vitro. Prenatal diagn 1984; 4:171-179.

2. Brackertz M, Kubbies M, Feige A, Salk D. Decreased oxygen supply enhances growth in culture of human mid-trimester amniotic fluid cells. Hum Genet 1983; 64:334-338.

3. Balin AK, Goodman DBP, Rasmussen H, Cristofalo VJ. The effect of oxygen tension on the growth and metabolism of WI-38 cells. J Cell Physiol 1976; 89:235-250.

4. Bradley TR, Hodgson GS, Rosendaal M. The effect of oxygen tension on hemopoietic and fibroblast cell proliferation in vitro. J Cell Physiol 1978; 97:517-522.

5. Brosemer RW, Rutter WJ. The effect of oxygen tension on the growth and metabolism of a mammalian cell. Exp Cell Res 1961; 25:101-113.

5. Brosemer RW, Rutter WJ. The effect of oxygen tension on the growth and metabolism of a mammalian cell. Exp Cell Res 1961; 25:101-113.

6. Guyton AC,Text Book of Medical Physiology. W.B. Saunders & Co., Philadelphia and London, Chap.39.

7. Elner A. Normal gas exchange in the human middle ear. Ann Otol Rhinol Laryngolog 1976; 85 suppl25:161-164.

8. Cantekin EI, Doyle WJ, Phillips DC, Bluestone CD. Gas absorption in the middle ear. Ann Otol Rhinol Laryngolog 1980; Suppl 68:71-75.

9. Morgenstern C. Oxygen supply of the middle ear mucosa under normal conditions and after Eustachian tube occlusion. Ann Otol Rhinol Laryngolog 1980; Suppl 68: 76-77

10. Ostfeld E, Blonder J, Szeinsberg A. The middle ear gas composition in air ventilated dogs. Acta Otolaryngol (Stockh) 1980; 89:105-108

11. Rice RH, Green H. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: Activation of the cross-linking by calcium ions. Cell 1979; 18:681-694.

12. Van Blitterswijk CA, Ponec M, van Muyen GNP, Wijsman MC, Koerten HK, Grote JJ. Culture and characterization of rat middle-ear epithelium. Acta Otolaryngol (Stockh) 1986; 101:453-466.

13. Lutgert HW, van Blitterswijk CA, Grote JJ. The effect of external stimuli on rat middle ear epithelium in culture.-Extracellular Carbon Dioxide Concentration-. Acta Otolaryng(Stockh) In Press.

14. Van Blitterswijk CA, Hesseling SC, Grote JJ. The effect of external stimuli on cultured rat middle ear epithelium. -extra cellular calcium concentration-.

CHAPTER V

THE INFLUENCE OF EXTRACELLULAR OXYGEN AND CARBON DIOXIDE ON MIDDLE EAR EPITHELIUM OF THE RAT IN CULTURE

- A CYTOMORPHOMETRIC STUDY -

HW Lutgert, CA van Blitterswijk, PJ Bijlsma, JJ Grote
E.N.T. Department, University Hospital Leiden, The Netherlands

SUMMARY

The effect of changes in the O_2 and CO_2 concentrations on growth and morphology of middle ear epithelium of the rat was studied. The CO_2 concentrations used were 0.03%, 5, and 10% with O_2 at a constant level of 21%. In the following experiment O_2 concentrations were 5, 10, and 21%, the CO_2 concentration was kept at 5% in the 5 and 10% O_2 cultures, and 10% in the 21% O_2 culture. The morphometric study was done with a digitizing tablet. Epithelium cultured at 0.03% CO_2 displayed the most striking changes in growth and morphology, e.g. cell number was reduced and the average cell area was significantly enlarged. This CO_2 concentration is present in the middle ear in cases with a tympanic membrane perforation. The influence of carbon dioxide on middle ear epithelium *in vitro* seemed to be more important than oxygen.

INTRODUCTION

In this study we have investigated the possible influence of shifts in the extracellular carbon dioxide and oxygen concentrations on growth and morphology of cultured rat middle ear epithelium. The normal and intact middle ear probably has a gas composition, of about 5% CO_2, 10% O_2, and 85% N_2, which differs from that of the ambient atmosphere i.e.; 0.03% CO_2 and 21% O_2.[1-3] In cases with a tympanic membrane perforation, it is conceivable that the O_2 and CO_2 concentrations will resemble those of the outer air. Some authors have reported the possible influence of a changed gaseous environment on the morphology of the human middle ear *in vivo*.[4-6] The effect for instance of grommets in cases of otitis media can be partially explained this way.[5]

Culture studies on different types of epithelia showed the influence of oxygen on cell metabolism *in vitro*.[7-10] The effect of CO_2 has hardly been investigated except in some explant studies on adenoid tissue and respiratory epithelium.[11-12] These studies showed that CO_2 may have an effect on the behavior of certain epithelia. Recently, we reported successively on the effect of CO_2 and O_2 on the proliferation and terminal differentiation of middle ear epithelium of the rat in culture.[6]

Especially the effect of a lowered CO_2 concentration (0.03%) on proliferation became evident from significant reduction in the proliferation rate. Terminal differentiation was not significantly influenced by CO_2 concentrations of 0.03, 5, and 10%, or O_2 concentrations of 5, 10, and 21%. In the study reported here we evaluated and compared the effect of changes in both CO_2 and O_2 concentrations by morphometric examination of the cultured middle ear epithelium.

MATERIALS AND METHODS

Culture conditions:Middle ear epithelium of male Wistar rats(body weight ca.200 g) was brought into cultivation.[18] This epithelium was originally obtained from outgrowths of explants, brought into serial cultivation, and later stored in liquid N_2. The cells were plated at a density of $2x10^4$ per cm^2 in combination with lethally irradiated 3T3 feeder cells($1.2x10^4$ per cm^2).The culture medium consisted of a mixture of Dulbecco's Vogt and Ham's F12 medium(3:1) supplemented with fetal calf serum(5%), hydrocortisone(0.4ug/ml), isoproterenol(10-6M). penicillin(100U/ml), and streptomycin(100ug/ml).

Epidermal growth factor(10ng/ml) was added on the fourth day. The culture medium was renewed twice a week. To create sufficient buffer capacity, we added HEPES(20mM) and $NaHCO_3$(0.85g/l) to this standard medium. In all experiments the cells were cultured in Petriperm Hydrophil® dishes (diameter 6 cm.) which have a gas-permeable bottom. The dishes were placed in Heraeus® incubators in which the gas conditions could be controlled in a water-saturated atmosphere. In the first set of experiments, epithelium was cultured at three CO_2 concentrations, i.e., 0.03, 5, and 10%. The oxygen concentration was kept at 21% (Table 1). In the second set of experiments cells were exposed to three O_2 concentrations, 5, 10, and 21%.

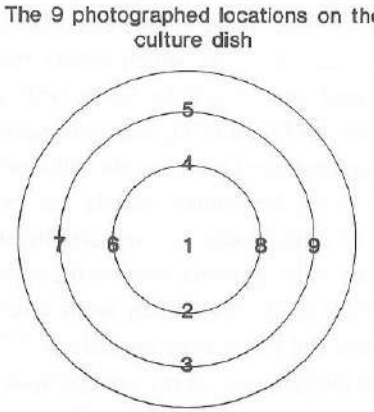


Fig. 1 : Location 1 represents *ring 0*. Locations 2,4,6, and 8 represents *ring 1*. Locations 3,5,7, and 9 represents *ring 2*.

The CO_2 concentration was 5% except in exp.6 where 10% CO_2 was used, because one of the three incubators could only operate at this concentration. This last experiment also served as a control experiment, since the gas composition was the same as that used in experiment 3 of the CO_2 study (Table 1). There was a time lapse of about one year between the two culture studies. Nitrogen was additionally supplied in all experiments.

Table I. *Gas concentrations used in the culture experiments*

	CO_2 %	O_2 %
Experiment 1	0.03	21
Experiment 2	5	21
Experiment 3	10	21
Experiment 4	5	5
Experiment 5	5	10
Experiment 6	10	21

The culture time was two weeks and cells were studied on days 1, 4, 6, 10, and 14. For each day three dishes were investigated per gas combination. The cultures were rinsed in phosphate-buffered saline(PBS), fixed in 1.5% glutaraldehyde in sodium cacodylate buffer (4°C, pH 7.4, 0.14 M) for two hours. Another rinse in PBS was followed by staining with toluidine blue and the epithelium was dried by exposure to the air, before examination with a light microscope.

Morphometric study: For each culture 9 photographs were taken of 9 predetermined areas at a magnification of 100 times (see Fig 1). On each photograph the morphology of 20 cells, also at fixed locations, was determined with the aid of a digitizing X/Y tablet connected to an MOB computer, so that average cell size and cell shape could be calculated. The cell shape was expressed as the so-called form factor, ranging from 0.00 to 1.00. In this context form factor 0.00 stands for a straight line, and 1.00 for a perfect circle, so the more closely a cell approximated 1, the more the cell shape resembled a circle. Besides the morphometric study we also counted the absolute number of cells on each photograph. This made it possible to obtain growth curves for each culture.

The results were also analyzed in relation to location on the culture dish. As indicated for Fig. 1, Photograph 1 was taken in the center(ring 0), Phots. 3,5,7, and 9 between the center and periphery (ring1) and Phots. 2,4,6, and 8 at the periphery (ring 2). The average values of rings 0, 1, and 2 were calculated and compared.

Statistical evaluation: The results of this experiment were evaluated statistically with the ANOVA test and Scheffé test (modified student's t test), and the level of significance was set at $p<0.05$.

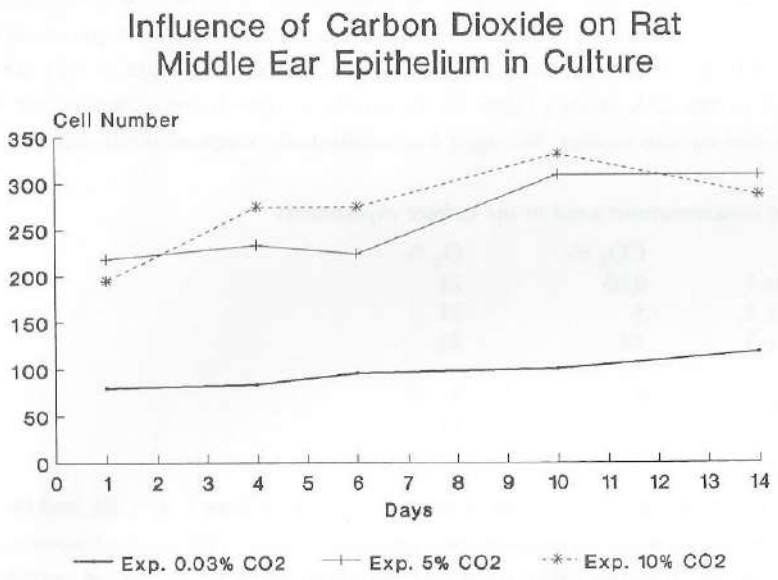


Fig. 2: The 0.03% CO_2 growth curve showed a statistically significantly lower cell number compared to the 5 and 10% CO_2 growth curves during the culture period of two weeks.

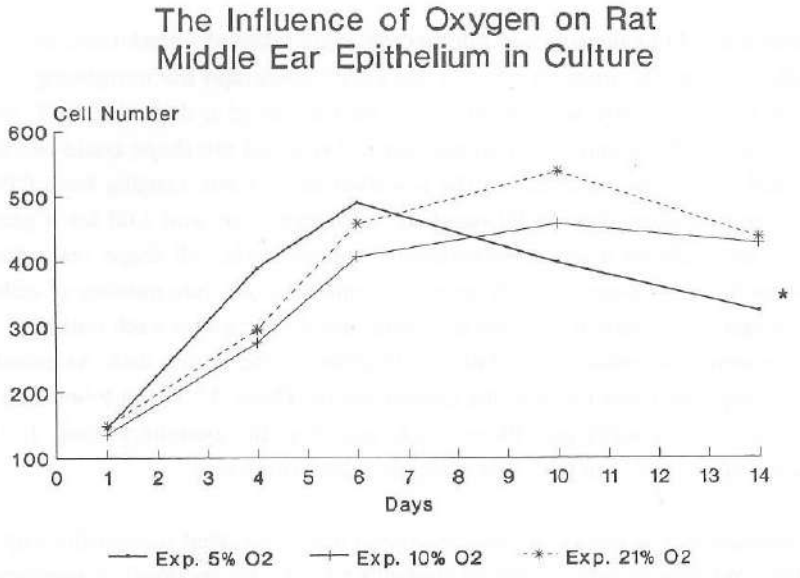


Fig. 3: *) The cell number on day 14 in the 5% O_2 experiment is statistically significantly lower than that in the 10 and 21% experiments.

RESULTS

General morphology

In all experiments the cell cultures were composed of flat polygonal cells, a multilayered epithelium was not observed. Confluence on the dish surface was generally reached between days 6 and 10. The 0.03% CO_2 culture displayed a different morphology, most of the cells were larger than those in the 5 and 10% CO_2 cultures, and the difference in cell size within the culture was more pronounced in the 0.03% CO_2 experiment. The O_2 experiments did not reveal any distinct morphological differences except on day 14. On that day the 5% O_2 culture showed, larger cells compared to those in the 10 and 21% cultures. All cultures displayed structures probably representing cornified envelopes, which were situated above the plane of the epithelium and were mostly related to areas with larger cells.

Growth curves

The cells on each photograph were counted, the results are shown in Fig. 2 and were considered as a basis for growth curves. The growth curve of the 0.03% CO_2 experiment lies at a lower level than that of the 5 and 10% curves(Fig. 2). Statistical analyzes showed that this difference in cell number was significant($p < 0.0001$) from the first day on and remained so throughout the culture period. No significant differences were found between the 5 and 10% CO_2 curves($p < 0.4953$). The course of the three curves in time, and the relative increase in cell number did not differ statistically. The growth curves of the O_2 experiment(Fig. 3) show a steady increase in cell number in all three experiments up to day 6. However, the 5% O_2 culture deviated from day 6, by a decrease leading to a lower cell number on day 14 compared to the 10 and 21% O_2 cultures. The overall results of the three curves are not statistically different($p < 0.1577$), but the end result of the 5% culture on day 14 differed significantly compared to the 10 and 21% O_2 experiments($p < 0.0090$, $p < 0.0070$, respectively).

Cell number related to the location on the culture dish

In Fig. 4 we see the 0.03% CO_2 growth curve subdivided according to the three rings 0, 1, and 2. An increase in cell number occurred in all three rings but on days 4 and 14 ring 0 showed a significantly higher cell number compared to rings 1 and 2. Concerning the 5 and 10% cultures an increase in cell number occurred in rings 1 and 2, and a decrease at the center, or ring 0.

In the O_2 cultures, as in most CO_2 cultures, there was also an increase of cell number in the periphery(rings 1 and 2) and a decrease or stabilization of cell number in the center(Fig. 5). In general, no statistical differences were found when the overall results of the individual rings were compared. In all experiments (CO_2 and O_2), however, values differed significantly between the three rings on one or more days.

Cell area

The average cell area of the 0.03, 5, and 10% CO_2 cultures is shown in Fig. 6. The cell area of the 0.03% CO_2 culture was statistically significantly larger compared to the 5 and 10% experiments ($p<0.0001$), for both, the overall results and the end result on day 14.

The difference in cell size was already present on the first day but increased in time. Fig. 7 shows the results of the O_2 experiment. The curves of the 10 and 21% O_2 cultures are almost identical, in both experiments a maximum in cell area was reached on day 4 after which cell size decreased up to day 10. The 5% curve shows, however, an increase in cell size from day 6, which gave a significantly larger average cell area on day 14 compared to the 10 and 21% cultures ($p<0.0042$ and $p<0.0001$, respectively). No significant differences were found between the 10 and 21% cultures($p<0.2860$).

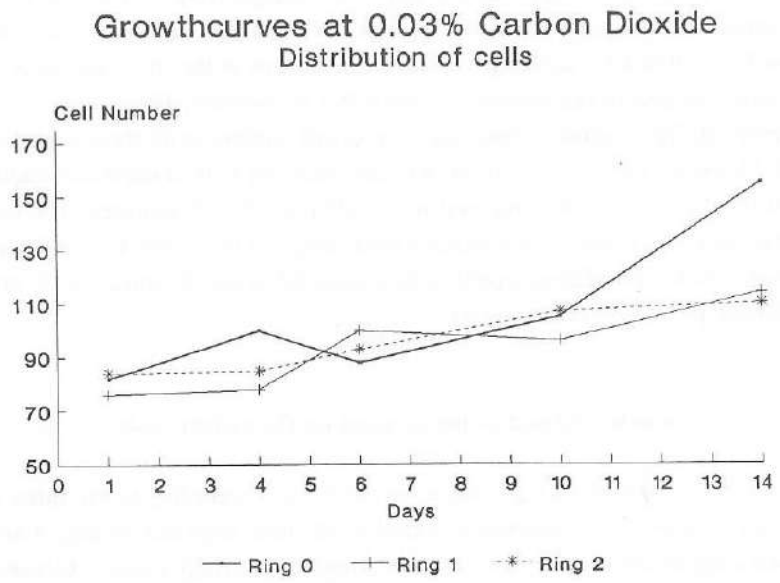


Fig. 4 : Subdivision of the 0.03% CO_2 growth curve into rings 0, 1, and 2.

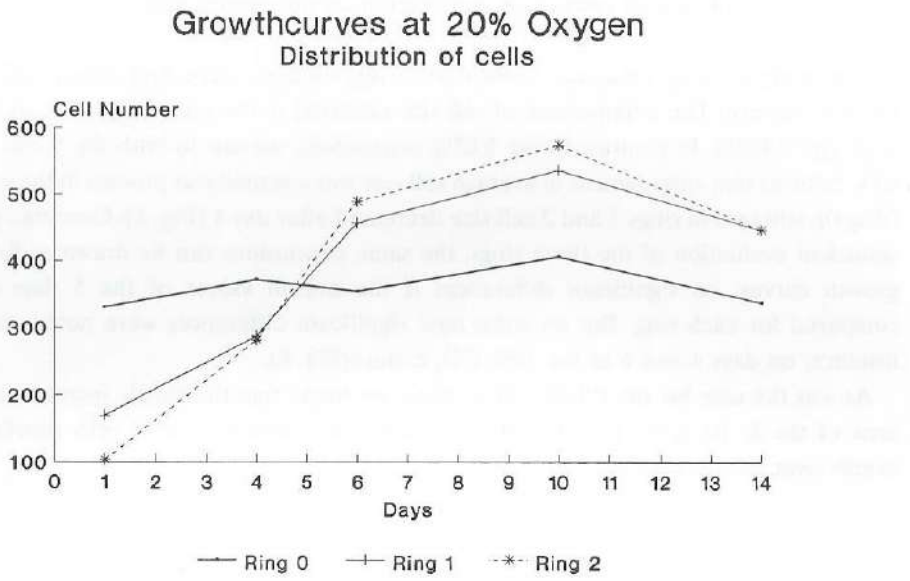


Fig. 5 : Subdivision of the 20% O_2 growth curve into rings 0, 1, and 2. Note the increase in cell number in rings 1 and 2.

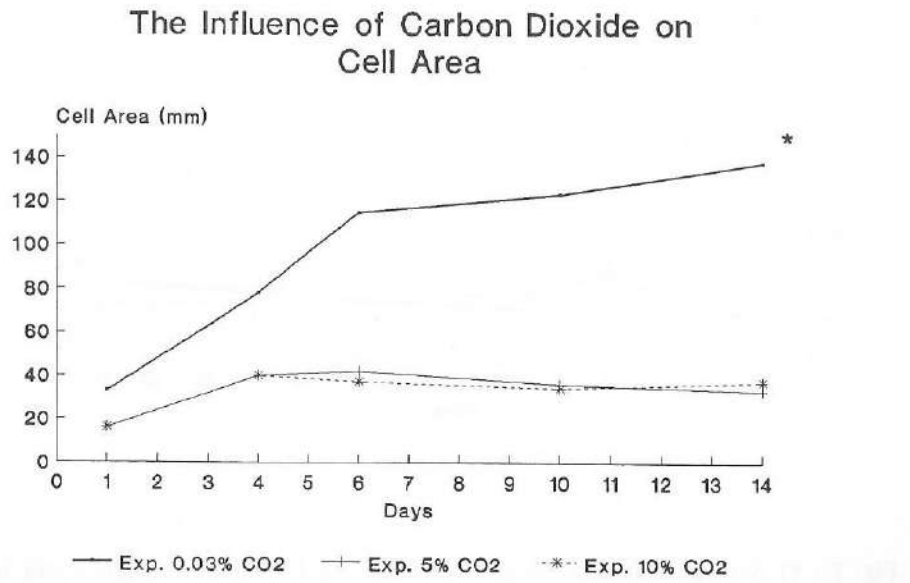


Fig. 6 : *) The average cell area of the 0.03% CO_2 culture is statistically significant larger during the 14-day period as compared to that in the 5 and 10% CO_2 cultures.

Cell area in relation to the location on the culture dish

The increase in cell area seen in the 0.03% CO_2 cultures, was evenly distributed over the dish surface. The enlargement of cell size occurred to the same degree in all three rings ($p<0.4826$). In contrast to the 0.03% experiment, we saw in both the 5 and 10% CO_2 cultures that enlargement of average cell size was a continuous process in the center (ring 0), whereas in rings 1 and 2 cell size decreased after day 4 (Fig. 8). Concerning the statistical evaluation of the three rings, the same conclusions can be drawn as for the growth curves: no significant differences if the overall values of the 5 days were compared for each ring. But on some days significant differences were noted, as, for instance, on days 4 and 6 of the 10% CO_2 culture(Fig. 8).

As was the case for the 0.03% CO_2 culture, we found that the steady increase in cell area of the 5, 10, and 21% O_2 cultures was due to enlargement of cells distributed evenly over the dish surface.

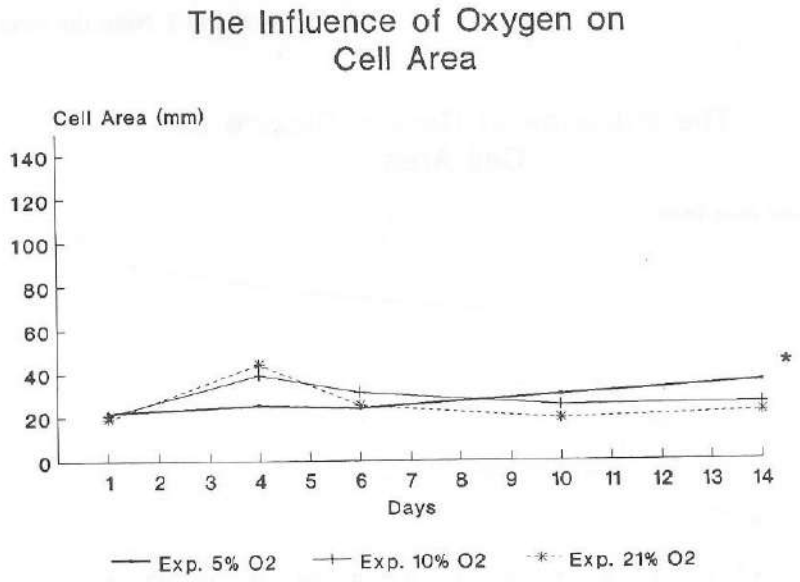


Fig. 7 : *) Average cell area on day 14 of the 5% O_2 culture is significantly larger compared to that in the 10 and 21% O_2 cultures.

Cell shape

As mentioned under materials and methods, the average cell shape could be calculated with the MOB computer, and was expressed in what is called the form factor, ranging from 0.00 to 1.00. Form factor 0.00 represents a straight line and 1.00 a perfect circle. The average cell shape of the CO_2 culture is shown in Fig. 9. The 0.03% CO_2 curve lies at a lower level. Statistical analysis showed that the average form factor of the 0.03% experiment was significantly lower than that of the 5 and 10% CO_2 curves($p<0.0017$ and $p<0.0056$, respectively). Between the 5 and 10% CO_2 cultures we found no significant differences ($p<0.6429$) in the form factor. The same holds for the O_2 experiments, since no significant differences were found.

Control experiment

Experiment 6 in the O_2 study served as control, because the same gas concentrations were used as in Exp. 3 of the CO_2 study. Both cultures showed confluence on the dish plate after day 6, and the average cell shape was identical in both experiments.

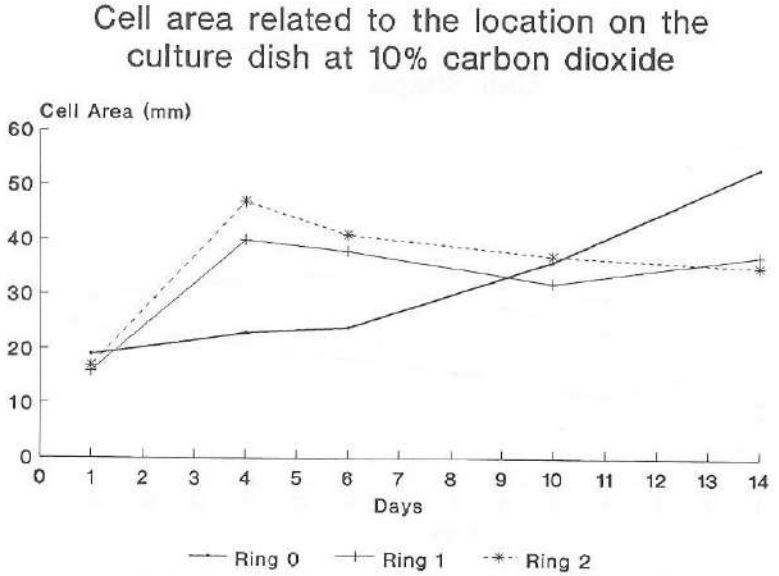


Fig. 8 : Ring 0 displays a different course of the enlargement of cell area compared to rings 1 and 2 in the 10% CO_2 culture.

Cell number reached a steady state in both experiments after day 6, but in Exp. 6 cell number was higher with, consequently, a smaller average cell size compared to Exp. 3. Statistical analysis showed that these differences were significant($p < 0.1430$).

DISCUSSION

We looked for any influence of extracellular CO_2 and O_2 on the morphology of cultured middle ear epithelium of the rat.[18] These two gasses were investigated separately and at least some of the gas combinations used are bound to occur in the clinical situation. For instance, Exp. 5 (5% CO_2 and 10% O_2) is the gas combination which probably represents the gas combination in the intact middle ear, whereas Exp. 1 (0.03% CO_2 and 21% O_2) is expected to occur in cases with a tympanic membrane perforation.[1-3]

A concentration of 0.03% CO_2 led to an altered morphology, which was confirmed by the morphometric study. Cells became extremely large compared to those in other cultures. Cell number was significantly lower at 0.03% CO_2, which could be observed from the first day on. Since all cultures started with the same cell number, we must conclude that fewer cells survived or became attached to the culture dish on the first day under 0.03% CO_2 conditions.

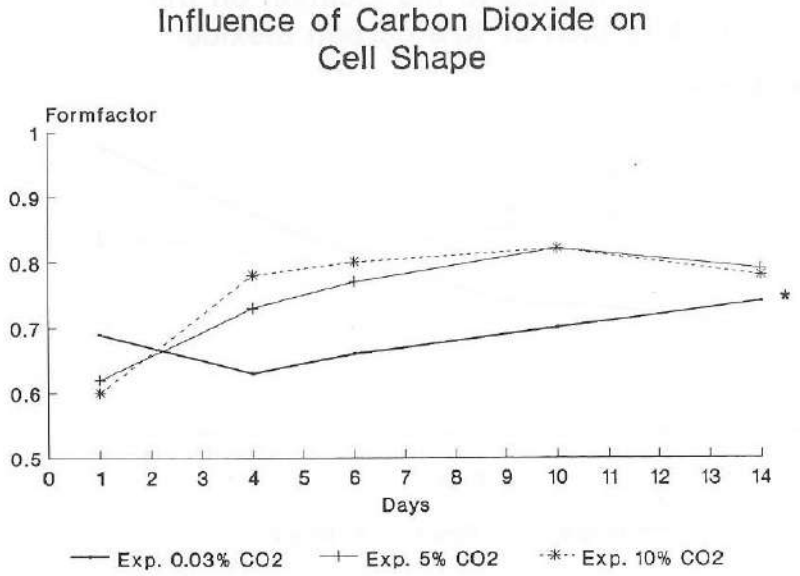


Fig. 9 : *) The 0.03% CO_2 culture displayed a statistically significantly lower form factor than those of the 5 and 10% CO_2 cultures.

The increase in cell number during the remainder of the culture time did not differ statistically from that with the 5 and 10% CO_2 cultures, which suggests that proliferation itself was not influenced by the lowered CO_2 concentration. This is in contrast with findings in earlier studies[6] where the growth curves (based on trypsinization of the culture and cell counting in a Bürker chamber) revealed that the proliferation retarded at 0.03% CO_2.

Furthermore, the 0.03% culture did not show confluence of cells even after 14 days, in contrast to the 5 and 10% CO_2 cultures, showing confluence from day 6 on. An explanation might be that the nine photographs did not accurately represent the actual cell number on the dish plate, but gave an overestimation of the cell number for the 0.03% CO_2 experiment.

The set of oxygen experiments showed less marked changes in morphology. If we consider an O_2 concentration of 10% as normal[1-3] then an elevation to 21% did not influence either the morphology or the proliferation rate of the epithelium. In contrast to cultures of cells of other types, e.g., amniotic fluid[7,8] cells or fibroblasts,[9,10] a lower oxygen concentration of 5%, did not lead to enhanced proliferation rate. On the contrary, at 5% O_2 the proliferation decreased after day 6 and cell size simultaneously increased. This resulted on day 14 in a statistically significantly lower cell number and larger cell area compared to the 10 and 21% O_2 cultures. The effect of 5% O_2 on the proliferation rate can be compared with the growth retardation of human keratinocytes cultured at 5% oxygen.[13]

Enlargement of cell size was found in keratinocyte cultures to be closely related to cornified envelope formation and terminal differentiation.[14-16]The process of terminal differentiation will lead *in vivo* to cell death and the formation of a stratum corneum[17] Our earlier studies[6] showed no such correlation in cultured middle ear epithelium of the rat, since the increase in average cell size of the 0.03% CO_2 or 5% O_2 cultures, did not lead to increased cornified envelope formation.

Except in the 0.03% CO_2 experiment, the morphology of cultured middle ear epithelium closely resembled the keratinocyte culture when performed under the same conditions.[14] In both cases flat polygonal cells with cornified envelopes lying above the plane of the monolayer were observed.[14] Such cornified envelopes indicate that middle ear epithelium can display spontaneous terminal differentiation *in vitro*.

Concerning the calculated average form factor we saw in all cultures that the lowest values occurred on day 1, probably due to the presence of the very irregular 3T3 feeder cells. Most cultures showed from day 6 on, an epithelium with regular polygonal cells and no differences in form factor were found except for the 0.03% CO_2 culture. The large cells of that culture varied in morphology and led to a statistically significantly lower form factor compared to the other cultures.

Because the cultures were consistently studied from center to periphery, we could find out whether certain phenomena had a relation to the location on the dish surface. Generally, on day 1 we observed, the highest cell number in the center, possibly due to the handling (swirling) of the culture dish and underscores the importance of examination of the entire culture.

There was a time lapse of one year between the two studies, and Exp. 6 of the O_2 study could serve as control experiment, because the same gas concentrations were used as in Exp. 3 of the CO_2 study. Both cultures showed regular polygonal cells with no differences in average cell shape. However Exp. 6 displayed significant differences in cell number and cell size, indicating a possible difference in culture conditions. For this reason, the results of the CO_2 and O_2 studies were not compared. A shift in the CO_2 concentration from 5%(Exps. 4 and 5) to 10% (Exp. 6) within the oxygen study was not considered to have influenced the cell cultures. This assumption was made because the carbon dioxide study revealed no differences in cultures grown at 5 and 10% CO_2 in combination with 21% O_2.

CONCLUSION

The morphology and growth of middle ear epithelium in culture is influenced by changes in the extra-cellular CO_2 concentration to a level, that will occur *in vivo* in case with a tympanic membrane perforation. The results of this study also indicated that a reduced O_2 concentration of 5% may led to less optimal culture conditions. In earlier studies we found no change in terminal differentiation of middle ear epithelium cultured under the gas conditions used in this experiment, which means that the changes in morphology and proliferation rate were not correlated with a change in the terminal differentiation.

REFERENCES

1) Grontved A, Moller A, Jorgensen L. Studies on gas tension in the normal middle ear. Acta Otolaryngol(Stockh) 1990;109:271-277.

2) Melvill Jones G. Pressure changes in the middle ear after altering the composition of contained gas. Acta Otolaryngol 1961; 53:1-11.

3) Shinkawa H, Sasaki Y, Inamura N et al. Middle ear pressure and gas diffusion. In:Lim DJ, Bluestone CD, Klein JO, Nelson eds. Recent advances in otitis media. Proceedings of the fourth international symposium. Kugler Publications Decker ,Toronto 1988 abstr.29

4) Sadé J, Weisman Z. The Phenotypic expression of middle ear mucosa. In: McCabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977; 52-61.

5) Smyth GDL, Hall S. Aetiology and treatment of persistent middle ear effusion. J. Laryngol Otol 1983; 97:1085-1089.

6) Lutgert HW, van Blitterswijk CA, Grote JJ. The effect of external stimuli on rat middle ear epithelium in culture. II -extracellular carbon dioxide concentration- Acta Otolaryngol(Stockh) In Press

7) Held KR, Sonnichsen S. The effect of oxygen on colony formation and cell proliferation of amniotic fluid cells in vitro. Prenatal diagn 1984; 4:171-179.

8) Brackertz M, Kubbies M, Feige A et al. Decreased oxygen supply enhances growth in culture of human mid-trimester amniotic fluid cells. Hum Genet 1983; 64:334-338.

9) Balin AK, Goodman DBP, Rasmussen H et al. The effect of oxygen tension on the growth and metabolism of WI-38 cells. J Cell Physiol 1976; 89:235-250.

10) Bradley TR, Hodgson GS, Rosendaal M. The effect of oxygen tension on hemopoietic and fibroblast cell proliferation in vitro. J Cell Physiol 1978; 97:517-522.

11) Weisman Z, Sadé J. Effect of environmental CO_2, O_2 and pH on the growth of respiratory epithelium in vitro. Ann Otol Rhinol Laryngol 1979;88:21-30.

12) Drucker I, Weisman Z, Sadé J. Tissue culture of human adenoids and of middle ear mucosa. Ann Otolog Rhinol Laryngol 1976;85:327-333

13) Horikoshi T, Balin AK, Carter DM. Effect of oxygen on the growth of human epidermal keratinocytes. J Invest Dermatol 1986; 86:424-427.

14) Watt FM, Green H. Stratification and terminal differentiation of cultured epidermal cells. Nature 1982;295:434-436.

15) Watt FM. Keratinocyte cultures: an experimental model for studying how proliferation and terminal differentiation are co-ordinated in the epidermis. J Cell Science 1988;90:525-529

16)Watt FM, Jordan PW, O'Neill CH. Cell shape controls terminal differentiation of human epidermal keratinocytes. Proc Natl Acad Sci USA 1988;85:5576-5580

17) Green H. Terminal differentiation of cultured human epidermal cells. Cell 1977;11:405-416

18) Van Blitterswijk CA, Ponec M, v Muijen GNP et al. Culture and characterization of rat middle ear epithelium. Acta Otolaryngol(Stockh) 1986;101:453-466

CHAPTER VI

PRIMARY ACQUIRED AND RECURRENT CHOLESTEATOMA *VERSUS* RESIDUAL CHOLESTEATOMA

HW Lutgert, CA van Blitterswijk, JJ Grote

E.N.T. Department, University Hospital Leiden, The Netherlands

SUMMARY

A comparative morphological study was performed between the primary acquired and recurrent cholesteatoma on the one hand and the residual type on the other. Between these two groups of cholesteatomas, one can distinguish differences in the pathogenesis and clinical features which may have therapeutic implications. This study, based on light- and electron microscopy, revealed no essential differences in morphology between the two groups of cholesteatoma. In particular, infiltration of matrix into subepithelial tissues could be found in cholesteatoma both with and without signs of inflammation or infection in the perimatrix, and this phenomenon could be applied to both types of cholesteatoma. This morphological uniformity suggests that the differences in clinical features and pathogenesis should not influence the otologist's choice of therapeutic approach. The results of this study emphasize the importance of removing as much as possible of the adjacent tissues during eradication of the cholesteatoma, regardless of clinical type of cholesteatoma or signs of infection

INTRODUCTION

Until the middle of this century, most cases of cholesteatoma were treated surgically either by radicalization or by transmeatal attico-antrotomy. In the 1950's, however, the intact canal wall technique or combined approach was introduced to preserve as much as possible of the hearing system and to avoid the problems associated with a non-self-cleaning cavity. The introduction of this new technique led to a new clinical type of residual cholesteatoma. Such residual cholesteatomas originating from a remnant of incompletely removed cholesteatoma matrix often develop behind an intact ear drum without evidencing signs of clinical infection or inflammation.[1] At present, five clinically distinct types of cholesteatoma are distinguished :

1) The primary acquired type, comprising cholesteatomas associated with a history of recurrent subacute middle ear infection. Induced proliferation of basal cells of Shrapnell's membrane is considered as the pathogenesis of these cholesteatomas. Clinically, a small attic perforation can be seen, via which the cholesteatoma communicates with the external ear canal.[2]

2) The secondary acquired cholesteatoma is associated with an acute necrotizing otitis media, leading to a posterosuperior perforation. Also, chronic functional obstruction of the Eustachian tube with subsequent retraction pockets in the posterosuperior or attic region can cause this type of cholesteatoma.[2,3]

3) The congenital cholesteatoma, which is considered to be the result of embryonic inclusion of squamous epithelium and develops behind an intact ear drum.[4]

4) The residual type, defined as an outgrowth of incompletely removed epithelium (matrix), occurring after attempted eradication of the lesion, mostly behind an intact ear drum.[1]

5) The recurrent cholesteatoma, which originates from retraction pockets of the ear drum into the middle ear cleft, generally after cholesteatoma surgery has been performed with a canal wall-up technique.[1]

Cholesteatoma after acute necrotizing otitis media and the congenital cholesteatoma are seldom seen and will not be discussed in this paper. Between the primary acquired and the recurrent cholesteatomas on the one hand and the residual type on the other, there are differences in the clinical features and the pathogenesis, as follows. *First*, the primary acquired and the recurrent cholesteatomas are continuous with the epidermis of the ear drum and the external ear canal, whereas the residual type has no direct connection with the existing epidermis. *Secondly*, the primary acquired and recurrent types are in direct contact with the external environment or the open air, whereas the residual type develops behind an usually intact ear drum. As many studies have indicated, the gas composition in the middle ear may differ from that of the open air, especially as to the proportions of CO_2 and O_2.[5,6] *Third*, the primary acquired and recurrent cholesteatomas arise due to active ingrowth of epidermis into the middle ear cleft. Chronic middle ear infections and tubal dysfunction are considered to be causative factors.[7,8] The residual type probably represents nothing more than an outgrowth of incompletely removed cholesteatoma matrix, often occurring in a non-infected environment. The differences in the pathogenesis and clinical features of the primary acquired and the recurrent cholesteatoma on the one hand and the residual type on the other raises the question as to whether different approaches to treatment are required. Since the answer to this question may lie in the histological and morphological appearances of the particular cholesteatoma, we attempted to define the morphology of each of the clinical types of cholesteatoma objectively by light and electron microscopy.

MATERIALS AND METHODS

Ninety cholesteatomas were obtained during surgery. Classification of the material according to primary acquired, recurrent, or residual types was based on the case history and clinical findings.

Table I. *Age and sex distribution (N=70)*

Type of Cholesteatoma	N	Male	Female	Age group in years 0-15	15-45	>45
Primary acquired	21	13	8	4	10	7
Recurrent	21	16	5	4	13	4
Residual	28	15	13	4	20	4

In this paper use is made of the terms matrix and perimatrix as defined by Lim.[9] Matrix refers to the cornifying epithelium itself and perimatrix to the subepithelial tissues. Twenty specimens were excluded because no matrix tissue was present; the material consisted of keratin masses and granulation tissue, indicating an origin from a cholesteatoma or epidermis, but a histological diagnosis could not be made. The other 70 specimens were classified as indicated in Table I.

All cholesteatomas were studied by light microscopy and five specimens of each group by transmission electron microscopy. Samples reserved for light microscopy were fixed immediately in a 1.5% glutaraldehyde solution in sodium cacodylate buffer (4 °C, pH 7.4, 0.14 M) for 16 h. After dehydration and celloidin treatment, the tissue was embedded in paraffin wax. Tissue section were cut 7μm thick and stained with hematoxylin-eosin.

For transmission electron microscopy the specimens were first fixed as for light microscopy and then rinsed with phosphate-buffered saline (ph 7.4), incubated in a 1% O_sO_4 PBS solution at room temperature for 30 min[10], dehydrated in graded alcohol series, and then embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Philips EM 201 electron microscope.

RESULTS

General morphology

The gross morphological appearance showed the following features. Only a few of the specimens had the classic pearl-like appearance, presenting a closed round sac composed of a thin matrix and perimatrix covering cornified debris[11] (Fig. 1A).

Six cholesteatomas of this type were seen, all in the residual group. Most of the other cholesteatomas showed an open lobed sac of matrix surrounded by subepithelial tissue (perimatrix) [11] (Fig. 1B).

Matrix

The thickness of the matrix was measured light microscopically, the number of cell layers being counted from the stratum basale up to the stratum granulosum. Individual cells of the granular cell layer can no longer be distinguished by light microscopy in this phase. A matrix was considered thin when the number of cell layers of the stratum basale and stratum spinosum did not exceed five. Intermediate thickness was taken as 5 to 15 cell layers, and thick as a matrix with more than 15 cell layers. As a reference, we took the skin of the external ear canal, where the number of cell layers forming the matrix ranges between 5 and 15. Thickness varied widely within cholesteatomas(Fig. 2), and each specimen showed at least two of the above-mentioned categories of matrix. According to our standards there were no differences in thickness of the basal layers of the three types of cholesteatoma (Table II).

The four cell layers of the matrix, i.e., the strata basale, spinosum, granulosum, and corneum will be discussed separately. In general, the morphology of all types of cholesteatoma was rather similar, and therefore no distinction will be made between the three types in the discussion of the results unless otherwise mentioned.

Basal layer: The cells of the basal layer were squamous, cuboidal, or cylindrical and the morphology varied within the same cholesteatoma. Most of the specimens showed at least two types of basal cells, mainly squamous and cuboidal. These cells generally occurred in a matrix with no more than 15 cell layers. Cylindrical cells were seldom seen in matrices with less than five cell layers(Table III). Locally in specimens with a thin matrix, squamous cells occurred in both the stratum basale and spinosum, which made it impossible to distinguish between these two layers light microscopically. Transmission electron microscopy(TEM) revealed that the cells of the basal layer were attached to the basal lamina by hemi-desmosomes, whereas the intercellular contact with the spinosal layer occurred via normal desmosomes. These desmosomes were rarely found between the basal cells themselves. Many pseudopodia were seen, especially among cuboidal and cylindrical cells. As a rule the basal lamina was intact, but locally, basal cells extended small finger-like protrusions into the perimatrix, and at such sites the basal lamina seemed to be disrupted. The cytoplasm of the basal cells contained bundles of tonofilaments, free polyribosomes, and occasionally mitochondria.

Spinosal layer: The cells of the spinosal layer can be readily distinguished from the other layers by their intercellular processes which are interconnected by numerous desmosomes, as was shown by TEM.

The cytoplasm had no special features distinguishing it from that of the basal cells. Tonofilaments seemed to be more abundant and tended to be more often aggregated into bundles which converged and terminated in the desmosomes. In the uppermost spinosal cell layers, Odland bodies were seen close to or fusing with the cell membranes adjoining the stratum granulosum.

Table II. *Thickness of matrices of the clinically differing cholesteatoma*

Type of Cholesteatoma	+	+ +	+ + +	Total
Primary Acquired	6	6	9	21
Residual	12	2	7	21
Recurrent	12	3	13	28

Grading:
+ = thin and intermediate matrix(1-5 and 5-15 cell layers resp.)
+ + = intermediate and thick matrix(5-15 and > 15 cell layers resp.)
+ + + = all three types of matrix(thin, intermediate and thick) present.

Occasionally, Langerhans cells were identified; these cells were not linked to other cells by desmosomes. The cytoplasm was more electrolucent than that of surrounding cells of the spinosal layer and contained "tennis rackets" and "rod-shaped" structures.[12] No tonofilaments were seen in the cytoplasm of the Langerhans cells.

Granular layer: The granulosum cells were identified by the presence of kerato-hyalin granules, visible by both light and electron microscopy. The cells were flattened and lay parallel to the surface. The occurrence of kerato-hyalin granules seemed to be closely associated with the dense clusters of tonofilaments.

Initially, the appearance of the cell membranes seemed to be normal, but gradually there emerged an electron-dense layer in close association with the cell membrane, forming the cornified envelope at the transition to the stratum corneum (Fig. 4). [13] The size of the kerato-hyalin granules increased and the normal cell contents disappeared.

Stratum corneum: This layer is characterized by dead squamous cells with a clear cornified envelope. The kerato-hyalin granules had vanished and the cells were filled with amorphous material showing a reticular pattern.

Perimatrix

The subepithelial tissue or perimatrix was examined with special attention for signs of infection or inflammation. TEM showed that the perimatrix of non-infected or non-inflamed cholesteatomas was composed predominantly of fibrocytes and an abundance of collagen and reticular fibers in the intercellular spaces.

Areas of inflammation generally showed an infiltrate containing round cells, i.e., clusters of lymphocytes, plasma cells, or macrophages, and/or combinations of these cell types. Polymorph nuclear leukocytes indicating an acute infection were seldom seen. Round cell infiltration was observed in all three types of cholesteatoma but, as Table IV shows, inflammation was seen more often in primary acquired and recurrent cholesteatomas than in the residual type. Cholesterol granulomas characterized by the typical needle-like clefts and surrounded by heavily infiltrated granulation tissue were not seen frequently (Table V).

In our material we were always able to identify giant cells in the immediate vicinity of the cholesterol clefts, but such cells were also detected in the absence of cholesterol granulomas. In a few cases a single or double layer of squamous or cuboidal cells covered the perimatrix and probably represented the middle ear mucosa. In most of the specimens, however a middle ear mucosa could not be recognized either light or electron microscopically. Ingrowth of matrix into the subepithelial tissues was studied too, e.g. papillary projections of the basal layers(Fig. 2) (strata basale and spinosum) and/or invasion of the perimatrix by the entire matrix. To establish correlation, if any, between signs of ingrowth of the matrix and the presence of inflammation in the perimatrix, we recorded cases of concurrence (Table IV).

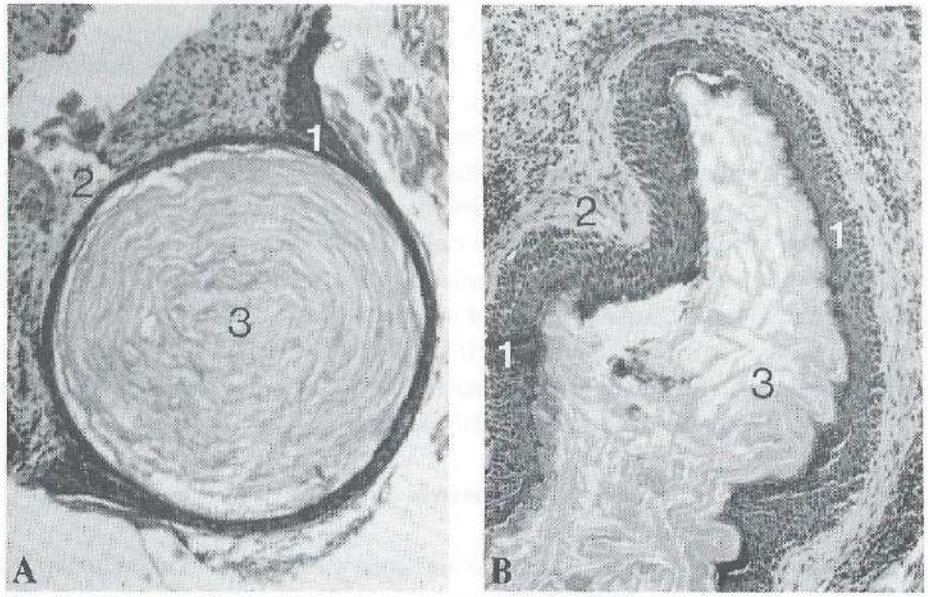


Fig. 1(A) Cholesteatoma presenting as a round, closed sac filled with epithelial debris, seen only in the residual group.
Fig. 1(B) Cholesteatoma showing an open, lobed sac, surrounded by perimatrix seen in all three types of cholesteatoma (1) matrix, (2) perimatrix, (3) keratin.

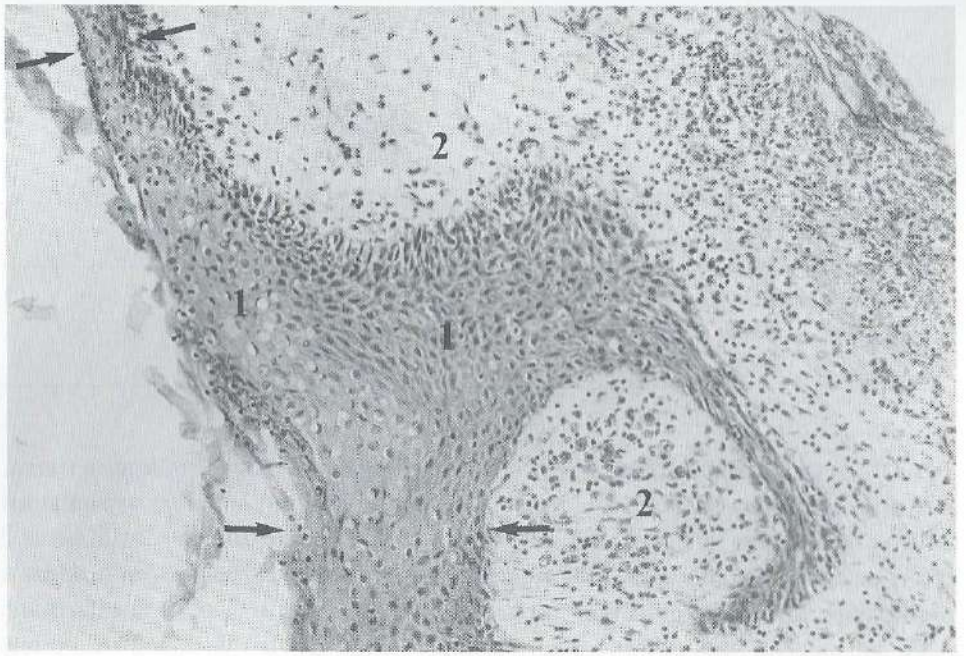


Fig. 2. Matrix (1) of cholesteatoma showing infiltration in perimatrix (2) and variation in thickness of matrix(*arrows*).

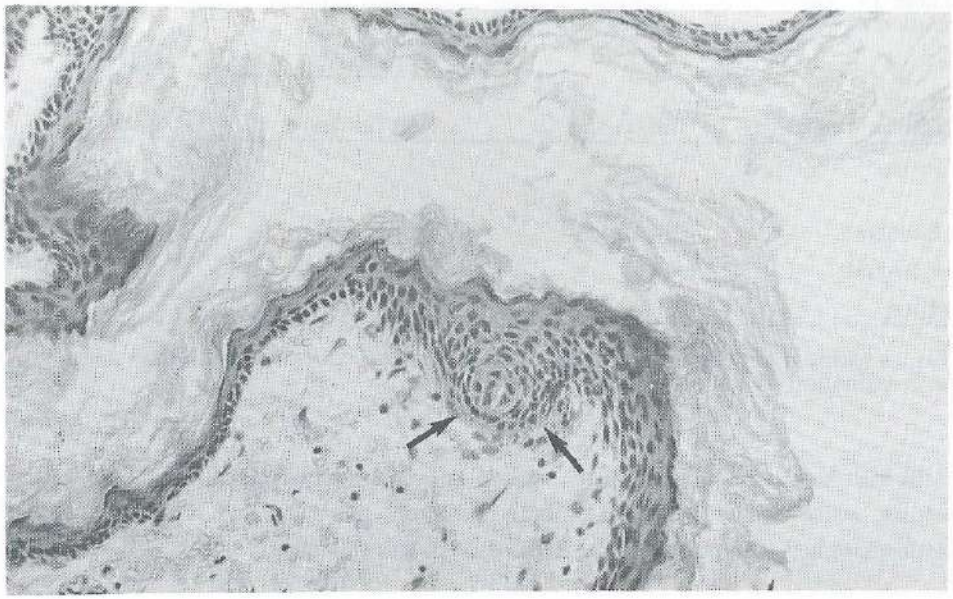


Fig.3. Whorl formation in cholesteatoma matrix.

Table III. *Distribution of morphological types of basal cells, in relation to thickness of matrix and type of cholesteatoma*

Type of basal cell	Number of cell layers in matrix								
	Prim. Acquired			Recurrent			Residual		
	1-5	5-15	>15	1-5	5-15	>15	1-5	5-15	>15
Squamous	15	6	1	15	2	0	21	4	0
Cubical	10	12	4	13	16	4	13	18	3
Cylindrical	1	10	7	1	9	5	2	6	5

Twelve out of 31 cholesteatomas with an inflamed perimatrix had an infiltrative matrix, but nine specimens showed papillary projections or infiltration in a non-inflamed environment. These phenomena were seen in the group of primary acquired and recurrent cholesteatomas and in the residual group. In some specimens with or without proliferation of basal layers, whorl formation was seen locally (Fig. 3). The cells of the basal and spinosal layers were now arranged circularly, and thus disrupted the normal structure of the matrix.

Table IV. *Distribution of inflamed cholesteatoma and occurrence of invasive matrix in relation to type of cholesteatoma and inflammation*

Type of Cholesteatoma	Perimatrix		
	Inflamed	Non-Inflamed	Absent
Primary Acquired	15 (3)	6 (1)	0
Recurrent	9 (4)	9 (4)	3
Residual	8 (5)	16 (4)	4
Total	32 (12)	31 (9)	7

Values within parentheses are numbers of cholesteatoma showing papillary projections or invagination of complete matrix in perimatrix.

Cells with kerato-hyalin granules or signs of keratinization were not found within these whorls, which occurred in one primary acquired, two recurrent, and two residual cholesteatomas. The perimatrix of each of them was inflamed.

Table V. *Cholesterol granuloma and occurrence of giant cells in the various types of cholesteatoma.*

Type of Cholesteatoma	Cholesterol Granuloma	Giant Cells
Primary Acquired	3	5
Recurrent	1	4
Residual	2	5
Total	6	14

DISCUSSION

A comparative study on the morphology of primary acquired and recurrent cholesteatoma on the one hand and of the residual cholesteatoma on the other has to our knowledge not been performed before. We wanted to find out whether the differences in the pathogenesis and clinical features of these two groups of cholesteatoma could have therapeutic implications, especially because the residual cholesteatoma is not caused by an active ingrowth of epithelium into the middle ear cleft, but is rather the result of an outgrowth of residual matrix not induced by a chronic middle ear infection or tubal dysfunction. To answer this question, we undertook a comparative morphological study based on light and electron microscopy.

Analysis of the chosen parameters showed no obvious differences except for two findings concerning the gross morphology. In the first place, the classic pearl-like appearance of a cholesteatoma composed of a matrix forming a closed round sac filled with concentric keratin layers, was only found in the residual cholesteatoma; and in the second place, signs of inflammation or possible infection were seen more often in the primary acquired and recurrent cholesteatomas than in the residual type. No features of the composition or thickness of the matrix were found to be specific for a given type of cholesteatoma. All of the specimens showed a cornifying epithelium in which the four cell layers(basale, spinosum, granulosum, and corneum) could be readily recognized.

Thickness varied widely within cholesteatomas, locally in cases of a thin matrix no distinction could be made between the basal or spinosal layer. Whorl formation occurring in some cholesteatomas has been described before,[11,13] but it is not certain whether this phenomenon belongs to the precursor stage of the micro-cholesteatoma. In our material, no granulosum cells or signs of keratinization were found within these whorls. Many authors suggest a correlation between inflammatory reactions in the perimatrix and signs of active ingrowth of matrix into the surrounding subepithelial tissues, e.g. papillary projections of basal layers or invasion by the complete cholesteatoma matrix.[14-17]

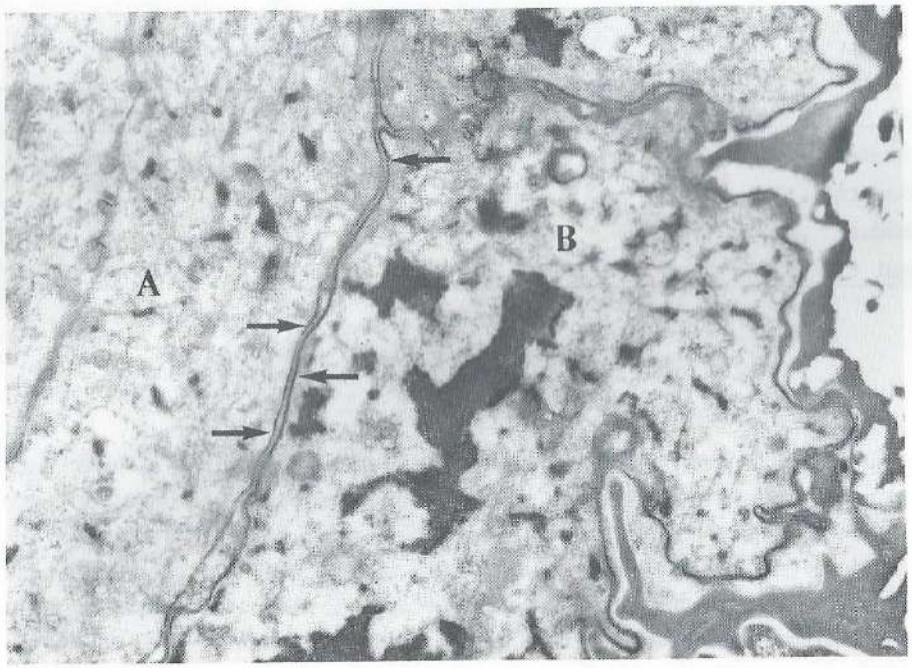


Fig. 4: TEM picture showing the transition of spinosal (A) to granular layer (B). Note the formation of the cornified envelope (*arrows*).

In our material, signs of an infiltrative matrix were not always restricted to cholesteatomas with an inflamed perimatrix, but also occurred in some of the specimens without marked round-cell infiltration. However, the presence of inflammation before surgery cannot be ruled out, and it is also possible that some specimens contained a non-representative part of the subepithelial tissues. Furthermore, it is not certain that the parameters we took as light- and electron microscopical signs of ingrowth are representative of clinically invasive cholesteatomas.

CONCLUSIONS

On the basis of the present results we must conclude that there are no essential differences between the morphology of the two groups of cholesteatoma. If the morphology is determined by pathogenesis, it could be concluded that the primary acquired, recurrent, and residual cholesteatomas arise from the same source, which would be in agreement with current opinions about cholesteatoma pathogenesis.

Most otologists believe that these lesions originate in some way or another from the existing epidermis of the external ear canal or ear drum.[3,7,15] The metaplastic origin of cholesteatoma[4] cannot be totally neglected. In our material, however, no observations could be made to sustain this theory.

The morphological uniformity means that the differences in clinical features and pathogenesis should not influence the otologist's choice of therapeutic approach. Although we cannot be certain that the parameters we applied for invasive behavior of the matrix are representative of the clinical situation, there is every reason to underscore the importance of removing as much subepithelial tissue as possible to decrease the risk of the development of residual cholesteatomas[18] regardless of either the presence of signs of infection or the clinical type of cholesteatoma.

REFERENCES

1) Sheehy JL, Brackmann DE, Graham MD. Cholesteatoma surgery. Residual and recurrent disease. Am Otol Rhinol Laryngol 1977;86:452-62.

2) Ruedi L. Pathogenesis and surgical treatment of the middle ear cholesteatoma. Acta Otolaryngol(Stockh) 1979;suppl 361:14-26

3) Fisch U. Tympanoplasty and stapedectomy. Stuttgart, New York: Thieme, Stratton, 1980;35-9

4) Sadé J, Babiaki A, Pinkus G. The metaplastic and congenital origin of cholesteatoma. Acta Otolaryngol(Stockh) 1983;96:119-29

5) Elner A. Normal gas exchange in the human middle ear. Am Otol Rhinol Laryngol 1976;Suppl 25:161-4

6) Ingelstedt S, Jonson B. Mechanisms of the gas exchange in the normal human middle ear. Ann Otol Rhinol Laryngol 1966;Suppl 224:452-61

7) Fernandez C, Lindsay JR, Moskowitz M. Some observations on the pathogenesis of middle ear cholesteatoma. Arch Otolaryngol 1959;69:537-46

8) Wolfmann DJ, Chole RA. Experimental retraction pocket cholesteatoma. Ann Otol Rhinol Laryngol 1986;95:639-44

9) Lim DJ, Saunders WH. Acquired Cholesteatoma, Ann Otol 1972:2-15

10) Van Blitterswijk CA, Ponec M, van Muijen GNP, Wijsman MC, Koerten HK, Grote JJ. Culture and characterization of rat middle ear epithelium. Acta Otolaryngol(Stockh) 1986;101:453-66

11) Lim DJ, Birck HG, Saunders WH. Aural cholesteatoma and epidermization; a fine morphological study. In: McGabe BF, Sade J, Abramson M, eds. Birmingham, Alabama:Aesculapius Publishing Co, 1977:233-52

12) Gantz BJ. Epidermal Langerhans cells in cholesteatoma. Ann Otol Rhinol Laryngol 1984;33:150-6

13) Broekaert D, Cooreman K, Couche P, et al. Keratinization of aural cholesteatoma. In: Sadé J, ed. Cholesteatoma and mastoid surgery. Amsterdam: Kugler, 1981;161-73

14) Rüedi L. Acquired cholesteatoma. Arch Otolaryngol 1963;78:44-53

15) Nager GT. Cholesteatoma of the middle ear; pathogenesis and surgical indications. In: McGabe BF, Sadé J, Abramson M,eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;193-203

16) Steinbach E. Die Pathogenese des Cholesteatoms. Arch Otorhinolaryngol 1981;231:547-82.

17) Jackson DG, Lim DJ. Morphology of the advancing front of cholesteatoma, experimental and human. Acta Otolaryngol(Stockh) 1978;86:71-88

18) Palve T, Makinen J. Why does middle ear cholesteatoma recur? Arch Otolaryngol 1983;109:513-8

CHAPTER VII

CYTOKERATIN PATTERNS OF TISSUES RELATED TO CHOLESTEATOMA PATHOGENESIS

CA van Blitterswijk, HW Lutgert, JJ Grote, SC Hesseling
CJ Out, GNP van Muijen, JAM Fransen
E.N.T. Department, University Hospital Leiden, The Netherlands

SUMMARY

Specimens of cholesteatoma matrix, meatal epidermis, and middle ear epithelium were removed during surgery, and immunohistochemical techniques were used to investigate cytokeratin expression. The use of five chain-specific anticytokeratin monoclonal antibodies and one broad specific anticytokeratin monoclonal antibody showed the divergent behavior of middle ear epithelium compared with the cytokeratin expression of the other two types of epithelium. Middle ear epithelium was characterized by the presence of cytokeratins 4,8,18, and 19, whereas in both cholesteatoma and meatal epidermis cytokeratin 10 predominated. Furthermore, cholesteatoma showed an infrequent focal presence of cytokeratins 4,18 and 19. The similarity between cholesteatoma and meatal epidermis with respect to morphology, and the presence of cytokeratin 10 support an epidermal origin of cholesteatoma. However, a metaplastic origin cannot be excluded, because of the infrequent occurrence of a small amount of cytokeratins 4, 18 and 19 in cholesteatoma matrix that was not found in meatal epidermis but was a component of the cytokeratin pattern of middle ear epithelium.

INTRODUCTION

The origin of cholesteatoma is still unknown. At present, three fundamentally different theories on the pathogenesis of this cornifying epithelium in the middle ear cavity are current, one concerning a congenital origin, one of an epidermal origin, and the third postulating a metaplasia of the middle ear epithelium. The congenital origin[1] seems to apply to only a small proportion of the total number of cholesteatomas. Many authors consider the epidermal origin as the most likely for cholesteatoma, but opinions differ on the exact mechanism by which the epidermis of either the meatal wall or the tympanic membrane invades the middle ear cavity.[2-6] The metaplasia theory[7,8] is supported by those who hold the view that certain stimuli to the middle ear epithelium lead to the development of cholesteatoma. All tissues of epithelial origin are characterized by the presence of cytokeratin. Cytokeratins belong to the intermediate filaments and comprise a family of at least 19 polypeptides with different molecular weights (40 to 68 kd) and isoelectric pH values (pH5.2-7.8).[9,10]

Since each type of epithelium expresses a characteristic set of cytokeratins,[10] which according to the current literature are affected only mildly, if at all, by neoplasia and metastasis,[11] it was thought that comparison of the cytokeratin patterns of cholesteatoma, meatal epidermis, and middle ear epithelium might shed some new light on the pathogenesis of cholesteatoma.

In the present study the cytokeratin pattern of these three tissues was investigated with five chainspecific monoclonal anticytokeratin antibodies directed against cytokeratins 4, 8, 18, and 19. In addition, a monoclonal antibody recognizing most cytokeratins was applied.

MATERIALS AND METHODS

Specimens were obtained from patients requiring middle ear surgery. Seven cholesteatoma, five auditory canal wall epithelium, and five middle ear mucosa specimens were analyzed with respect to cytokeratin expression. For each of these three categories, one specimen from each patient was evaluated. The specimens were placed immediately in phosphate-buffered physiologic saline (pH 7.4) at 4° C and processed within 2 hours after the operation.

Routine transmission electron microscopy: Specimens were fixed in a 1.5% glutaraldehyde solution in sodium cacodylate buffer (0.14 M, pH 7.4, 4° C) for 2 hours, rinsed, and postfixed in 1% osmium tetroxide for 30 minutes at room temperature. Embedding techniques and ultra thin sections were essentially the same as described elsewhere.[12]

Immunohistochemistry: Material was embedded in Tissue-Tek OCT compound, frozen in liquid nitrogen, and stored at -70° C. Cryosections were cut 6μm thick in cryostat and attached to albumin/glycerin-coated object glasses. The object glasses were kept at -20° C and processed for immunohistochemistry within 24 hours. Before the immunostaining procedure the material on the object glasses was thawed for 1 hour at room temperature and fixed in acetone for 10 minutes. Cryosections were incubated in the presence of the anticytokeratin antibody before incubation in a rabbit anti mouse IgG/peroxidase complex. The complex was incubated with 20% pooled human serum to reduce aspecific staining.

Antibodies were visualized by immersion in a 3,3-diaminobenzidine tetrahydrochloride solution containing hydrogen peroxide. For the controls the first incubation step was omitted. After the immunostaining the sections were stained in hematoxylin for 30 seconds, dehydrated in a graded alcohol series, and embedded in DPX mountant.

Table I. *Cytokeratins and corresponding chain-specific monoclonal antibodies*

Cytokeratin	Monoclonal Antibody
Cytokeratin 4	6B10
Cytokeratin 8	M20
Cytokeratin 10	RKSE60
Cytokeratin 18	M9
Cytokeratin 19	LP2K
Broad specific	C80

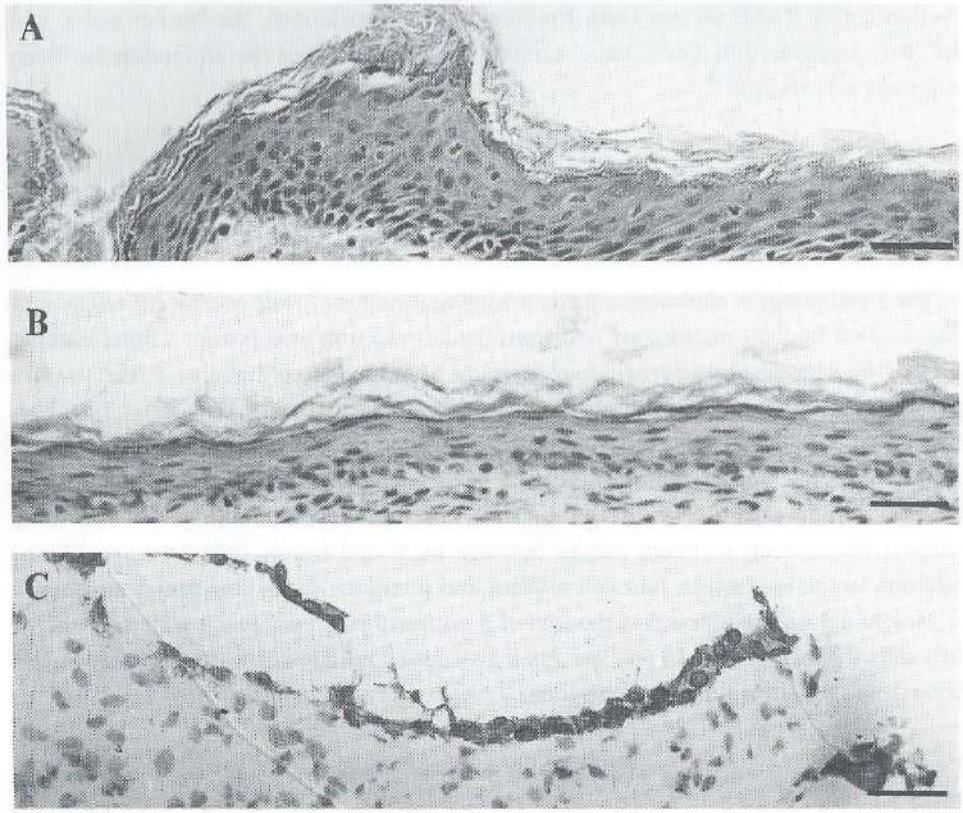


Fig. 1 Light micrographs showing morphology of A) cholesteatoma matrix, B) meatal epidermis, and C) middle ear epithelium(Bars-8 μm).

Transmission electron microscopic immunocytochemistry: Some of the specimens were fixed for 2 hours in a phosphate-buffered (pH 7.4, 4° C) 1% formaldehyde solution to which 0.1% glutaraldehyde had been added. This was followed by fixation and storage in a similar solution, but without glutaraldehyde. Shortly before cryosection, the specimens were impregnated with a 2.3 M sucrose solution for 30 minutes and then frozen in liquid nitrogen. Ultrathin cryosections were cut at -90° C in a Sorvall MT 2B ultramicrotome with a cryoattachment. These cryosections then were incubated with the broad specific anticytokeratin antibody (clone 80) followed by incubation with rabbit anti mouse IgG and protein A gold (10nm). Finally, the cryosections were stained and embedded in 0.3% uranyl acetate with 2% methyl cellulose. These procedures were performed according to Fransen et al.[13]

Monoclonal anticytokeratin antibodies: These antibodies were used as shown in Table I. Monoclonal antibodies M9 and clone 80 were obtained from Sanbio BV (Uden, the Netherlands), RKSE 60 was from Eurodiagnostics (Apeldoorn, the Netherlands), and LP2K was provided by Dr E. Lane, London. The specificity of the antibodies has been reported elsewhere.[14-18]

RESULTS

Morphology

The morphology of cholesteatoma matrix, meatal epidermis, and middle ear epithelium was studied by light microscopy and transmission electron microscopy. Cholesteatoma matrix showed the characteristic four layers of a cornifying epithelium, ie, the stratum basale, stratum spinosum, stratum granulosum. and stratum corneum (Fig. 1A). The overall thickness of the matrix without the stratum corneum ranged between two and twenty cells, the average being eight cells. Meatal epidermis, too, showed the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Fig. 1B). Thickness without the stratum corneum ranged between eight and twenty cells (average 13). In addition to the four strata, hair cell follicles and secretory glands sometimes were seen.

Middle ear epithelium was composed of a noncornifying epithelium generally one to two cells thick (Fig. 1C). In one specimen a stratified epithelium was found (seven cell layers). All cells were flat and polygonal.

Cytokeratin demonstration

Routine transmission electron microscopy revealed the presence of intermediate-like filaments in all three tissues studied (Fig. 2).

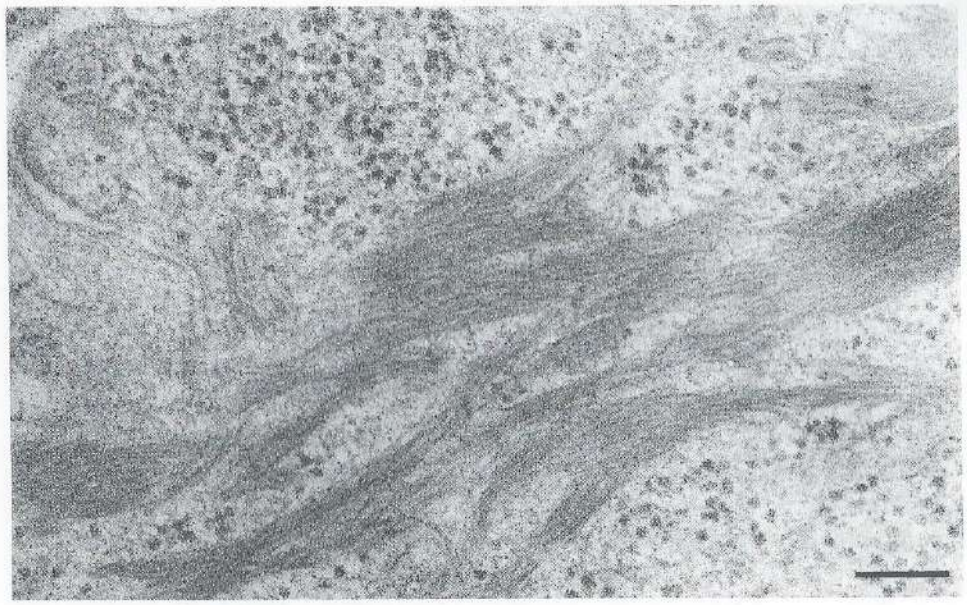


Fig. 2 Transmission electron microscopy showing intermediate-like filaments in cholesteatoma matrix(bar-0.2 μm).

In cholesteatoma and meatal tissues these filaments were most prominent, occupying a large proportion of the cytoplasmic area. Similar filaments rarely were seen in middle ear epithelium. Ultrathin cryosections of cholesteatoma incubated with monoclonal antibody clone 80, which has a broad specificity against cytokeratin, showed positive staining of these filaments, indicating the presence of cytokeratin (Fig. 3). In the immunohistochemical studies, all investigated tissues showed positive staining after incubation with clone 80. Divergence of staining characteristics was found with the other monoclonal antibodies. The exact distribution of these antibodies in the different types of tissue is given in table II. Control studies did not reveal any noteworthy aspecific staining. Cholesteatoma matrix in general did not react positively with monoclonal antibodies 6B10, M20, M9, or LP2K (specific for cytokeratins 4, 8, 18, and 19, respectively).

In two of seven cholesteatomas, however, a minor focal presence of cytokeratins 4, 18, and 19 was seen in the cholesteatoma matrix (Fig. 4A). Cytokeratin 10 occurred in all cholesteatomas; it was seen in the stratum spinosum, stratum granulosum, and stratum corneum, but not in the stratum basale (Fig. 4B).

Meatal epidermis also showed a positive reaction product with RKSE 60 in all strata except the stratum basale (Fig 5). In contrast to the cholesteatoma tissue, no foci of cytokeratins 4, 18, and 19 were observed. Middle ear epithelium , unlike cholesteatoma and meatal epidermis, did not show a positive staining for cytokeratin 10. A positive reaction product was found in all epithelial cells for cytokeratins 4, 8, 18, and 19 (Fig 6). The specimen with a stratified epithelium comprising approximately seven cell layers showed the same staining behavior as the other middle ear epithelium specimens.

DISCUSSION

Several hypotheses have been proposed to explain the pathogenesis of cholesteatoma. When these hypotheses are subdivided on the basis of the epithelium giving rise to the cholesteatoma, a distinction can be made between theories postulating a congenital,[1] epidermal,[2-6] or middle ear epithelium origin.[7,8] The morphologic similarity between cholesteatoma epidermis and the epidermis of the auditory canal wall shown by this and other studies,[19,20] both being stratified cornifying epithelia in contrast to the non cornifying middle ear epithelium, suggests an epidermal origin of cholesteatoma.

Fig. 3 Transmission electron micrograph of cholesteatoma cryosection. Electron-dense gold particles indicate presence of monoclonal antibody clone 80 in intermediate-like filaments. Bar-0.2 μm.

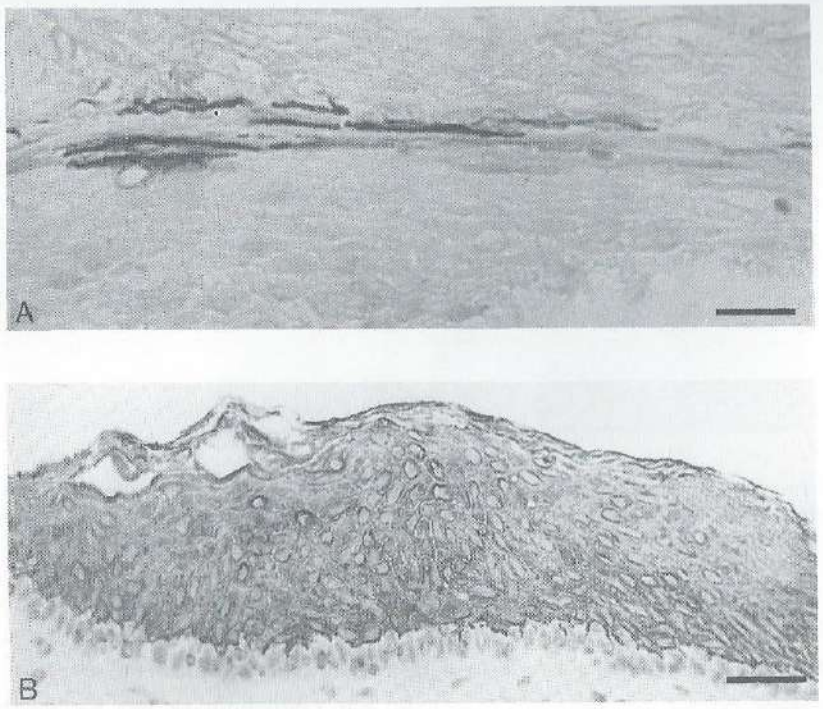


Fig. 4 Cryosections of cholesteatoma. Bars-8 μm. A) positive staining for cytokeratin 4 in small foci, which was seen infrequently. B) reaction product indicating presence of cytokeratin 10. Note absence of staining in stratum basale.

However, according to Sadé et al,[7,8] metaplasia of middle ear epithelium would cause this epithelium to become a stratified cornifying epidermis that could form the basis of cholesteatoma. While this hypothesis seems to be partially supported by experiments in which middle ear epithelium showed terminal differentiation in culture,[21] it has been rejected by Palva et al,[22] who claim that the occurrence of metaplastic middle ear epithelium is limited to the advancing front of cholesteatoma and does not play a major role in the genesis of cholesteatoma.

Since each type of epithelium is characterized by a specific set of cytokeratins[10] and the overall type of cytokeratin expression is not altered after transformation,[11] it may be assumed that the tissue along the area of origin of cholesteatoma will be characterized by a cytokeratin expression similar to that of cholesteatoma. On these grounds it seemed possible that analysis of the cytokeratin pattern of cholesteatoma epidermis, meatal epidermis, and middle ear epithelium would shed some light on the controversy between adherents of the epidermis theory and the metaplasia theory.

The presence of cytokeratin in cholesteatoma and middle ear epithelium has been reported by several authors.[8,21-25]

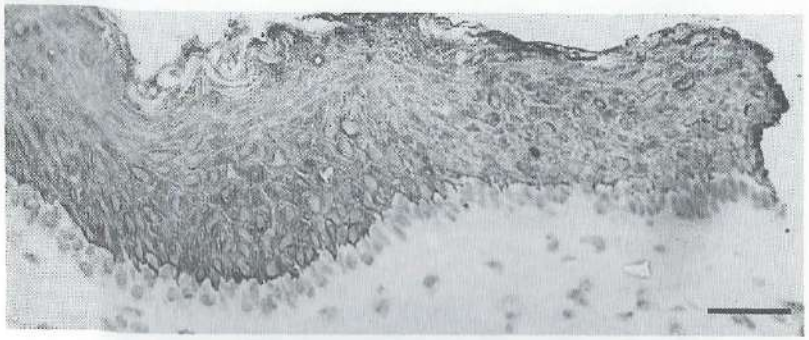


Fig. 5 Immunoperoxidase staining for presence of cytokeratin 10 in meatal epidermis showed same staining characteristics as were found for cholesteatoma. Bar-8 μm.

Only Palva et al [22] observed a difference in cytokeratin pattern between these two types of epithelium, but since these authors used only a polyclonal and one monoclonal antibody, this difference might reflect a single deviating cytokeratin and therefore would not exclude completely the role of middle ear epithelium in the pathogenesis of cholesteatoma.

An effort to determine the cytokeratin pattern of cholesteatoma in relation to human epidermis was made by Gillis et al,[24] who described a less complicated cytokeratin pattern for cholesteatoma on the basis of one-dimensional gel electrophoresis. Unfortunately, the human epidermis used as reference was not meatal epidermis, hence their findings are difficult to interpret in relation to the pathogenesis of cholesteatoma. Results obtained for other epithelial tissues[10] strongly suggest that the morphologic differences between stratified cornifying cholesteatoma and meatal epidermis[19,20] on the one hand and the noncornifying middle ear epithelium on the other would be reflected also by a divergence in cytokeratin pattern.

Our results show that although both cholesteatoma and auditory canal wall epidermis possess cytokeratin 10, this cytokeratin was not expressed by the non-cornifying middle ear epithelium. This is in agreement with the data published by Moll et al,[10] who found cytokeratin 10 in cornifying stratified epidermis, eg, foot-sole epidermis and anal canal epithelium, and not in non-cornifying epithelia such as that of the trachea and esophagus.

The presence of cytokeratin 4, which was found by us occasionally in cholesteatoma matrix and invariably in middle ear epithelium, has been described for stratified epiglottis and esophageal epithelia.[10]

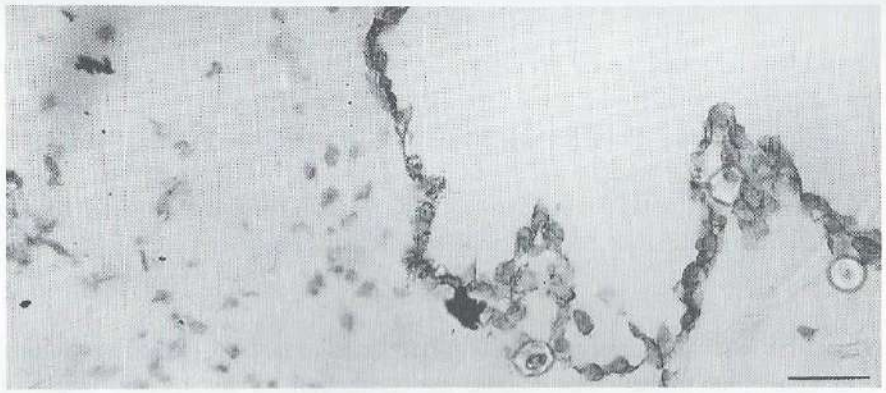


Fig. 6 Cryosection of middle ear epithelium showing presence of cytokeratin 18. Bar-8 μm.

Cytokeratins 18 and 19, which we found in addition to cytokeratin 4 in middle ear epithelium and in focal concentrations in some cholesteatomas, are characteristic features of a number of non-cornifying epithelia such as that of the trachea. Cytokeratin 8, found in middle ear epithelium, also can be demonstrated in the trachea.

In sum, the results of the present study on the expression of cytokeratins in cholesteatoma, meatal, and middle ear epithelia are in general consistent with the cytokeratin patterns of epithelia reported in the literature, although the infrequent observation of minor focal concentrations of cytokeratins 4, 18, and 19 seems to be exceptional.

An evaluation of the cytokeratin expression of cholesteatoma matrix in relation to the cytokeratin patterns in meatal and middle ear epithelia permits three conclusions:

Table II. *Staining characteristics of five chain-specific anticytokeratin monoclonal antibodies*

Epithelia	6B10	M20	RKSE 60	M9	LP2K	C80
Cholesteatoma	F	-	+	F	F	+
Meatus	-	-	+	-	-	+
Middle Ear	+	+	-	+	+	+

F Indicates focal staining
\- Indicates no staining
\+ Indicates positive staining

1. The staining characteristics of cytokeratin observed in cryosections of most cholesteatomas are similar to those seen for meatal epidermis.
2. The cytokeratin pattern in middle ear epithelium differs distinctly from the pattern occurring in most cholesteatomas.
3. Two of the cholesteatomas studied showed a minor focal presence of cytokeratins 4, 18, and 19. These cytokeratins are characteristic of middle ear epithelium and some noncornifying epithelia.

Although on this basis cholesteatoma would be expected to have a relation to auditory canal wall epithelium rather than middle ear epithelium, a metaplastic origin of cholesteatoma cannot be excluded.

Acknowledgments- The authors are indebted to P. van der Hoek, B. van der Lans, J.J. Beentjes, and L.D.C. Verschragen for technical assistance, and to the medical staff and the operating room personnel of the Department of Otolaryngology for providing the surgical specimens.

REFERENCES

1. Derlacki EL, Clemis JD. Congenital Cholesteatoma of the middle ear and mastoid. Ann Otol Rhinol Laryngol 1965;74:706-27

2. Ruedi L. Cholesteatosis of the attic. J Laryngol Otol 1958;72:593-609

3. Abramson M, Gantz BJ, Asarch RG, Litton WB. Cholesteatoma pathogenesis: evidence for the migration theory. In: McCabe B, Sadé J, Abramson M, eds. Cholesteatoma: first international symposium. Birmingham, Ala: Aesculapius Publishing Co, 1977;176-186.

4. Buckingham RA. Etiology of a middle ear cholesteatoma: Kodachrome study of pathogenesis. Ann Otol Rhinol Laryngol 1968;77:1054-8

5. Palva T, Karma P, Makinen J. The invasion theory. In: Sadé J, ed. Cholesteatoma and mastoid surgery. Amsterdam: Kugler, 1982:249-64

6. Lim DJ. Epithelial invasion, contact guidance and contact inhibition. In: Sadé J, ed. Cholesteatoma and mastoid surgery. Amsterdam: Kugler, 1982: 141-52

7. Sadé J. Pathogenesis of attic cholesteatoma: the metaplastic theory. In: McCabe B, Sadé J, Abramson M, eds. Cholesteatoma: first international symposium. Birmingham, Ala: Aesculapius Publishing Co, 1977:212-32.

8. Sadé J, Babiacki A, Pinkus G. The metaplastic and congenital origin of cholesteatoma. Acta Otolaryngol (Stockh) 1983;96:119-29.

9. Steinert PM, Jones JCR, Goldman RD. Intermediate filaments. J Cell Biol 1984;99:22-7

10. Moll R, Franke WW, Schiller DL, Geiger B, Keppler R. The catalogue of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.

11. Moll R, Moll I, Franke WW. Differences of expression of cytokeratin polypeptides in various epithelial skin tumors. Arch Dermatol Res 1984;276:349-63.

12. Grote JJ, van Blitterswijk CA. Acute otitis media: an animal experimental study. Acta otolaryngol (Stockh) 1984;98:239-49.

13. Fransen JAM, Ginsel LA, Hauri HP, Sterchi E, Blok J. Immuno-electronmicroscopical localization of a microvillus membrane disaccharidase in the human intestinal epithelium with monoclonal antibodies. Eur J Cell Biol 1985;38:6-15

14. Van Muyen GNP, Ruiter DJ, Franke WW et al. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos.4 and 13. Exp. Cell Res 1986;162:97-113.

15. Van Muijen GNP, Ruiter DJ, Ponec M, Huiskens-van der Mey C, Warnaar SO. Monoclonal antibodies with different specificities against cytokeratins. Am J Pathol 1984;114:9-17

16. Van Muijen GNP, Warnaar SO, Ponec M. Differentiation related changes of cytokeratin expression in cultures keratinocytes and in fetal, newborn, and adult epidermis. Exp Cell Res 1987;71:331-45.

17. Ramaekers FCS, Putts JJG, Moesker et al. Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumors. Histochemistry 1983;15:691-713.

18. Lane EB, Bartek J, Purkis PE, Leigh IM. Keratin antigens in differentiating skin. Ann NY Acad Sci 1985;455:241-58.

19. Friedmann I. Epidermoid cholesteatoma and cholesterol granuloma. Experimental and human. Ann Otol Rhinol Laryngol 1959;68:57-79.

20. Lim DJ, Saunders WH. Acquired cholesteatoma: light and electron microscopic observations. Ann Otol Rhinol Laryngol 1972;81:2-12.

21. Van Blitterswijk CA, Ponec M, van Myuen GNP, Wijsman MC, Koerten HK, Grote JJ. Culture and characterization of rat middle ear epithelium. Acta Otolaryngol (Stockh) 1986;101:453-66.

22. Palva T, Letho VP, Virtanen I, Mäkinen J. Junctions of squamous epithelium with middle ear epithelium. Acta Otolaryngol (Stockh) 1985;99:297-304.

23. Broekaert D, Cooreman K, Coucke P, et al. Keratinization of aural cholesteatoma (keratoma): a quantitative histophotometric study of the sulphydryl and disulphide content. In: Sadé J, ed. Cholesteatoma and mastoid surgery. Amsterdam: Kugler Publications, 1982:161-73.

24. Gillis E, Kluyskens P, Broekaert D, Coucke P, Nsabumukunzi S, Reyniers P. Structural and topical studies of cholesteatoma proteins, in relation to the keratinization process. Acta Otolaryngol Belg 1980;34:23-33.

25. Kluyskens P, Gillis E, Broekaert D, Coucke P, Nsabumukunzi S, Reyniers P. Further research on solubilization of cholesteatoma keratins. Acta Otolaryngol (Stockh) 1981;91:585-7.

CHAPTER VIII

MORPHOLOGY AND CYTOKERATIN PATTERN OF HUMAN MIDDLE EAR MUCOSA WITH AND WITHOUT A TYMPANIC MEMBRANE PERFORATION

HW Lutgert, CA van Blitterswijk, MJM Smits, GNP van Muijen, JJ Grote
E.N.T. Department, University Hospital Leiden, The Netherlands

SUMMARY

The cytokeratin expression of middle ear epithelium in the presence and absence of a tympanic membrane perforation was studied with the use of monoclonal antibodies against cytokeratins 1,4,5,6,7,8,10,13,18, and 19. Light microscopy, showed a metaplastic change of middle ear mucosa in 8 out of 12 cases with a perforation of the tympanic membrane. In those specimens the mucosa showed three or more cell layers locally. Biopsy specimens of ears with an intact tympanic membrane showed in all cases a mucosa composed of at most two cell layers. The cytokeratin expression was not affected by the presence of a perforation. The terminal differentiation of middle ear epithelium seemed not to be influenced by a change in the gaseous environment. The presence of a tympanic membrane perforation alone, did not lead to a metaplastic change of the middle ear mucosa into a cornifying epithelium.

INTRODUCTION

The pathogenesis of cholesteatoma auris is still unknown. One subject of discussion concerns a postulated metaplastic origin of this disease. The general opinion is that cholesteatoma originate from the existing epidermis of the external ear canal or tympanic membrane. How this cornifying epithelium will invade the middle ear cleft is not completely known yet. The three theories concerning an epidermal origin refer to; migration,[1] retraction,[2] and proliferation,[3] all three modes might occur alone or in combination.

The metaplasia theory assumes that the middle ear mucosa can differentiate into a stratified squamous cornifying epithelium. This process would be induced by changes in its direct environment.[4] Some of the possible factors that might influence the differentiation of middle ear epithelium, were investigated in, *in vitro* studies, done in our laboratory.[5] Cultured middle ear epithelium of the rat showed an altered morphology and proliferation rate when exposed to a gas composition comparable with that of the ambient atmosphere, as may occur *in vivo*, in cases with a tympanic membrane perforation. Cells became very large and the proliferation rate was slowed down, when compared to cultures exposed to gas values assumed to occur in the intact middle ear.

However, the terminal differentiation was not significantly influenced.[6] Sadé[7] reported that *in vivo*, a metaplastic shift of the mucosa into a cornifying epithelium was found when a tympanic membrane perforation was present. A change in the environmental gas composition was considered responsible for this phenomenon. The gaseous atmosphere in the intact middle ear concerning the CO_2 and O_2 concentrations differs strongly from that of the open air.[8,9] The CO_2 concentration is much higher (±5% versus 0.03%) and the O2 concentration a twofold lower (± 20.9% versus 10%).

A metaplastic change of mucosa into a cornifying epithelium may be coupled with a change in the cytokeratin pattern. Cytokeratins are cytoplasmic proteins belonging to the intermediate filaments, and occur in all tissues of epithelial origin.[10,11] At present 19 different cytokeratins have been identified, and each type of epithelium expresses a characteristic set of these cytokeratins. There are essential differences in cytokeratin patterns between simple, non-cornifying and cornifying epithelia.[11] Recently, van Blitterswijk et al.,[12] reported on the cytokeratin patterns of middle ear mucosa, cholesteatoma and epidermis. This study showed a different cytokeratin expression between cornifying(epidermis) and non-cornifying epithelia(middle ear mucosa).

A shift in the environmental CO_2 and O_2 concentrations may influence the differentiation and subsequently the cytokeratin pattern of middle ear epithelium. In this experiment we studied the morphology and cytokeratin pattern of human middle ear mucosa in the presence and absence of a tympanic membrane perforation. The cytokeratin expression was determined by incubating the specimens with 10 different monoclonal antibodies against cytokeratins 1,4,5,6,7,8,10,13,18, and 19.

MATERIALS AND METHODS

Biopsy specimens of the mucosa of the promontory were taken from 7 patients with an intact tympanic membrane, and from 12 patients with a central perforation. In the former group two specimens proved to have insufficient material and were excluded from this study. Age, sex distribution and other parameters are showed in Table I and will be discussed in due course. The first group was operated on otosclerosis or ossiculoplasty and had no history of cholesteatoma. For the second group the following conditions were considered obligatory:

1) No history of middle ear infection or otorrhoea during the last three months.
2) No history of cholesteatoma or previous middle ear operations for eradication of that disease.
3) No marginal perforations.
4) Ears with retraction of the tympanic membrane or adhesions between the eardrum and the medial wall of the middle ear cleft were excluded.

After removal, the specimens were immediately placed in 4°C phosphate-buffered physiological saline(pH 7.4). Within 2 hours these biopts were embedded in Tissue-Tek OCT compound, frozen in liquid N_2 and stored at -70°C. Cryosections were cut 4-6μm thick in a cryostat and attached to albumin/glycerin-coated object glasses, which were kept at -20°C and were further processed for immunohistochemistry within 24 hours. Before the immuno staining procedure, the object glasses were thawed, dried for 1 hr at room temperature, and fixed in acetone for 10 minutes. Cryosections were incubated for 1 to 2 hrs, with 50μl anticytokeratin antibody or 50μl PBS serving as negative control, followed by incubation in rabbit anti-mouse IgG/peroxidase complex or goat anti-rabbit IgG/peroxidase complex, depending on the first antibody used. The complexes were incubated in 20% pooled human serum to reduce aspecific staining.
Antibodies were visualized by immersion (10 min) in 3,3-diaminobezidine tetrahydrochloride.The slides were weakly stained in Mayer's haematoxylin (30 sec), dehydrated in graded alcohol and embedded in DPX mountant. The following monoclonal antibodies(MoAb) and the cytokeratins(CK) they recognize were used:

AF 87	specific for	CK 1	RKSE 60	specific for	CK 10
6B10	specific for	CK 4	2D7	specific for	CK 13
RCK 102	specific for	CK 5+8	M9	specific for	CK 18
AF 124	specific for	CK 6	2C8	specific for	CK 18
RCK 105	specific for	CK 7	Ks19.1	specific for	CK 19
M20	specific for	CK 8	M80	broad spectrum	

The numbers given for the cytokeratins are according to Moll[11]: M 80 is a broad specific antibody used as a positive control for the presence of cytokeratins. For negative controls, the same procedure was followed except for the incubation with the anti-cytokeratin antibodies. Since RCK 102 recognizes both CK 5 and 8, the presence of CK 5 could only be determined if M 20 showed, by negative staining, the absence of CK 8.

RESULTS

Morphology

The biopsy specimens stained with haematoxylin were studied by light microscopy. The mucosa of the promontory with an intact tympanic membrane, showed predominantly a single-layered cuboidal epithelium (Fig. 1) in three patients, and the remaining two specimens also had areas with a bi-layered cuboidal epithelium.

Metaplastic signs such as an epithelium with more than two layers were not encountered in ears with an intact tympanic membrane. The morphological findings in the 12 biopts from ears with a perforation could be divided into two groups:
One comprising 8 specimens of patients with a perforation, in which focal stratification varying between 3 and 10 layers occurred. In 7 specimens this stratification was visible between normal areas of single- or bi-layered cuboidal epithelium (Fig. 2). In one ear this stratification was a general feature, occurring between focal areas of a bi-layered epithelium. The borders between metaplastic and normal epithelium were abrupt and without special characteristics. The cells of the multi-layered epithelium kept their cuboidal appearance, in all but one case. In that specimen we saw a locally stratified squamous epithelium. None of the biopts studied showed a prickle cell or granular cell layer or metaplastic areas with signs of keratinization.

In the second group, with 4 specimens, no stratification was found, but 3 of the 4 specimens showed a predominantly bi-layered epithelium. All of these biopts showed cuboidal epithelium. The underlying stroma showed a connective tissue, with incidental focal occurrence of round cell infiltration, increased vascularization and gland-like structures. However, in none of these cases we found distinct signs of acute or chronic inflammation. There was no correlation between increased cell infiltration in the stroma and the number of cell layers of the middle ear epithelium.

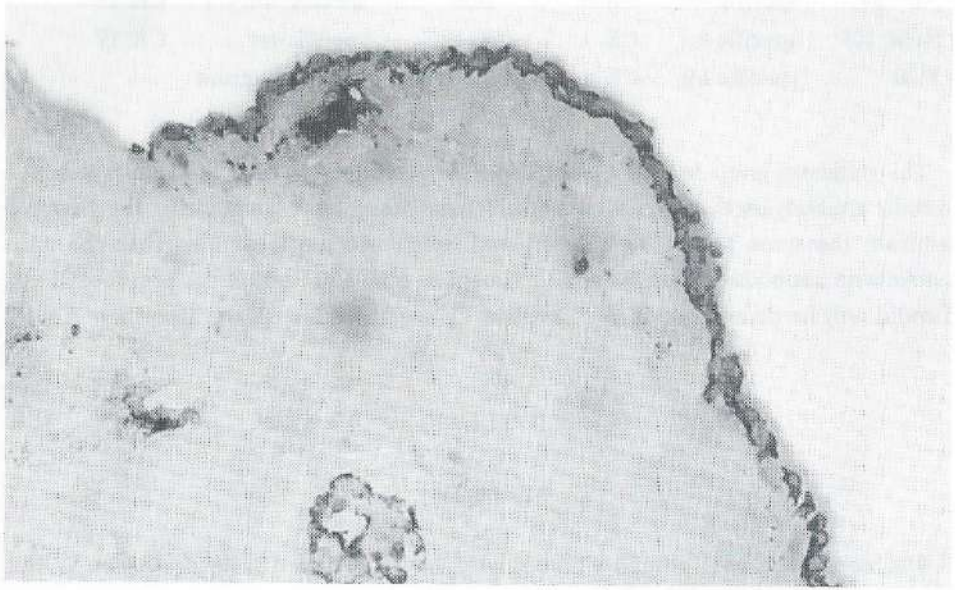


Fig. 1 Epithelium of the middle ear without the presence of a tympanic membrane perforation, in this case presenting as a mono-layered mucosa. The mucosa stained positive with monoclonal antibody RCK 102.

Table I

Middle ear mucosa with a perforation of the tympanic membrane

n = 12

biopt No	1	2	3	4	5	6	7	8	9	10	11	12
age(yrs)	13	11	14	25	24	49	70	49	27	57	40	58
sex m/f	f	m	m	f	f	m	m	m	m	f	f	m
perf. in years	5	6	5	1	3	22	1	10	12	20	8	1
previous[1] oper.	-	-	+	-	+	+	-	+	+	+	+	+
previous[2] inf.	+	+	+	+	+	+	+	+	+	+	+	+
meta-plasia[3]	-	+	+	+	-	+	+	-	-	+	+	+

Middle ear mucosa without a perforation

n = 5

biopt No	13	14	15	16	17
age(yrs)	34	39	41	25	54
sex m/f	f	m	m	m	f
perf. in years	-	-	-	-	-
previous[1] oper.	+	+	-	+	+
previous[2] inf.	+	-	+	+	+
meta-plasia[3]	-	-	-	-	-

ad 1) Previous operations included placement of grommets, myringoplasty and ossiculoplasty.

ad 2) The last middle ear infection healed at last 3 months before the biopsy was taken.

ad 3) Criterium for metaplasia was the (focal) presence of three or more cell layers

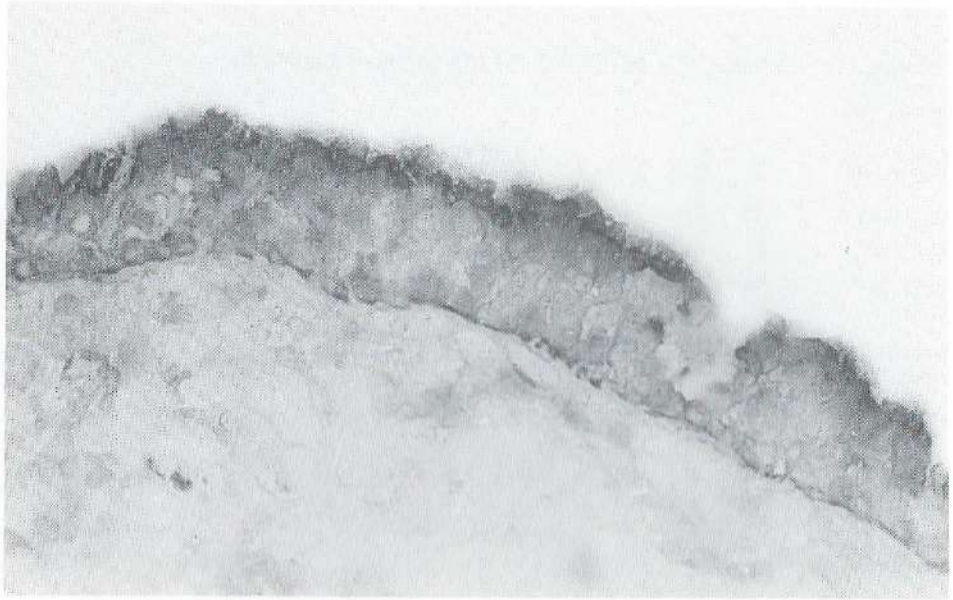


Fig. 2 Epithelium of the middle ear with a perforation of the tympanic membrane, showing a multi-layered epithelium. The mucosa stained positive with monoclonal antibody RCK 105

Although the ears with and without a tympanic membrane perforation had to satisfy certain criteria, we checked the patients' histories for any clues that would permit differentiation of the group in which stratification was found. The numbers are, of course, small but such parameters as otitis media, previous middle ear operations (placement of grommets, myringoplasty, ossiculoplasty), age of patient or duration of perforation did not point to any definite preference for one of the groups (Table I).

Cytokeratin staining

All biopts challenged with antibodies for cytokeratins 4,6-8,18, 19 and the broad specific antibody showed positive staining. Antibody RCK 102 can identify cytokeratins 5 and 8 and was positive for all biopts on which it was used. The presence of cytokeratin 5 could not be established because antibody M 20 showed positive staining for cytokeratin 8 in all cases. CK 18 was not detected by antibody 2C8 but could be visualized by antibody M9.

Cytokeratins 1, 10, and 13 were not found in any of the biopts incubated with these antibodies. The presence of stratification, even up to 10 cell layers did not alter the cytokeratin composition. All layers of the epithelium were stained in cases with positive recognition of the antibody. Some variation was observed in the degree of staining in cases with a multi-layered epithelium.

Some specimens showed stronger staining in the basal cell layers, others in the upper layers. However, no correlation with the antibody used and location in the cell layers was found. Specimens incubated with antibody AF 124 for CK 6 showed in many cases a faint background staining of the subepithelial tissue. However, in most biopts recognition of CK 6 could be considered positive. Gland-like structures situated in the subepithelial layer, were encountered in many biopts, and often showed positive staining, especially for cytokeratins 4,5,6,8, and 19. The staining in these structures was most pronounced in the periluminal cells. Due to the focally restricted presence of stratification in the biopts, it was not possible to produce sufficient sections of such metaplastic areas for incubation with all antibodies within the same specimen. On the whole, however, it was possible to study the stratified mucosa with the 10 chosen monoclonal antibodies(MoAb) several times, as can be seen from Table II.

Table II

Monoclonal antibody	Specific for Cytokeratin	Number of times this monoclonal a'body was used in metaplastic epithelium.
6B10	CK 4	3
RCK 102	CK 5+8	3
RCK 105	CK 7	3
M20	CK 8	3
RKSE 60	CK 10	3
2D7	CK 13	3
M9	CK 18	2
Ks19.1	CK 19	6
AF 87	CK 1	1
AF 124	CK 6	3
M80	Broad specific	All

DISCUSSION

In this study we wanted to find out whether an alteration of the environmental gas composition would influence the morphology and cytokeratin pattern of the epithelial lining of the middle ear cavity. We assumed that the gas composition of the middle ear would change in cases with a tympanic membrane perforation.

As a result of the direct connection between the tympanum and the outer air the carbon dioxide concentration would be lowered from about 5 to almost zero % and the oxygen concentration increased from 10 to 21%.[8,9]

Light microscopy indicated that the morphology may change under atmospheric conditions, because 8 out of 12 specimens taken from ears with a tympanic membrane perforation, showed a multi-layered epithelium of at least three cell layers. The mucosa of the promontory normally has 1 to 2 cell layers[13] as we found in the biopts with an intact ear drum.

It is interesting to note that despite the stratification of the mucosa, the cells of all but one specimen kept a cuboidal appearance. Metaplasia of respiratory epithelium is generally characterized not only by stratification but the cells also become more flattened, ultimately resulting in the squamous epithelium as described by Michaels[14] and Auerbach.[15] The change in morphology in our biopts can be considered a hyperplastic reaction representing the first stage in the process of metaplasia(Auerbach[15]). Except for the presence of a defect in the tympanic membrane we could not identify any other factor that could explain the morphological changes in this group. It therefore seems obvious to attribute this mild metaplastic reaction to the shift in the oxygen and carbon - dioxide concentrations. Of course, other environmental factors such as temperature, air humidity, or air flow, might also change in cases with a tympanic membrane perforation.

A change in the environmental conditions to which the epithelium of the middle ear is exposed can be accompanied by a change in the morphological appearance, as reported, for instance, by Vennix et al.,[16] who found locally in the infected middle ear of the rat a metaplastic cornifying epithelium. As already mentioned, middle ear epithelium can be considered an extension of the respiratory epithelium,[4,13] and we questioned whether a shift in the environmental gas composition could change the cytokeratin pattern of the middle ear mucosa. For this study we used monoclonal antibodies against cytokeratins 1, 4-8, 10, 13, 18, and 19 to establish the cytokeratin composition of middle ear mucosa in patients with and without a tympanic membrane perforation. Cytokeratins 7, 8, 18, and 19 are specific for a simple non-stratified epithelium,[11,17] whereas 4, 5, 6, and 13 belong to the more complex non-cornifying stratified epithelia,[11,17] e.g. the lining of the trachea or pharynx.

Cytokeratins 1 and 10 are typical of a cornifying epithelium such as the skin of the external ear canal.[11] All biopts we examined were positive for 4, 6, 7, 8, 18, and 19. Cytokeratins 1, 10, and 13 could not be detected and the presence of CK 5 remained uncertain, because antibody RCK 102 can recognize both CK 5 and 8. Cytokeratin 8 was, however, visualized by antibody M20, and we cannot be certain that the staining pattern of RCK 102 included both cytokeratins.

Others have concluded that CK 5 is present in normal middle ear mucosa as LE 41[18] or M20[12] did not or only incidentally expressed the presence of CK 8, in combination with positive staining by RCK 102. In our material, however, CK 8 was detected consistently and we believe that this cytokeratin can apparently be considered to belong to the normal set of cytokeratins in middle ear epithelium.

In this study CK 6 was positively identified. According to Moll,[11] this cytokeratin can be found in, for instance, tracheal epithelium which is also a respiratory epithelium. Cytokeratins 1 and 10 could not be found in any of the specimens, which indicates that middle ear epithelium does not belong to the group of cornifying epithelia. The stratification of the middle ear epithelium did not exert an influence on the cytokeratin set or the staining pattern of the cell layers. Even a stratification of more than 10 layers did not offer any evidence pointing to the presence of cytokeratins 1 and 10, which mean that this metaplastic change was not related to a significant change in the differentiation of the epithelium.

The identification of CK 4 by monoclonal antibody 6B10 and the failure to demonstrate the presence of CK 13 by monoclonal antibody 2D7 was unexpected but has been reported by others.[18,19] Cytokeratins are generally present pair-wise,[20] one CK belonging to the acidic and one to the basic group, as exemplified by CK 4 and 13. The results of this study in combination with the observations of others[12,18] indicate that the cytokeratin set of middle ear epithelium at the moment is at least composed as follows: 4, 5, 6, 7, 8, 14, 18, and 19.

The middle ear epithelium is considered to be an extension of the respiratory epithelium, and its cytokeratin pattern is almost similar to that of tracheal epithelium, i.e.: 6, 7, 8, 13, 14, 15, 17, 18, and 19.[11]

CONCLUSIONS

The presence of a tympanic membrane perforation may induce mild metaplastic changes in the middle ear epithelium, probably due to an altered gaseous environment. The metaplastic change, stratification, did not influence the cytokeratin pattern of the epithelium. In sum the terminal differentiation of middle ear epithelium is not influenced by a gaseous environment like that of the ambient atmosphere. An eventual metaplastic change of the middle ear mucosa into a cornifying epithelium cannot be ascribed to the presence of a tympanic membrane perforation alone.

REFERENCES:

1) Habermann E. Zur Entstehung Cholesteatom des Mittelohrs. Arch für Ohrenheilk 1888; 27:42-50.

2) Bezold F. Uber das Cholesteatoms des Mittelohrs. Zeitschr für Ohrenheilk 1891;21:252-264.

3) Rüedi L. Pathogenesis and surgical treatment of the middle ear cholesteatoma. Acta Otolaryng(Stockh) 1979; suppl 361.

4) Sadé J, Weisman Z. The phenotypic expression of middle ear mucosa. In:McCabe BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publ Co, 1977: 52-61.

5) Van Blitterswijk CA, Hesseling SC, Grote JJ. The effect of external stimuli on cultured rat middle ear epithelium. 1 Extracellular calcium concentration. Acta Otolaryngol(Stockh) 1990;109:101-111

6) Lutgert HW, Blitterswijk van CA, Grote JJ. The effect of external stimuli on rat middle ear epithelium in culture - Extra cellular carbon dioxide concentration-. Acta Otolaryng(Stockh) 1990;109:431-437

7) Sadé J. Retraction pockets and attic cholesteatoma. Acta Otorhinolaryngol(Belgica) 1980;34:62-84.

8) Melvill Jones G. Pressure changes in the middle ear after altering the composition of the contained gas. Acta Otolaryngol(Stockh) 1961;53: 1-11.

9) Cantekin EI, Bluestone CD. Membrane ventilating tubes for the middle ear. Ann Otol Rhinol Laryngol 1976;suppl 23:270-276.

10) Steinert PM, Jones JCR, Goldman RD. Intermediate Filaments. J of Cell Biology 1984;99 No1:22-27

11) Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.

12) Blitterswijk van CA, Grote JJ, Lutgert HW et al. Cytokeratin patterns of tissues related to cholesteatoma pathogenesis. Ann Otol Rhinol Laryngol 1989;98:635-640.

13) Lim DJ. Normal and pathological mucosa af the middle ear and Eustachian tube. Clinic Otolaryngol 1979;4:213-234.

14) Michaels L. The metaplastic process in epithelial strata. In: Sadé J, ed. Cholesteatoma and Mastoid Surgery. A' dam: Kugler Publ, 1982;299-303.

15) Auerbach O, Cuyler Hammond E, Garfunkel L. Histologic changes in the larynx in relation to smoking habits. Cancer 1970;25:92-98.

16) Vennix PPCA, Kuypers W, Tonnaer ELGM, Peters TA, Ramaekers FCS. The cytokeratin expression in the normal rat middle ear and during induced ome. In: Tos M. Thomson J, Peitersen E, eds. Cholesteatoma and Mastoid Surgery. A'dam. Berkeley, Milano: Kugler & Ghedini Publ, 1989:111-115.

17) Nagle RB, Moll R, Weidauer H, Nemetschek H, Franke WW. Different patterns of cytokeratin expression in the normal epithelia of the upper respiratory tract. Diff 1985;330:130-140.

18) Broekaert D, Cornille A, Eto H et al. A comparative immunohistochemical study of cytokeratin and vimentin expression in middle ear mucosa and cholesteatoma, and in epidermis. Virch Arch Pathol Anat 1988;413:39-51

19) Muijen van GNP, Ruiter DJ, Franke WW et al. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins No 4 and 13. Exp Cell Res 1986;162:97-113.

20) Sun TT, Eichner R, Nelson BA et al. Keratin classes: Molecular markers for different types of epithelial differentiation. J Investig Derm 1983;81:109-115.

CHAPTER IX

GENERAL DISCUSSION

In the study reported here an attempt was made to determine to which degree changes in the gas composition of the middle ear play a role in the pathogenesis of cholesteatoma. The gas composition in the middle ear cleft is related to the aeration or ventilation of the middle ear, and in the physiologic situation higher CO_2 and lower O_2 concentrations are found as compared with the gas composition of the ambient air.[1,2]

In case of direct communication of the middle ear with the outer air due to a perforation of the tympanic membrane, it is likely to assume that the gas composition in the middle ear cleft will be altered and will resemble that of the ambient air. Likewise, cholesteatoma too are often associated with defects in the tympanic membrane or external ear canal.

Under perforation conditions, middle ear mucosa and cholesteatoma tissue may be exposed to gas concentrations occurring in the ambient atmosphere. *In vitro* and *in vivo* studies have indicated that the morphology and cell metabolism of several cell types can be influenced by changing the extracellular concentrations of CO_2 and O_2 (Chapter II). To answer the main question posed above, we may put forward two questions:

1) Will the morphology and cell metabolism of the middle ear epithelium be influenced by changes in the extracellular CO_2 and O_2 concentrations?

2) Is there a relationship between any influence exerted on the middle ear mucosa by CO_2 and O_2 and cholesteatoma pathogenesis?

The influence of the CO_2 level on middle ear epithelium has been discussed in Chapter III, on the basis of an *in vitro* study in which we used serially cultured middle ear epithelium of the rat. The epithelium was exposed to three gas concentrations, i.e., 0.03, 5, or 10% CO_2. Other culture conditions were kept similar, the O_2 concentration was 21% and the nitrogen concentration 79%.

The epithelium cultures displayed a monolayer of flat predominantly polygonal cells, among which clusters of cells covered with microvilli and locally cornified envelopes were seen. It was found that cells grown under gas conditions similar to those of the ambient air, i.e., 0.03% CO_2, 21%O_2 and 79% N_2, exhibited differences in morphology and proliferation rate if compared with the 5 and 10% CO_2 cultures.

Clusters of very large cells were seen at 0.03% CO_2. The growth curves showed that there was a statistically significant retardation of the proliferation rate at 0.03% CO_2.

Middle ear epithelium cultured at 5 or 10% CO_2 showed no differences in either the proliferation rate or the morphology.

In contrast with the 5 and 10% CO_2 cultures, confluence of the epithelium on the dish was not reached at the end of the culture period at 0.03% CO_2. The changes in the morphology and proliferation rate were not accompanied by any change in the degree of terminal differentiation of the cells. The percentage of cells able to generate cornified envelopes after the addition of Ionophore X 537A did not differ statistically from that found for the epithelia cultured at 5, or 10% CO_2.

In Chapter IV we have discussed the middle ear epithelium of the rat cultured at different O_2 concentrations i.e., 5, 10, or 21%. In contrast to the effects of CO_2, morphology of the cultured epithelia was generally the same when cultured under the three O_2 concentrations. The differences in O_2 concentration did not affect the proliferation rate or terminal differentiation of the cells.

Chapter V dealt with the morphometric evaluation of the cultured epithelia of both the CO_2 and O_2 experiments. The marked divergence of the morphology of cells grown at 0.03% CO_2 was reflected by a statistically significantly enlargement of the average cell area, and the average cell shape was more irregular, as reflected by the statistically significantly lower value of form factor. Morphometric analysis revealed no differences between the 5 and 10% CO_2 cultures or the 5, 10, and 21% O_2 cultures except, that the average cell size of the 5% O_2 culture on day 14 was significantly larger than that of the average cell size of the 10 and 21% O_2 cultures on the same day.

In sum, the culture studies reported in Chapters III-V, showed that at the gas concentrations used, the morphology and proliferation rate were most clearly affected by changes in the carbon dioxide concentrations; in the present context, oxygen seems to play a less important role.

In vitro studies with keratinocytes showed that progress in terminal differentiation was related to enlargement of the cell size.[3,4] This relation was not seen in our culture experiments, because the evident increase in the size of the cells in the 0.03% CO_2 culture was not associated with a significant increase of cornified envelope formation or the degree of terminal differentiation.

If our culture results may be extrapolated to the clinical situation than it does not seems likely that a decrease of the extracellular CO_2 concentration, such as might occur in cases with tympanic membrane perforation, will play a role in the possible transformation of middle ear mucosa into a cornifying epithelium.

In Chapter VI we studied the morphology of clinically different types of cholesteatoma, i.e. the primary acquired, recurrent, and residual cholesteatoma. In this study the residual types developed behind an intact tympanic membrane and the middle ear was not clinically infected. It is conceivable that this type of cholesteatoma developed in a gaseous environment of the kind normally present in the healthy middle ear.

All primary acquired and recurrent cholesteatoma were associated with defects in the tympanic membrane and /or bony wall of the external ear canal, permitting a direct communication with the ambient atmosphere and in many of these cases a concomitant infection was present.

Despite these differences in clinical appearance, pathogenesis, and possibly gaseous environment, we found no significant morphological differences between the three types of cholesteatoma. The matrix of the cholesteatoma consisted of a cornifying epithelium identical to that of normal epidermis. An interesting phenomenon seen in the cholesteatoma matrix was the extremely local variation in the thickness of this cornifying epithelium which is normally not seen in epidermis. The perimatrix or the subepithelial tissue of the cholesteatoma matrix also varied in thickness, consisted mainly of fibrous tissue with or without signs of chronic inflammation or infection. We did not find any relation between the morphology of the perimatrix and that of the matrix. In several specimens we found a one- or two-layered cubical epithelium probably representing the middle ear mucosa. In none of these cases did we see metaplastic changes of this epithelium or a transition from middle ear mucosa to the cornifying epithelium of the cholesteatoma.

In sum, this morphological study did not provide any indications that metaplasia of middle ear epithelium must be considered as a factor in cholesteatoma pathogenesis.

Chapter VII reports an investigation into the cytokeratin pattern of cholesteatoma matrix, meatal epidermis, and middle ear mucosa. Because all tissues of epithelial origin express a characteristic set of cytokeratins,[5] it was thought that analysis and comparison of the cytokeratin pattern of the above mentioned epithelia might shed some new light on the pathogenesis of cholesteatoma. In this study the middle ear mucosa showed the presence of cytokeratins 4, 18, and 19 which are known to occur in non-cornifying epithelia. Cytokeratin 10, characteristic of cornifying epithelia, was found in cholesteatoma and meatal epidermis but was not detected in middle ear mucosa. Most of our cholesteatoma showed a cytokeratin staining pattern identical to that of the meatal epidermis, but in two cholesteatoma focal staining of cytokeratins 4, 18, and 19 was seen. The focal staining of cytokeratins 4, 18, and 19 in cholesteatoma can be explained by the change in local environment and the hyperproliferative state in which the meatal epidermis has entered, a phenomenon described by several authors.[6,7]

In sum, on the basis of these findings a relation between cholesteatoma and meatal epidermis seems to be more probable, but a metaplastic origin cannot be excluded.

In the studies reported in Chapters III-V we did not find any significant influence of CO_2 and O_2 on middle ear epithelium with respect to cholesteatoma pathogenesis. Neither the morphological investigation of cholesteatoma described in Chapter VI or the cytokeratin experiment discussed in Chapter VII provided indications pointing to a possible metaplastic origin of the cholesteatoma.

Chapter VIII reports an *in vivo* study, to determine whether a tympanic membrane perforation and eventual a change in the extracellular gaseous environment could induce changes in morphology or metaplasia and/or a change in the differentiation of the epithelium of the human middle ear.

The morphological study was done with light microscopy and to detect any shift in the differentiation of the mucosa we determined its cytokeratin pattern. Middle ear mucosa exposed to the ambient atmosphere showed in the majority of cases focal areas of a multi-layered cubical epithelium with more than three cell layers; this can be considered a mild metaplastic reaction.[8]

Determination of the cytokeratin set was done with ten monoclonal antibodies, however, we did not detect any shift in the cytokeratin set of middle ear mucosa exposed to the ambient atmosphere in comparison with epithelium grown in its physiologic gaseous environment. Especially cytokeratins 1 and 10, which are specific for a cornifying epithelium, were not detected in the metaplastically changed middle ear mucosa. Even a metaplastic stratification with more than ten cell layers did not provide any evidence pointing to the presence of cytokeratins 1 and 10, thus indicating that this metaplastic change was not related to a significant change in the differentiation of the epithelium. We shall now return to the two main questions put forward above:

1) Will the morphology and cell metabolism of the middle ear epithelium be influenced by changes in the extracellular CO_2 and O_2 concentrations?

2) Is there a relationship between any influence exerted on the middle ear mucosa by CO_2 and O_2 and cholesteatoma pathogenesis?

The first of the questions can be answered affirmatively because the morphology of the middle ear epithelium was indeed influenced both *in vitro* and *in vivo*, especially by changes in the CO_2 concentration. Cell metabolism was affected by changes in ratio of CO_2 to O_2 because we found a reduced proliferation rate under gas conditions resembling those of the ambient atmosphere. Terminal differentiation, however, was not influenced by CO_2 or O_2.

The second question must be answered in the negative because the *in vitro* and *in vivo* studies showed that the degree of terminal differentiation of middle ear epithelium remained unchanged. Hence, we may conclude that the influence of CO_2 and O_2 on middle ear epithelium is of little importance within the context of cholesteatoma pathogenesis. Of course, it must be kept in mind that in our *in vivo* studies we did not include or examine other environmental factors that might change in the presence of a tympanic membrane perforation, such as, air humidity, air flow, temperature, or any influence of other gasses present in the ambient atmosphere.

Furthermore, it is unknown to what degree a change in the gas composition of the middle ear, after perforation of the tympanic membrane, will actually occur near the cell membrane of the middle ear epithelium. In the intact middle ear the CO_2 concentration was found to be about 5%, this percentage probably being the result of the rapid diffusion of CO_2 into the middle ear[9] and this value shows correlation with the gas pressure of CO_2 in the blood tissue compartment.[10,11]

If an extracellular CO_2 concentration of 0.03% (ambient atmosphere) occurs, it is conceivable that a rapid diffusion of CO_2 from the blood-tissue compartment would decrease the eventual effect of a lowered CO_2 concentration on the middle ear epithelium *in vivo*. The gas diffusion itself would be dependent on the degree of perfusion and vascularization of the subepithelial tissues, and the values of gas pressures in the blood and middle ear.[12,13]

Another factor that might influence a possible effect of the extracellular gas composition is the presence of the mucus layer covering the middle ear mucosa. The consistency or composition of this mucus may be altered due to the different atmosphere.

Sadé,[14] Weisman,[15] and Drucker[16] performed culture experiments with different CO_2 and O_2 concentrations and pH values, and concluded that CO_2 can be considered a non-specific inducer in mucosal cell differentiation. However, Sadé advised that his conclusions be considered with reservation, because the pH level in the culture media was difficult to control, due to the different CO_2 concentrations. Furthermore he did not have access to culture dishes with a gas permeable bottom. In his opinion, a low CO_2 concentration such as would occur in case of tympanic membrane perforations could be responsible for squamous metaplasia in the middle ear and an increase of the number of keratinizing cells of the middle ear epithelium. This possible role of the CO_2 to O_2 ratio in squamous metaplasia and cell differentiation is, however, not confirmed by our findings.

The authors who adhere to the metaplasia theory have reported high incidences, i.e. up to 60%, of metaplastic middle ear epithelium in their biopsy specimens. Several authors (Sade,[17] Bendek,[18] Bodelet,[19] Palva[20]) have suggested the transformation of normal middle ear mucosa via metaplasia into a cornifying epithelium in ears with chronic infection and cholesteatoma. In our material we did not find any evidence pointing to a metaplastic process of this kind. In reports of *in vivo* experiments, however, squamous metaplasia of middle ear epithelium leading to a cornifying epithelium has been described, and ascribed to vitamin A deficiency,[21] tobacco smoke, and tobacco tar.[22]

Kuypers and Vennix[23] studied the cytokeratin pattern of non-infected and infected middle ear mucosa of the rat. In one case after healing of both the middle ear infection and a tympanic membrane perforation, they found an isolated area of a cornifying epithelium.

Unfortunately due to embedding in paraffin, it was not possible to challenge this epithelium with antibodies against cytokeratins characteristic for a cornifying epithelium. A positive staining product was, however, found for antibodies against cytokeratins occurring in middle ear epithelium. Lim[24] examined mucosal biopsy specimens of 180 human middle ears of patients suffering from otitis media with effusion. None of these patients had a history of tympanic membrane perforation, otorrhoea, or ear surgery. In three middle ears he found cornifying epithelia, but none of these patients showed the clinical picture of cholesteatoma.

In sum, there is little evidence that squamous metaplasia with subsequent formation of cornifying epithelium will occur in the human middle ear, and it remains uncertain whether such metaplasia will lead to cholesteatoma at all.

The most accepted view of cholesteatoma pathogenesis is that the origin must be sought in the existing epidermis of the tympanic membrane or external ear canal. Experiments and clinical observations (reviewed in Chapter II) indicate that this epidermal hypothesis might be correct. Epidermis may invade the middle ear cleft by migration, retraction or proliferation, and a congenital, traumatic, or iatrogenic origin may incidentally lead to cholesteatoma. The results of the cytokeratin study also point to an epidermal origin.

Vennix and Kuypers[6] performed extensive cytokeratin studies in rat and human middle ear mucosa. As in our study the cytokeratin pattern of middle ear mucosa resembled the cytokeratin pattern of upper and lower respiratory epithelium.

Future research

If we assign little or no importance to the metaplasia theory in the pathogenesis of cholesteatoma the question as to what factors or conditions cause the invasion of epidermis into the middle ear cleft must still be answered. As several authors have pointed out, a common element of the proposed epidermal theories is loss of or damage to the lamina propria, before ingrowth of the epidermis takes place.[25] The lamina propria is composed of connective tissue and is situated between the epidermis of the ear drum and the mucosal lining of the middle ear. When the integrity of the lamina propria is lost due, for instance, to chronic or acute infection, the epidermis and mucosa may come into direct contact with each other. I believe that we must focus our attention on the processes that take place when this contact of different epithelia occurs. As is known from several morphological studies, dealing specifically with the transition zone between epidermis and middle ear epithelium, there is generally some kind of contact inhibition between the two epithelia.[26,27]

It is still not known which factors disturb this armed truce, permitting the invasion of cornifying epithelium into the middle ear. The above mentioned epithelia can be cultured at present and it would be of interest to perform encounter experiments with middle ear mucosa and epidermis *in vitro*. These encounter experiments could take place under different conditions, taking into account, for instance, the influence of endotoxines,[28,29] the extra-cellular calcium[30] concentration, and bacterial infection, on the transition zone between the two types of epithelium. These experiments might elucidate why a cornifying epithelium can invade the middle ear cleft.

REFERENCES

1. Felding JU, Rasmussen JB, Lildholt T. Gas composition of the normal and the ventilated middle ear cavity. Scand J Clin Lab Invest 1987;47 suppl 186:31-41

2. Grontved A, Moller A, Jorgensen L. Studies on gas tension in the normal middle ear. Acta Otolaryngol(Stockh) 1990;109:271-277

3. Watt FM, Green H. Stratification and terminal differentiation of cultured epidermal cells. Nature 1982;295:434-436

4. Watt FM, Jordan PW, O'Neill CH. Cell shape controls terminal differentiation of human epidermal keratinocytes. Cell Biolog 1988;85: 5576-5580.

5. Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24

6. Vennix PCA, Kuypers W, Tonnaer ELCM, Peters TA, Rameakers FCS. Cytokeratin profiles of induced epidermoid formations in the rat middle ear and human cholesteatoma lesions. Arch Otolaryngol 1990 in Press.

7. Broekaert D, Cornille A, Eto H et al. A comparative immunohisto-chemical study of cytokeratin and vimentin expression in middle ear mucosa and cholesteatoma, and in epidermis. Virch Arch Pathol Anat 1988;413:39-51

8. Auerbach O, Cuyler Hammond E, Garfunkel L. Histologic changes in the larynx in relation to smoking habits. Cancer 1970;25:92-98.

9. Elner A. Normal gas exchange in the human middle ear. Ann Otol Rhinol Laryngol 1976;85 suppl 25:161-164

10. Ingelstedt S, Jonson B, Rundcrantz H. Gas tension and pH in middle ear effusion. Ann Otol 1975;84:198-201

11. Cantekin EI, Doyle WJ, Phillips DC et al. Gas absorption in the middle ear. Ann Otol Rhinol laryngol 1980;89:suppl 68:71-75

12. Shinkawa H, Sasaki Y, Inamura N et al. Middle ear pressure and gas diffusion. In:Lim DJ, Bluestone CD, Klein JO, Nelson eds. Recent advances in otitis media. Proceedings of the fourth international symposium. Decker ,Toronto 1988 abstr.29

13. Ostfeld E. Middle ear gas composition. Ann Otol Rhinol Laryngol 1985;94 suppl120:32-33

14. Sadé J, Weisman Z. The phenotypic expression of middle ear mucosa. In: McGabe,BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;52-61

15. Weismann Z, Sadé J. Effect of environmental CO_2, O_2 and pH on the growth of respiratory epithelium in vitro. Ann Otol Rhinol Laryngol 1979;88:21-30

16. Drucker I, Weismann Z, Sadé J. Tissue culture of human adult adenoids and of middle ear mucosa. Ann Otol Rhinol Laryngol 1976;85:327-333

17. Sadé J. Pathogenesis of attic cholesteatoma: The metaplasia theory. In: McGabe,BF, Sadé J, Abramson M, eds. Cholesteatoma. Birmingham, Alabama: Aesculapius Publishing Co, 1977;212-223

18. Bendek A. Histopathology of transudatory-secretory otitis media. Arch Otolaryng;78:33-38

19. Bodelet B. Wayoff M. Métaplasie et cholesteatome. Ann Otolaryngol 1972; 89:411-418

20. Palva T, Palva A, Dammaert K. Middle ear mucosa and chronic ear disease. Arch Otolaryng 1968;87:21-29.

21. Chole RA. Squamous metaplasia of the middle ear mucosa during vitamin A deprivation. Otolaryngol Head Neck Surg 1979;87:837-844

22. Greenberg SD, Dickey JR, Neely JG. Effects of tobacco on the ear. Ann Otol Rhinol Laryngol 1973;82:311-317

23. Vennix PPCA, Kuypers W, Tonnaer ELGM, Peters TA, Ramaekers FCS. The cytokeratin expression in the normal rat middle ear and during induced OME. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma an Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;111-115

24. Lim DJ. Normal and pathological mucosa of the middle ear and Eustachian tube. Clin Otolaryngol 1979;4:213-234.

25. Marquet J. Cholesteatoma or keratoma. Acta Oto Rhino Laryngol(Belgica) 1980;34;5-10

26. Lim DJ. Epithelial invasion, contact guidance and contact inhibition. In: Sadé J, ed. Cholesteatoma and Mastoïd surgery. A'dam : Kugler Publ, 1982;141-152.

27. Palva T, Lehto VP, Virtanen I et al. Junctions of squamous epithelium with middle ear mucosa. Acta Otolaryngol (Stockh) 1985;99 297-304

28. Sugita T, Huang CC, Abramson M. Effect of endotoxin on keratin production of keratinocytes in vitro. Am J Otolaryngol 1986;7:42-46

29. Sugita T, Huang CC, Abramson M. The effect of endotoxin and prostaglandin E_2 on the proliferation of keratinocytes. Arch Otorhinolaryngol 1986 243;211-214

30. Van Blitterswijk CA, Hesseling SC, Grote JJ. The effect of calcium on terminal differentiation, proliferation and morphology of cholesteatoma matrix, meatal epidermis and middle ear epithelium. In:Tos M, Thomson J, Peitersen E,eds. Cholesteatoma and Mastoid Surgery. A'dam, Berkeley, Milano: Kugler&Ghedini Publ, 1989;77-81

SUMMARY

The pathogenesis of cholesteatoma is still a subject of discussion. At present, three fundamentally different theories concerning the origin of cholesteatoma can be distinguished. First, the epidermal theory, according to which the origin must be sought in the existing epidermis of the external ear canal or tympanic membrane. This theory is adhered to by most otologists, although there is considerable discussion as to how this epidermis invades the middle ear cleft. Second, the congenital theory, which postulates that inclusion of ectodermal tissue during fetal life will ultimately lead to cholesteatoma In the third place there is the metaplasia theory, supported by those who hold the view that certain stimuli to the middle ear epithelium lead to the development of cholesteatoma. Several *in vitro* and *in vivo* studies have suggested that changes in the extracellular carbon dioxide and oxygen concentrations can influence the morphology and metabolism of cells.

In this study we attempted to find out whether changes in the extracellular gas composition of the middle ear can play a role in the pathogenesis of cholesteatoma, especially within the context of the metaplasia theory. The gas composition in the intact middle ear cleft differs strongly from that of the open air, by having higher carbon dioxide and lower oxygen concentrations. In cases with a tympanic membrane perforation, it is reasonable to assume that the gas composition in the middle ear will be altered and more closely resemble the gas composition of the open air. Furthermore, cholesteatoma are often associated with defects in the tympanic membrane or external ear canal. In such cases middle ear mucosa and cholesteatoma tissue may be exposed to gas concentrations resembling that of the ambient atmosphere. We assesed the eventual influence of a change in the extracellular CO_2 and O_2 concentrations on the morphology, proliferation rate, and terminal differentiation of middle ear mucosa *in vitro* and *in vivo*.

The *in vitro* studies comprised culture experiments in which the middle ear epithelium of the rat was exposed to different CO_2(0.03, 5, and 10%) and O_2(5,10,and 21%) concentrations. The morphology and proliferation rate of cultured middle ear epithelium was distinctly affected by exposure to a gaseous environment resembling that of the ambient atmosphere, i.e. 0.03% CO_2 and 21% O_2. The degree of terminal differentiation of the cells, however, was not altered relative to the findings in the other experiments. No differences were found between the 5 and 10% CO_2 cultures or between the cells cultured at the three O_2 concentrations. In sum these *in vitro* experiments did not suggest that the influence of extracellular CO_2 and O_2 levels is of importance in relation to cholesteatoma pathogenesis.

The morphology of three clinical types of cholesteatoma was also studied i.e. the primary acquired, recurrent, and residual cholesteatoma.

Between the primary acquired and the recurrent cholesteatoma, considered as a single group and the residual type differences in clinical appearance, pathogenesis, and possibly the gaseous environment under which these cholesteatoma developed can be distinguished. However, despite these differences we found no significant alterations in morphology between the three types of cholesteatoma by either light or electron microscopy. Histological or morphological features indicating a possible metaplastic origin were not found in our material.

A postulated relationship between middle ear epithelium, epidermis of the external ear canal, and cholesteatoma can be detected by studying the cytokeratin patterns of the above-mentioned tissues. Each type of epithelium expresses a characteristic set of cytokeratins (CK), and comparison of these patterns may shed some light on the pathogenesis of cholesteatoma. In our study we used monoclonal antibodies against CK 4, 8, 10, 18, and 19.

CK 10 specific for a cornifying epithelium was expressed in epidermis and cholesteatoma matrix but not in middle ear mucosa. CK 4, 8, 18, and 19 specific for non-cornifying epithelia were not detected in epidermis, but were invariably present in middle ear epithelium and occasionally in cholesteatoma. The results of this immunohistochemical study point to a relationship between cholesteatoma and auditory canal wall epithelium, but do not rule out a metaplastic origin of cholesteatoma.

In the last of the studies reported here, we looked for evidence whether morphology and cytokeratin pattern of middle ear epithelium can be influenced *in vivo*, under exposure to changes in the extracellular gaseous environment. For this purpose, we challenged human middle ear mucosa with and without a tympanic membrane perforation with 10 monoclonal antibodies against cytokeratins; 1, 4, 5, 6, 7, 8, 10, 13, 18, and 19. Light microscopy showed mild metaplastic changes of the middle ear mucosa in a majority of the cases with a tympanic membrane perforation. The cytokeratin expression was not affected despite the changed morphology in the presence of a perforation. None of the biopsy specimens showed the presence of cytokeratins 1 and 10, which are characteristic of a cornifying epithelium.

The presence of a tympanic membrane perforation alone did not lead to a metaplastic change of the middle ear mucosa into a cornifying epithelium. These *in vitro* and *in vivo* studies showed that the degree of terminal differentiation of middle ear epithelium is not influenced by the above-mentioned CO_2 and O_2 concentrations. Hence, we may conlude that the influence of CO_2 and O_2 levels, is of little importance in relation to cholesteatoma pathogenesis. The results of the morphological study of cholesteatoma and middle ear epithelium did not point to a possible metaplastic origin. In sum, we consider a metaplastic genesis of cholesteatoma as very unlikely.

SAMENVATTING

De pathogenese van het cholesteatoom is reeds meer dan honderd jaar een onderwerp van discussie. In feite kan men momenteel drie wezenlijk verschillende theorien betreffende de ontstaanswijze van het cholesteatoom onderscheiden. Ten eerste de epidermale theorie, die ervan uitgaat dat de bestaande epidermis van de uitwendige gehoorgang of het trommelvlies het middenoor binnendringt en aldus tot cholesteatoom aanleiding geeft. Deze theorie heeft de meeste aanhangers en lijkt op klinische gronden zeer reëel. In de tweede plaats is er de mogelijkheid van een congenitale origine. Het cholesteatoom zou ontstaan uit embryonaal of ectopisch ectodermaal weefsel. Ten derde bestaat er de metaplasie theorie, die ervan uitgaat dat door bepaalde stimuli middenoor epitheel kan differentiëren tot een verhoornend plaveisel epitheel en aldus tot cholesteatoom aanleiding geeft. Een van de factoren die mogelijk een rol zou kunnen spelen binnen de metaplasie theorie is een verandering in de extra-cellulaire CO_2 and O_2 concentratie. Vooral *in vitro* experimenten toonden aan dat morfologie en cel metabolisme van gekweekte cellen of weefsels kan worden beinvloed door veranderingen in de concentraties van CO_2 en O_2. Het doel van dit proefschrift was om te onderzoeken of een verandering in de CO_2/O_2 ratio, van belang kan zijn binnen het kader van de cholesteatoom pathogenese.

De gas samenstelling in het intacte middenoor vertoont duidelijke verschillen met die van de buitenlucht, de CO_2 concentratie is 5% en de O_2 concentratie is ongeveer 10%. Men mag aannemen dat door een directe communicatie met de buitenlucht, het middenoor epitheel zal worden blootgesteld aan gas concentraties gelijkend op die in de ons omringende atmosfeer i.e.; 0.03% CO_2 en 20.9% O_2. Een dergelijke situatie doet zich voor bij een trommelvlies perforatie en mogelijk bij cholesteatomen met een defect in de uitwendige gehoorgang of het trommelvlies. Teneinde de eventuele invloed van CO_2 en O_2 op middenoor epitheel te bepalen werden zowel *in vitro* als *in vivo* studies verricht.

Het *in vitro* experiment bestond uit kweek studies, waarbij middenoor epitheel van de rat werd gekweekt onder verschillende CO_2 (0.03%,5%,en 10%) en O_2 (5%,10%,en21%) concentraties. Morfologie en groei werd statistisch significant beinvloed door de celkweek bloot te stellen aan een gas samenstelling gelijkend op die van de buitenlucht. De mate van terminale differentiatie van het epitheel bleef echter onveranderd ten opzichte van de andere celkweken. Er werden geen verschillen gevonden, tussen de 5 en 10% CO_2 kweek of tussen de verschillende O_2 kweken. Uit deze kweek experimenten kan men concluderen dat de CO_2 concentratie, morfologie en groei van middenoor epitheel *in vitro* kan beinvloeden. Echter, gezien het feit dat de terminale differentiatie onveranderd bleef bij de verschillende CO_2 en O_2 concentraties, lijkt het onwaarschijnlijk dat deze gassen een rol spelen binnen de cholesteatoom pathogenese.

In de klinische situatie zijn verschillende typen cholesteatoom te onderscheiden die mogelijk verschillen in pathogenese, en zich ontwikkeld hebben onder verschillende gas condities. Het residu cholesteatoom bijvoorbeeld ontstaat uit een niet geheel verwijderde cholesteatoom matrix en kan groeien in een atmosfeer zoals die voorkomt in het intacte middenoor. Daar tegen over staat het primair verworven en recidief cholesteatoom, dat meestal gepaard gaat met defecten in de uitwendige gehoorgang of het trommelvlies, waardoor een directe communicatie met de buitenlucht mogelijk is. Deze cholesteatomen ontstaan waarschijlijk door een actieve ingroei van huid in het middenoor.

De morfologie van bovengenoemde typen cholesteatoom werd middels licht- en electronen microscopie onderzocht en onderling vergeleken. Het bleek dat geen essentiele verschillen tussen het residu cholesteatoom enerzijds en het primair verworven en recidief cholesteatoom anderzijds konden worden aangetoond. Evenmin vonden wij aanwijzingen in dit materiaal die zouden kunnen duiden op een metaplastische origine.

Concluderend kunnen we stellen dat de morfologie van het cholesteatoom niet lijkt te worden beinvloed door mogelijke verschillen in pathogenese of extra-cellulaire gas samenstelling.

Men kan een eventuele relatie tussen het cholesteatoom en middenoor mucosa enerzijds of tussen het cholesteatoom en epidermis van de uitwendige gehoorgang anderzijds, onderzoeken door het cytokeratine patroon van bovengenoemde epithelia te bestuderen. Ieder type epitheel heeft een eigen set van cytokeratines en vergelijking van deze cytokeratine patronen kan meer inzicht verschaffen in de origine van het cholesteatoom. Voor dit onderzoek werd gebruik gemaakt van monoclonale antilichamen gericht tegen de cytokeratines(CK) 4, 8, 10, 18 en 19.

CK 10 is specifiek voor een verhoornend plaveisel epitheel, en werd uitsluitend aangetoond in cholesteatoom en de epidermis van de uitwendige gehoorgang. De CK's 4, 8, 18 en 19 zijn specifiek voor niet verhoornend epitheel en werden zonder uitzondering aangetoond in het middenoor epitheel. De CK's 4, 18 en 19 bleken focaal aanwezig te zijn in enkele cholesteatomen. Op grond van dit immunohistochemisch onderzoek lijkt een epidermale origine van het cholesteatoom het meest waarschijnlijk.

Middenoor epitheel bleek *in vitro* niet essentieel te veranderen door verschuivingen in de CO_2/O_2 ratio. Het morfologisch en immunohistochemisch onderzoek gaven evenmin aanleiding metaplasie van middenoor epitheel als een oorzaak voor cholesteatoom vorming te beschouwen.

Teneinde de eventuele invloed van CO_2 en O_2 op humaan middenoor epitheel, *in vivo*, te onderzoeken werd het cytokeratine patroon van middenoor epitheel onderzocht bij patienten met en zonder de aanwezigheid van een trommelvlies perforatie. Hiervoor werden monoclonale antilichamen gebruikt gericht tegen de cytokeratines 1, 4, 5, 6, 7, 8, 10, 13, 18 en 19.

Middenoor epitheel genomen uit oren met een trommelvlies perforatie liet in de meerderheid der gevallen focaal metaplastische veranderingen zien. Echter, het cytokeratine patroon van dit middenoor epitheel werd niet beinvloedt door de aanwezigheid van een trommelvlies perforatie en zelfs metaplastisch epitheel van meer dan 10 cellagen, vertoonde geen verandering in het cytokeratine patroon. CK 1 en 10 zijn specifiek voor een verhoornend plaveisel epitheel en werden in geen van de middenoor biopten aangetoond.

Conclusie: een trommelvlies perforatie op zich, geeft geen aanleiding tot dusdanige veranderingen in de differentiatie van middenoor epitheel dat hierdoor een verhoornend plaveiselcel epitheel zal ontstaan. Boven beschreven *in vitro* en *in vivo* studies tonen aan dat CO_2 en O_2 geen wezenlijke invloed hebben op de (terminale) differentiatie van middenoor epitheel. De CO_2 en O_2 concentraties zoals hier gebruikt lijken dan ook geen rol te spelen binnen de cholesteatoom pathogenese. Zowel het morfologisch als het immunohistochemisch onderzoek gaven geen aanwijzingen voor een metaplastische origine, en de metaplasie theorie lijkt klinisch van geen belang.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 12 juni 1954 te 's-Gravenhage. Het middelbaar onderwijs werd gevolgd op het Christelijk Atheneum te Arnhem, en het H.B.S.-B diploma werd behaald in 1972. Van 1972 tot 1979 studeerde hij Geneeskunde aan de Rijksuniveristeit te Utrecht. Het artsexamen werd april 1979 behaald, waarna de militaire dienst vervuld werd bij de Koninklijke Marine, hij werd gedetacheerd als arts bij het Corps Mariniers te Doorn. Van oktober 1980 tot mei 1981 was hij werkzaam als keurings- en waarnemend bedrijfsarts bij de Nederlandse Spoorwegen. Van mei 81 tot mei 85 werd hij opgeleid tot keel-, neus-, en oorarts in het Academisch Ziekenhuis te Leiden (Hoofd: Prof. Dr. P.H. Schmidt). Sedertdien bleef hij als staflid aan de afdeling Keel-, neus- en oorheelkunde van het Academisch Ziekenhuis te Leiden verbonden.