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Mutagen Sensitivity: a phenotype reflecting susceptibility to head and neck squamous cell carcinoma Jacqueline Cloos

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a phenotype reflecting susceptibility to head and neck squamous cell carcinoma

MUTAGEN SENSITIVITY: A PHENOTYPE REFLECTING SUSCEPTIBILITY TO HEAD AND NECK SQUAMOUS CELL CARCINOMA

Jacqueline Cloos

The research described in this thesis was performed at the Department of Otolaryngology/Head and Neck Surgery. University Hospital Vrije Universiteit, Amsterdam. Financial support was provided by the Scientific Counsel of Smoking and Health, the Netherlands.

Zambon Nederland BV, producent van o.a. Fluimucil 600^{R} en Panotile^{R}, is acknowledged for the financial support in the printing of this thesis.

Cover design: Ton Cloos

-Naar een gevelsteen van apotheek Barentsen te Drachten-

Drukkerij Elinkwijk BV, Utrecht

ISBN 90-9009052-5

VRIJE UNIVERSITEIT

MUTAGEN SENSITIVITY: A PHENOTYPE REFLECTING SUSCEPTIBILITY TO HEAD AND NECK SQUAMOUS CELL CARCINOMA

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr E. Boeker,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der geneeskunde
op woensdag 28 februari 1996 te 13.45 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105

door

Jacqueline Cloos

geboren te Amsterdam

Promotor: prof.dr G.B. Snow
Copromotor: dr B.J.M. Braakhuis
Referent: prof.dr A. Westerveld

DNA neither cares
nor knows
DNA just is
and we dance to its music
- *Richard Dawkins* -

Unraveling the mysteries of DNA might make us composers?

voor mijn ouders
voor Ronald

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Part of this chapter will be submitted as a review

HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

Squamous cell carcinoma of the head and neck has an incidence of about 5% of all newly diagnosed cancers in North Western European countries and the United States [Muir, 1995]. These carcinomas arise from the mucous membrane of the upper aerodigestive tract. Currently these types of tumor occur predominantly in 50-70 years old males. The male:female ratio varies from 2:1 to 6:1 depending on several variables [Schottenfeld, 1992]. In this thesis head and neck squamous cell carcinoma (HNSCC) refers to tumors of the oral cavity, larynx and pharynx (except nasopharynx) (Figure 1). Squamous cell carcinoma of the nasal cavity and paranasal sinuses are not included as it has been suggested that these tumors have a different etiology and represent a heterogenous group of neoplasms [Barnes *et al.*, 1985].

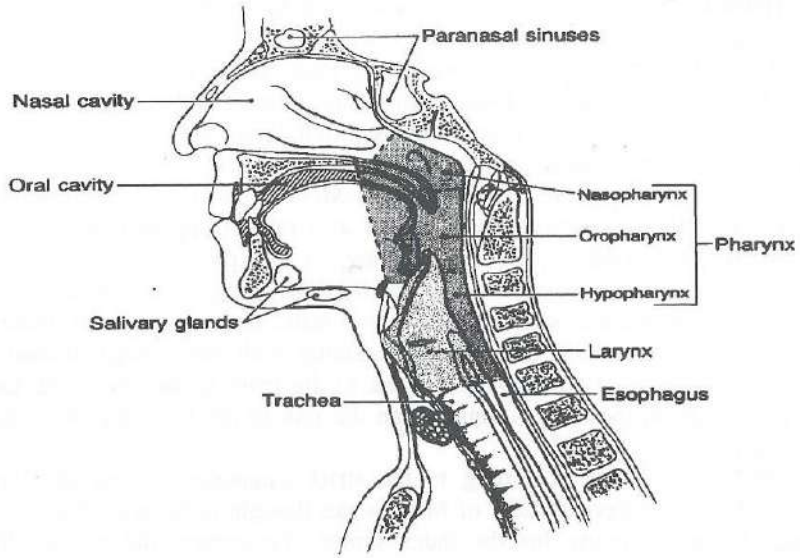

FIGURE 1 Sagittal section of the aerodigestive tract

The treatment of HNSCC is dependent on the tumor site and stage of the disease [Vokes, 1993], but commonly consists of surgery, radiotherapy or a combination of both. Especially for the more advanced stages of disease involving large tumors, usually with lymph node metastases, the five years survival figures have not improved significantly during the last decades [Blitzer, 1988; Boring *et al.*, 1994]. This is despite of the development of more sophisticated surgical and

radiotherapeutical treatment modalities. In an attempt to improve survival for these patients, new treatment modalities are being investigated such as the use of radioimmunotherapy [Van Dongen *et al.*, 1994]. This approach aims at the eradication of minimal residual disease at the local/regional level or micro-metastases at distant sites.

Another important risk factor which influences particularly the survival of patients with small tumors is the occurrence of multiple primary tumors (MPT) in the mucous membranes of the head and neck, esophagus and lung [De Vries, 1990; McCally, 1995].

Multiple primary tumors in HNSCC patients

HNSCC, lung and esophageal cancer can be grouped together by using the term respiratory and upper digestive tract (RUDT) cancer. Patients successfully treated for HNSCC cancer are at relatively high risk to develop other new tumors in the RUDT area. The development of multiple malignant epithelial tumors in this area is a major clinical concern. The reported incidence varies from 15% to as high as 30%, being dependent on the site of the first (or index) tumor and the length of follow-up [Shapshay *et al.*, 1980; McGuirt *et al.*, 1982; De Vries, 1990]. Overall, for HNSCC patients, MPT occur at a rate of approximately 3% per year for the first five years. MPT usually carry a bad prognosis because they often occur either at notoriously bad sites, like the lung or esophagus, or within previously treated areas within the head and neck, defying effective treatment. For some categories of HNSCC patients presenting with early stage disease, such as T_1N_0 glottic cancer of the larynx, the risk to die from an as yet undetected second primary cancer in the lung is higher than the risk to die from the index tumor just diagnosed [Vikram, 1984].

MPT are defined according to the strict diagnostic criteria of Warren and Gates [1932]. The development of MPT is not thought to be related to induction of damage by radiotherapy for the index tumor. To explain the risk of MPT, the concept of "field cancerization" is generally accepted [Slaughter *et al.*, 1953; Hittelman *et al.*, 1991], which assumes that the entire mucous membrane of the RUDT is on its way to neoplasia -though to a varying degree for the several sites within the RUDT- due to a similar exposure to the same carcinogens, like tobacco and alcohol.

Two approaches may be pursued to reduce morbidity and mortality related to MPT: early detection and prevention. The detection of cancer at an earlier stage may improve treatment efficacy as a more tissue-sparing approach may become feasible [Edell *et al.*, 1992]. Until now, early detection has been associated with controversies such as the question as to how frequently patients should be followed

up and what further investigations should be undertaken at each follow-up visit [Snow, 1992]. Ideally, regular panendoscopy including bronchoscopy and esophagoscopy should be carried out in every patient, but clearly this is not feasible for practical reasons. New methods of early detection are required which are less disturbing for the patient and which can preferably be performed on a large population basis. Since such techniques are not available yet it is of the utmost importance to focus on the population at the highest risk for MPT and therefore investigate new methods to identify these high risk individuals.

Chemoprevention

It has to be emphasized that preventive strategies are important in reducing the risk of MPT such as avoidance of carcinogen exposure particularly through life style changes. This type of primary prevention is important since the patients who continue smoking are more at risk to develop MPT than patients who stop. However, after quitting smoking, the chance of developing MPT only gradually decreases over the course of years. Furthermore, some pre-neoplastic changes may already have occurred. Thus, additional methods to reduce the risk of MPT in these patients are desirable.

The process of carcinogenesis may be delayed or inhibited through the administration of synthetic or natural compounds. Indications that this so-called chemoprevention is feasible originate from epidemiologic studies which reveal an increased risk of cancer when low levels of certain nutrients or fibers are present in the diet [Kune *et al.*, 1993; DeLuca, 1993]. *In vitro* and animal studies have shown that for instance anti-oxidants can be used to protect against carcinogenic assaults. N-acetylcysteine (NAC), a precursor of glutathione, is proposed to act in the early phase of DNA damage induction [De Flora *et al.*, 1986; Izzotti *et al.*, 1995]. Its mechanism of action is mainly due to its anti-oxidant/detoxification properties. In addition, inhibition of malignant progression of initiated cells has also been reported [Albini *et al.*, 1995]. Other nutrients which are considered to have protective potential are vitamins. It has been found in animal studies that vitamin A and its synthetic and natural analogues, the retinoids, such as retinyl palmitate and 13-cis retinoic acid (isotretinoin) can inhibit carcinogenesis [Moon *et al.*, 1983; Hill and Grubbs, 1992]. It has been suggested that vitamin A may even be effective after transformation of the target cell. This is probably due to induction of differentiation [Shalinsky *et al.*, 1995].

A milestone in the field of HNSCC has been the study by Hong *et al.* [1990], who reported that daily treatment with high doses of 13-cis retinoic acid (isotretinoin: 50-100 mg/m^2) was effective in preventing the development of MPT in curatively treated HNSCC patients. A long-term follow-up of this trial still

showed a statistical significant lower incidence of second primary tumors in the treatment arm [Benner *et al.*, 1994].

An ongoing chemoprevention trial in Europe to prevent MPT in HNSCC patients is a multicenter EORTC study: Euroscan [De Vries *et al.*, 1992]. The subjects are curatively treated early stage HNSCC patients. A 2 x 2 factorial design is used to test 2 agents and in order to achieve this the patients were randomized in four groups:

1) 300,000 IU Retinyl Palmitate (RP, vitamin A) daily during 1 year and half this dose during the second year
2) 600 mg N-acetylcysteine (NAC) daily for 2 years
3) both RP and NAC
4) no treatment

Endpoints of Euroscan are the number and time of occurrence of second primary tumors in the RUDT, local/regional recurrences and distant metastases as well as long term survival. The long duration these trials need to allow for conclusions is a major disadvantage. Therefore, high priority is now given to identify biomarkers that indicate early carcinogenesis and which can be used as surrogate (intermediate) endpoints [Bongers *et al.*, 1995].

Some other aspects have to be considered when conducting a chemoprevention trial. The subjects to whom treatment is offered are healthy persons who are cured of their first primary tumor. Moreover, for the great majority of these subjects treatment is redundant since they will never develop MPT anyhow. Therefore it has to be emphasized that compounds which are selected for such trials should have only minimal side-effects. In addition, besides for the acute toxicity of the compound it has to be emphasized that the supplementation of compounds such as anti-oxidants can exert adverse effects on the long course. This latter phenomenon was found in a Finnish chemoprevention trial [Alpha-tocopherol group, 1994]. In this trial approximately 30,000 "healthy" male smokers were either supplemented with β-carotene or α-tocopherol for 5 to 8 years and compared to a placebo-treated group. The group supplemented with β-carotene were found to even have 18% higher frequency of lung cancer compared to placebo-treated group. This negative outcome of chemoprevention illustrates the importance of thorough research on the mechanisms of action of the preventive agents.

By selecting groups of HNSCC patients with an increased risk for MPT in the RUDT, chemoprevention has much better opportunities [Morse and Stoner, 1993; Lippman *et al.*, 1994]. Moreover, it would be a logical approach to tailor the screening procedures in each individual to the degree of risk for the development of RUDT carcinoma. However, our knowledge of individual risk factors is insufficient and it is of great importance that more research will be carried out into the etiology of cancer of the RUDT.

In conclusion, identification of high risk individuals is thus essential for both early detection and chemoprevention of MPT to be successful.

RISK FACTORS FOR HNSCC

Traditional epidemiology has revealed some important environmentally related risk factors associated with the development of HNSCC. Infection with viruses such as herpes simplex virus [Larsson *et al.*, 1991] and human papillomavirus [Fouret *et al.*, 1995] has been associated with oral cancer. Exposure to occupational risk factors, such as asbestos [Muscat and Wynder, 1991], organic compounds and coal products [Maier *et al.*, 1991] have been found to increase risk for HNSCC. Dietary factors, particularly lack thereof, have also been related to risk for HNSCC [La Vecchia *et al.*, 1993; Zheng *et al.*, 1993; Garewal, 1993].

The most important risk factors in the etiology of HNSCC, however, are tobacco smoking [Wynder and Hoffman, 1994] and alcohol abuse [Kato and Nomura, 1994; Ng *et al.*, 1993]. Moreover, the combination of these latter factors have been shown to render relative risks that are more than multiplicative [Spitz *et al.*, 1988; Maier *et al.*, 1992]. Although these factors seem to be important for the carcinogenesis of the entire RUDT there are large differences between risk estimates for the varying tumor sites within the RUDT and even within the head and neck [Brugere *et al.*, 1986; Baron *et al.*, 1993].

Although the relationship between the incidence of HNSCC and life style factors is very strong, only a small fraction of persons who are exposed to tobacco and alcohol develops HNSCC. It is thus very likely that host specific factors that determine a certain intrinsic susceptibility are of importance. This supports the hypothesis that a genetic predisposition exists for the development of HNSCC. Several descriptive epidemiological studies have provided additional evidence on familiar clustering of RUDT cancer [Sellers *et al.*, 1994; Goldstein *et al.*, 1994; Law *et al.*, 1990]. Copper *et al.* [1995] found that having a first degree relative with HNSCC or lung carcinoma increased the risk for developing HNSCC (odds ratio 3.5). For siblings of the cancer patients this risk estimate was even as high as 14.6. Furthermore, it could be demonstrated that familial clustering is a risk factor for the development of MPT [Bongers *et al.*, 1995].

Cancer predisposition

Cancer predisposition is extremely present in particular syndromes, such as Fanconi's anemia, Xeroderma Pigmentosum and Bloom's syndrome [Digweed, 1993]. A well known cancer prone syndrome is Ataxia telangiectasia (AT). Homozygote AT patients have a 70- to 250- fold risk for developing cancer. The great majority (64%) of cancers developing in AT patients are of the hematopoietic system such as leukemias and malignant lymphomas [Gatti and McConville, 1994]. The rest are epithelial cancers, including oral cancer [Hecht and Hecht, 1990]. At

the cellular level AT is characterized by an increased sensitivity to radiation. AT cells are also unusually sensitive to radiomimetic, double strand breaks (DSB) inducing drugs, like bleomycin and neocarcinostatin [Lehmann and Stevens, 1979]. Despite all the experimental data, the underlying defect in AT has not been elucidated. It is most likely a defect in DNA-processing or a defect in the DNA damage surveillance network [Taylor, 1994].

The AT syndrome is an interesting syndrome for cancer research in general, since AT heterozygotes are estimated to represent 0.5 to 2.8% of the general population [Swift, 1991]. The chromosomal instability of AT heterozygotes is milder as compared to homozygotes [Parshad *et al.*, 1985]. The risk for cancer in AT homozygotes is much more pronounced as compared to the AT heterozygotes. However, AT heterozygotes have an increased risk to develop cancer compared to the normal population, in particular breast carcinomas [Swift, 1990; Cortessis *et al.*, 1993]. It was suggested that this high incidence of breast cancer was related to former exposure to radiation. For a relatively low accumulative dose an odds ratio of 5.8 ($p<0.005$) could be calculated for AT heterozygotes [Swift, personal communication, 1995]. This additional influence of external factors besides genetic make-up was not only found for breast cancer, but also for other cancer types. In conclusion, for AT heterozygotes an increased sensitivity to carcinogenic assaults may be the cause of the enhanced susceptibility to cancer.

Cancer susceptibility

Besides for these well known cancer prone syndromes it is becoming increasingly accepted that also for the occurence of common cancers, which was earlier thought to be predominantly due to environmental exposure, an intrinsic susceptibility is an important underlying factor. This susceptibility is present in a latent fashion and only becomes apparent after carcinogenic exposure. At present, it is acknowledged that between induction of DNA damage and malignant transformation several specific changes have to occur [Harris, 1991]. At any level of this multistep carcinogenic process putative factors can be identified, which may be important for cancer susceptibility. For instance, enzymes which are involved in the activation of procarcinogens into active carcinogens may play an important role in the first stage. The cytochrome P-450 (CYP) enzymes have been shown to play a role in this respect [Hlavica, 1994]. Genetic polymorphism in these CYP-genes has been associated with the risk for certain types of cancer [Gonzalez, 1995]. Currently, a lot of investigations are focussing on polymorphism in the N-acetyltransferase genes in relation to several environmentally induced cancers [Vineis *et al.*, 1994]. At the level of detoxification, enzymes such as glutathione-S-transferases play a major role and have been associated with intrinsic susceptibility

to several cancers [Bell *et al.*, 1993; Nakajima *et al.*, 1995].

In addition to this genotypical identification of intrinsic cancer susceptibility more phenotypically related assays have been developed. One such type of assay is based on a hidden chromosomal instability similar to what is found in the cancer prone syndromes. It should be emphasized that these syndromes represent the extreme end of the spectrum of chromosomal instability in the population. The cells of patients with most of these syndromes are already suffering damage in a spontaneous fashion without external DNA damage inducers. The overwhelming majority of individuals in the human population do not have such severely defective DNA maintenance mechanisms. It has been postulated that a degree of mutagen susceptibility may exist among persons as a consequence of minor imperfections in DNA maintenance systems which in combination with specific exposures leads to an increased cancer risk. To identify this sensitive group in the population, assays are used, which measure persistence of DNA damage after challenging cells *in vitro* with a relevant model compound.

Chromosomal instability after damage induction in the S-G2 phase of the cell cycle has been found to be related to cancer susceptibility. Gantt and colleagues claim that high sensitivity to radiation induced chromosomal instability is related to the development of several types of cancer [Gantt *et al.*, 1986]. Scott and colleagues [1994] use the same assay but focus mainly on the development of breast cancer. For the development of HNSCC a similar association to G2-phase sensitivity has been found. Hsu and colleagues [1983] measured sensitivity to the clastogenic activity (induction of chromatid breaks) of bleomycin (BLM) in peripheral blood lymphocytes of a large number of control persons and cancer patients. They referred to this BLM sensitivity as mutagen sensitivity. Since they measured clastogenic sensitivity this term "mutagen sensitivity" is not entirely correct. However, since this term has become well established in the literature, it is commonly accepted to be used in future communications.

Mutagen sensitivity

For the investigation of latent or "hidden" chromosomal instability the mutagen sensitivity assay is used as it is reliable and simple. Since a genetic factor should be present in all normal somatic cells, peripheral blood lymphocytes (PBL) are the best choice for the assay. These cells can easily be obtained and can be cultured under mitogen stimulation. For the induction of DNA damage BLM was chosen for its clastogenic properties and for its mechanism of action which resembles that of environmental carcinogens [Pryar, 1982]. The final assay developed by Hsu [1983] is performed on cultured lymphocytes which are challenged with BLM in the late S-G2 phase of the cell cycle. After harvesting the

cells, metaphase spreads are made and the chromosomal damage is scored under a microscope at 1250 x magnification. The mean number of chromatid breaks per cell (b/c) in 50-100 metaphases is a measure for the chromosomal instability [Hsu *et al.*, 1985; Hsu, 1987]. Figure 2 gives an example of such a metaphase spread in which clearly four breaks are indicated.

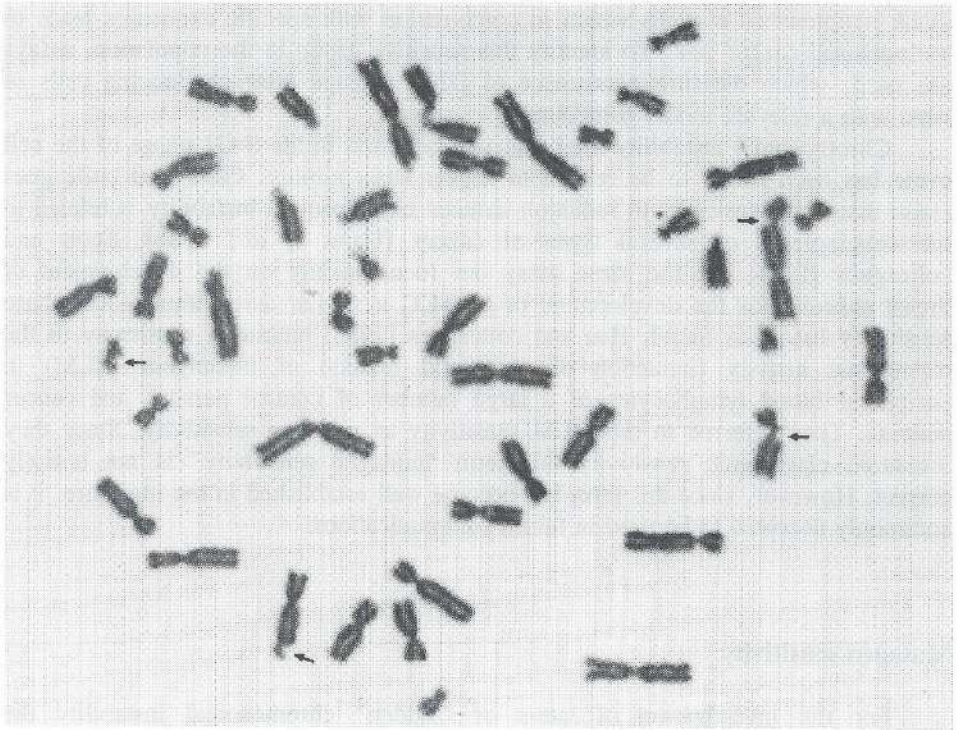

FIGURE 2 Metaphase spread of peripheral blood lymphocytes challenged with bleomycin. The arrows indicate the chromatid breaks

Using this assay, a large number of healthy persons and cancer patients (Table 1) were screened. It was found that in the normal population a large variation exists in mutagen sensitivity [Hsu *et al.*, 1989], showing a normal (Gaussian)

distribution. Based on this normal distribution, it appeared that a hypersensitive borderline could be set at a b/c level of 1.0 (mean value of the control persons plus one time the standard deviation). Theoretically, about 16% of the normal population should be at the hypersensitive end of the profile.

TABLE 1 *Overview of the b/c score of control persons and several classes of cancer patients*

Group (n)	mean b/c[1] score ± standard deviation	Percentage hypersensitive phenotype[2]	Reference
Control (335)	0.60 ± 0.35	12	Hsu *et al.*, 1989
HNSCC (77)	1.03 ± 0.51	48	Hsu *et al.*, 1989
Lung cancer (71)	0.98 ± 0.41	48	Hsu *et al.*, 1989
Colon cancer (83)	1.00 ± 0.41	46	Hsu *et al.*, 1989
Central Nervous System cancer (10)	0.55 ± 0.27	20	Schantz and Hsu, 1989

[1]b/c= chromatid breaks per cell after challenging lymphocytes with 30 mIU/ml BLM.
[2]hypersensitive phenotype is defined as a b/c score >1.0

A large variation in mutagen sensitivity was shown between the subjects within the various groups (table 1) reflecting an individual susceptibility to environmental mutagens. The key finding in these investigations was that only cancer patients with tumors that originate from tissues directly exposed to the external environment had an increased mutagen sensitivity. This indicates that both a sensitivity phenotype and exposure are important for the development of cancer.

Others were able to support this concept of hidden chromosomal instability using slightly different assays [Hagmar *et al.*, 1994; Li and Lin, 1990; Ning *et al.*, 1992]. In addition, Sanford and colleagues investigated radiosensitivity in skin fibroblasts and keratinocytes of control persons and cancer patients [Gantt *et al.*, 1986; Parshad *et al.*, 1983]. They found an increased sensitivity to irradiation in terms of an increased number of induced chromatid breaks and gaps in individuals predisposed to cancer, such as family members of patients with Wilm's tumor and retinoblastoma.

Whether high mutagen sensitivity has a distinct hereditary basis is not proven yet. However, small family studies show an increased mutagen sensitivity in cancer patients from cancer prone families compared to their first degree relatives without cancer [Knight *et al.*, 1993; Liang *et al.*, 1989; Bondy *et al.*, 1993].

Implications of mutagen sensitivity for HNSCC patients

The data on mutagen sensitivity in HNSCC patients underscore the importance of considering interindividual susceptibility when determining cancer risk. It should be expected that if HNSCC is related to a genetic factor, tumors will also occur in relatively young individuals. Schantz and co-workers found that young adults with HNSCC showed an increased mutagen sensitivity, while elderly men without tumors, who had extensively been exposed to tobacco and alcohol during their life, were predominantly insensitive [Schantz *et al.*, 1989]. This again points towards a different individual genetic susceptibility to carcinogens.

Another important aspect of the mutagen sensitivity data is the fact that they might be very valuable for the identification of HNSCC patients who are at high risk for the development of MPT. Schantz *et al.* [1990] were the first to assess the predictive value of the b/c level in this respect in a small prospective study. They found a relative risk of 4.4 for hypersensitive patients (b/c level >1.0) to develop second primary malignancies as compared to non-sensitive persons. This was later substantiated by enlarging the cohort and extending the duration of follow-up [Spitz *et al.*, 1994].

Recently a large case-control study has been performed in which mutagen sensitivity and exposure to wood dust and smoking were related to lung cancer risk [Spitz *et al.*, 1995]. Stratified analysis showed a greater than multiplicative interaction of the combined odds ratios of wood dust exposure, smoking and mutagen sensitivity. This finding indicates that both exposure to carcinogenic agents and susceptibility to DNA damage determine cancer risk.

Intensive study of the breakpoints of BLM induced chromatid breaks in lymphocytes of lung cancer patients showed a preferential breakpoint at 5q13-22, the region where the adeno polyposis coli (APC) gene is located [Wu *et al.*, 1995]. These authors argue that the analysis of chromosomal instability in lymphocytes could be a promising potential biomarker for the identification of high risk persons for lung cancer. Whether the same or other specific breakpoints will be found for HNSCC and other cancer types is currently under investigation. Cytogenetic studies to compare the breakpoints in the tumors to those in the lymphocytes of the same patients may provide further insight into the role of chromosomal instability in carcinogenesis.

Underlying mechanism(s) of mutagen sensitivity

The chromosomal breakage, which is used as an endpoint in the mutagen sensitivity assay, represents only a fraction of the total mutation output. Most gene mutations are not microscopically detectable. However, the b/c level as measured with the mutagen sensitivity assay has been shown to predict a person's sensitivity to DNA damaging compounds. The advantage of an assay with such an overall endpoint is that persons with any deficiency in the DNA maintenance pathway will

be identified. The disadvantage of this same phenomenon is that the mechanism of mutagen sensitivity may consist of multiple underlying factors. These factors may become of importance when specific prevention to high risk individuals can be offered. For instance, when detoxification is failing because of a lack of anti-oxidant protective properties, prevention of carcinogenesis may be offered by supplementation of anti-oxidants to the patients.

Defective DNA repair systems can also be the basis of mutagen hyper-sensitivity similar to what is found for the chromosomal instability syndromes. This impaired DNA repair capability can be caused by factors at several levels such as damage recognition, cell cycle arrest, DNA repair-enzymes. Especially for the repair of double strand breaks, which are capable of inducing chromatid breaks, recombination repair is essential. Some investigators claim that the chromatin structure is of importance [Pandita and Hittelman, 1995].

Another way to investigate underlying mechanisms of mutagen sensitivity is to look more closely at the model compound used to induce the damage. Knowledge regarding its mode of action may explain variability in sensitivity to this compound.

Mechanism of action of bleomycin

BLM is an antibiotic and cytotoxic drug which is considered a very useful test compound for the mutagen sensitivity assay. The commercial preparation of BLM consists of a mixture of natural products, which differ in the terminal amine, and which are isolated from *Streptomyces verticillus*. Figure 3 gives the backbone structure of bleomycin(s). The BLM stock of Lundbeck which was used in all the experiments in this thesis had a copper content of 0.005% ($\pm$ 1.2 μM) which originates from the bacteria of which it has been isolated.

This Cu-BLM has the advantage of being quite stable to ligand substitution reactions and is able to reach the nucleus and bind the DNA without being irreversibly inactivated. Controversy exists as to whether Cu-BLM by itself is able to produce DNA-damage or whether the copper exchanges with iron first [Stubbe and Kozarich, 1987].

An important mechanism for the genotoxic effects of BLM is the formation of single-strand breaks (SSB), double-strand breaks (DSB) and apurinic/apyrimidimic (AP) sites containing oxidized deoxyribose moieties [Stubbe and Kozarich, 1987]. This DNA damage may be induced directly by the activated BLM and possibly in part by hydroxyl radicals generated via free-radical-mediated reactions [An and Hsie, 1993].

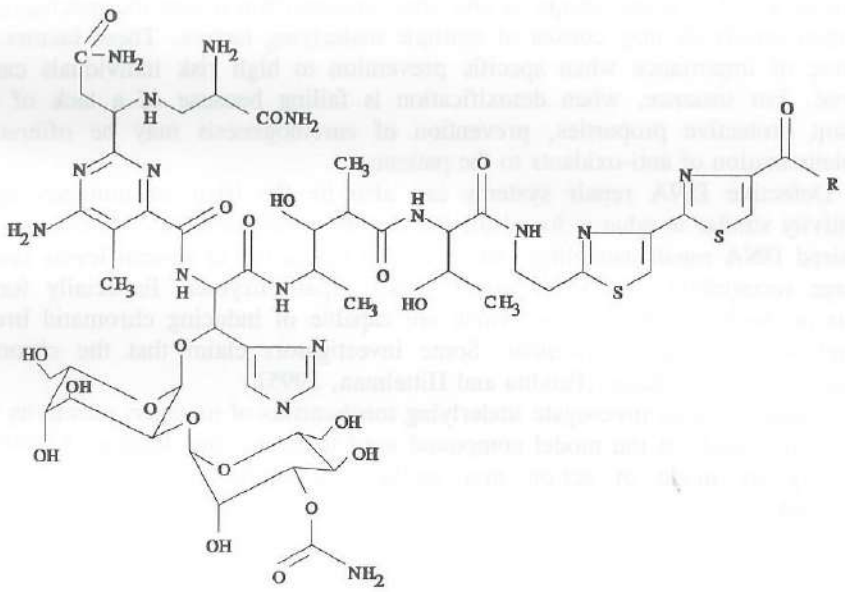

FIGURE 3 Backbone structure of bleomycin. Terminal R differs between natural identified compounds.

It has been well established that BLM has to bind DNA before it can be activated [Burger *et al.*, 1981]. Fe^{3+}-BLM-DNA complex may then be reduced to Fe^{2+}-BLM-DNA, and subsequently activated by O_2 [Fulmer and Petering, 1994]. After the first scission, BLM bound to the DNA can become reactivated and another break can be made. When this occurs in the opposite strand of a SSB, a DSB is introduced leading to a ratio of DSB:SSB of 1:8. For radiation induced damage, which does not involve DNA binding and reactivation, this ratio is about 1:100.

BLM can be inactivated by bleomycin-hydrolases. In addition, compounds such as ascorbic acid are able to render BLM irreversibly inactive in the absence of DNA [Buettner and Moseley, 1992].

So far it is not known what exactly determines BLM toxicity, however, the broad spectrum of DNA-damaging properties are thought to be comparable to carcinogens to which the patients are exposed [Pryar, 1982]. BLM is a useful model compound for chromosomal instability assays such as the mutagen sensitivity assay due to its ability to induce a high number of DSB (clastogenic properties) in a cell cycle independent manner.

AIM OF THIS STUDY AND OUTLINE OF THE THESIS

It has become more and more accepted that besides exogenous factors also endogenous factors may determine the risk for HNSCC. The aim of this project was to investigate whether the "hidden" chromosomal instability, similar to AT heterozygotes, is an individual endogenous risk factor for the development of HNSCC. To assess this "hidden" chromosomal instability we adopted the mutagen sensitivity assay. The hyper mutagen sensitive sub-population may be at the highest risk for the development of a second primary malignancy. This finding would enable us to identify those patients at risk of MPT, who can then be targeted for intensive behavioral interventions, surveillance through screening, and enrolment in chemopreventive programs. Knowledge about the mechanisms underlying mutagen sensitivity may be helpful to tailor prevention according to individual requirements. Therefore investigations to explore the basis of mutagen sensitivity were also undertaken.

The study commences with the validation of the mutagen sensitivity assay for the individual patient. In chapter 2 this validation is explored by determining whether the b/c levels can reproducibly be measured. In addition, the outcome of the assay was correlated to other risk factors in order to prove that mutagen sensitivity is a constitutional factor. After evaluation of the assay, a case-control study was performed (chapter 3). Besides control persons and HNSCC patients with one primary tumor, patients who had already developed MPT were also included. From these results a model was postulated in how mutagen sensitivity interacts with carcinogen exposure to determine cancer risk. This was further substantiated in a multicenter case-control analysis in which the role of mutagen sensitivity in HNSCC carcinogenesis could be proven (chapter 4).

Furthermore, investigations to examine the mechanism underlying mutagen sensitivity were undertaken. Chapter 5 deals with the relation between mutagen sensitivity and the ability to repair DNA damage induced by radiation. This DNA repair study was performed using an elegant immunochemical assay, with an antibody directed towards single stranded DNA, after partial alkaline unwinding of the DNA.

It has been suggested that mutagen sensitivity can be associated with a deficiency in detoxification and therefore might be influenced by supplementation of anti-oxidants. In chapter 6 the direct influence of several anti-oxidants, such as NAC to bleomycin induced damage is tested in a cell-free system. The effect of NAC supplementation on the b/c value in Euroscan patients is described in chapter 7. General discussion and future perspectives are addressed in chapter 8.

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Chapter 2

ASSOCIATION BETWEEN BLEOMYCIN GENOTOXICITY AND NON-CONSTITUTIONAL RISK FACTORS FOR HEAD AND NECK CANCER.

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Cancer Letters, 74, 161-165, 1993

Abstract

Sensitivity of phytohaemagglutinin-stimulated lymphocytes to bleomycin clastogenicity is increased in patients with tumors in organs and tissues that are in direct contact with the external environment, like the mucosa of the head and neck [Hsu *et al.*, 1989]. Sensitivity to bleomycin may reflect a genetically determined hypersensitivity to certain genotoxicants and therefore may be important in the carcinogenic process. In this study the applicability of bleomycin genotoxicity was investigated in cultured lymphocytes of 40 individuals without a tumor history. No correlations were observed with increasing age, and the well-kwown head and neck cancer risk factors alcohol and tobacco consumption. Since inter-individual variation in sensitivity greatly exceeded intra-individual variation, our results suggest that an elevated bleomycin clastogenicity score may identify individuals who have a constitutional hypersensitivity towards certain genotoxicants, and may show an increased cancer susceptibility.

Introduction

It can be hypothesized that the development of a tumor is related to an increased sensitivity to genotoxic compounds. Mutagen sensitivity, which is defined by Hsu *et al.* [1989] as a mild chromosomal instability after induction of DNA damage with the genotoxic agent bleomycin was increased in lymphocyte blood cultures of patients with head and neck squamous cell carcinoma (HNSCC). This bleomycin genotoxicity assay can be used to reveal DNA damage induced in the G2 phase of the cell cycle which has been claimed to be important for cancer susceptibility [Gantt *et al.*, 1986; Price *et al.*, 1991]. To make this assay operable to identify individuals at risk for HNSCC, it first had to be determined whether bleomycin clastogenicity is a constitutional factor which can be assessed for the individual person.

In this study we first determined the reproducibility of the bleomycin genotoxicity assay. Second, whether the bleomycin clastogenicity score is associated with non-constitutional risk factors for HNSCC like age, alcohol consumption and smoking habits was evaluated in subjects without a tumor history to exclude possible systemic effects caused by the presence of a tumor.

Materials and Methods

Subjects

Heparinized blood samples were obtained from healthy volunteers and hospitalized persons without a tumor history. These control persons were selected based on their age, current and cumulative smoking habits and alcohol-consumption as assessed by an extended questionnaire. As a measure of cumulative smoking the number of pack years was calculated for each subject defined as the number of years, multiplied by the number of cigarette-packs smoked daily (assuming that one pack contains 25 cigarettes). A similar calculation was used for alcohol drinking history, in which one unit is defined as one standard glass of alcoholic beverage consumed daily.

Cell culture and bleomycin treatment

Human peripheral blood lymphocytes were cultured at 37°C, 5% CO_2, for 72 hr. One half ml heparinized whole blood was diluted in 4.5 ml RPMI 1640 medium with 2 mM L-glutamine (Gibco, Paisley, UK) supplemented with 15% fetal calf serum (Flow Laboratories, Irvine, UK.), 1.5% phytohaemagglutinin (Wellcome Diagnostics, Dartford, UK), 100 IU/ml penicillin and streptomycin (ICN Biomedicals ltd, Irvine, UK). Whole blood was either cultured the same day it was obtained, or stored at 4°C for two days maximum before culturing. Of each subject duplicate cultures were used. Bleomycin (Lundbeck, Copenhagen, Denmark) was added 5 hours before cell harvest, ensuring that damage induced in the late S- and G2-phase of the cell cycle could be evaluated at metaphase. A stock solution of bleomycin (1.5 IU/ml in sterile water) was stored in aliquots of 1 ml at -20°C. Cells were arrested in metaphase by adding 100 μl, 50 μg/ml Colcemid (Sigma, St Louis, USA) 1 hour before harvesting. The cells were harvested by centrifugation (300*g*, 5 min) and treated with a hypotonic solution (0.06 M KCl) for 20 min. For fixation and washing of the cells Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) was used. Fixed cells could be stored at -20°C.

The influence of bleomycin dose and incubation time on chromosomal breakage rates was first evaluated in several persons having different clastogenicity scores, showing that a dose of 30 mIU/ml and an incubation time of 5 hours were suitable conditions (data not shown), which is in agreement with studies by Hsu *et al.* [1987].

Slide preparation and scoring

Cells were washed with fresh fixative and dropped on wet slides. After air drying, slides were stained with 5% Giemsa solution (Merck, Darmstadt, Germany), coded and scored under a light microscope with a magnification of 1250 x. From each slide, 50 metaphases were evaluated for the presence of chromatid breaks. Since it is important to use standardized criteria for detection of breaks, the breaks were scored according to guidelines of Hsu [1987]. In conformity with their findings, only a small proportion of chromatid gaps were seen after bleomycin treatment, which were omitted from the analysis. Cells containing more than 12 breaks were scored as having 12 [Hsu *et al.*, 1989]. The mean number of breaks per cell, based on the evaluation of 100 metaphases, was taken as a measure for clastogen sensitivity.

Statistical analysis

The reliability coefficient of the clastogen sensitivity value, was calculated as (SD between subjects)^2 / [(SD between subjects)^2 + (SD within subjects)^2] [Fleiss, 1986]. Because the SD between subjects expresses biological variability, it is not dependent on the number of cells scored. The SD within subjects, however, does depend on the number of cells scored. Analyzing 100 metaphases revealed a reliability coefficient of 0.70 which was a 30% increase compared to the reliability coefficient based on 50 metaphases (0.54). Therefore, it was decided to combine independent duplicate samples of 50 scored metaphases.

Correlation coefficients were calculated using the Spearman rank analysis. To compare breaks per cell values (b/c) between groups the Student's t-test was used.

Results

Before the bleomycin clastogenicity score can be considered as a cancer risk factor, the b/c value should be consistent for each individual person. To assess the reproducibility of the assay, multiple samples were tested from the same person with various time intervals (1 to 12 weeks), in a total of 9 individuals, 5 of whom were HNSCC patients.

Reproducibility was high, reflected by a coefficient of variation (standard deviation expressed as a percentage of the mean) of 8.9% (Table 1). These data show a mean intra-individual variation of 0.08 and a mean inter-individual variation of 0.35. A correlation could not be found between the intra-individual variation and the interval time between 2 experiments.

The b/c value of the cultures without bleomycin varied from 0 to 0.06 b/c (n=10). The mean b/c value $\pm$ standard deviation (sd) after incubation with 30 mIU/ml bleomycin for 5 hours was 0.78 $\pm$ 0.20 (n=40) and varied from 0.44 to 1.24. The b/c values of males (0.78 $\pm$ 0.21; n=28) and females (0.81 $\pm$ 0.17; n=12) were not significantly different ($p>0.2$). Moreover, there was no significant difference between healthy volunteers and hospitalized persons (0.71 $\pm$ 0.21; n=11 and 0.82 $\pm$ 0.19; n=29 $p>0.1$).

TABLE 1 *Reproducibility of the chromosomal aberration assay*

Patient number	Exp. I (b/c)	Exp. II (b/c)	Mean (b/c)	Standard deviation	Interval (weeks)
1	0.52	0.47	0.50	0.04	4
2	0.65	0.47	0.56	0.13	4
3	1.08	1.15	1.12	0.05	12
4*	0.69	0.77	0.73	0.06	1
5	1.24	1.01	1.13	0.16	8
6*	1.25	1.26	1.26	0.01	2
7*	0.82	0.78	0.80	0.03	1
8*	1.14	0.93	1.04	0.15	2
9*	1.39	1.48	1.44	0.06	3

Blood from several individuals was cultured, and challenged with 30 mIU/ml bleomycin for 5 hours. Chromosomal breakage is expressed as number of breaks per cell (b/c) based on 2 independent scores of 50 metaphases. Time intervals between the experiments I and II varied between 1 to 12 weeks. Coefficient of variation: 8.9%. Intra- and inter-individual variations as calculated by sd were 0.08 and 0.35, respectively.
* HNSCC patient

The inter-individual variation in b/c score between subjects was highly correlated with percentage of aberrant metaphases ($r=0.85$, $p<0.0001$), and with b/c values using a cut-off point of 6 breaks instead of 12 breaks per cell ($r=0.93$, $p<0.0001$). A weaker correlation was found between b/c and breaks per aberrant metaphase ($r=0.7$ $p<0.0001$).

To estimate the influence of environmental factors, the possible correlation of the b/c value was assessed with age and smoking- and alcohol drinking habits. Results are summarized in Figure 1a, 1b and 1c. No correlation was found between the b/c value and age ($r=0.25$, $p=0.12$), pack years ($r=0.22$, $p=0.17$) or unit years ($r=0.23$, $p=0.16$). The b/c value was also not influenced by current smoking and alcohol use. A mean b/c value of 0.76 ± 0.20 ($n=16$) was found for

non-smokers versus 0.81 ± 0.20 ($n=24$) for current smokers ($p=0.5$). For current alcohol use the b/c value in users and non-users was 0.80 ± 0.21 ($n=27$) and 0.76 ± 0.18 ($n=13$) ($p>0.5$), respectively. In addition, current smoking and alcohol abuse together did not influence the clastogenicity score (b/c value: 0.84 ± 0.20 ($n=16$)).

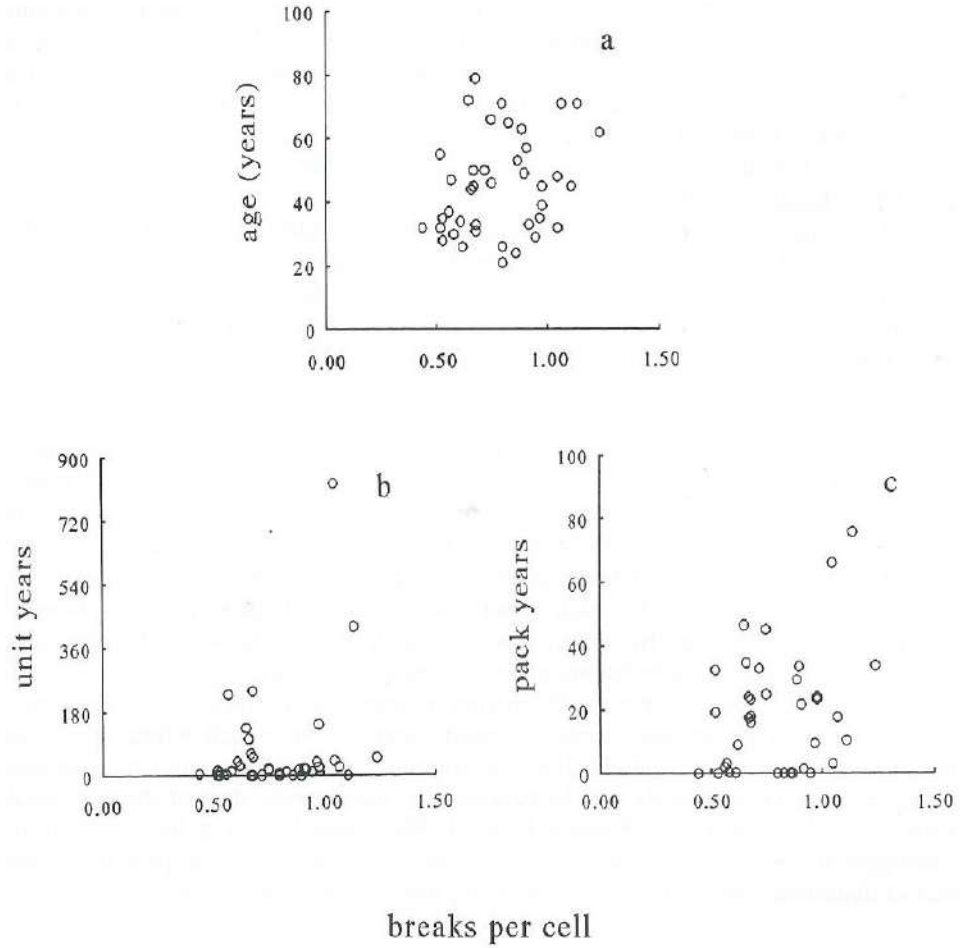

FIGURE 1 Bleomycin-induced breaks per cell values of 40 control persons showing lack of correlation with age, and cumulative alcohol (unit years) and tobacco (pack years) consumption.

Discussion

HNSCC patients show found an increased chromosomal instability compared to control persons after challenging phytohaemagglutinin-stimulated lymphocyte cultures with bleomycin in the late S- and G2-phase [Hsu *et al.*, 1989]. This phenomenon was referred to as "mutagen sensitivity" and was hypothesized to be related to a genetic predisposition for cancer. It is important to identify patients with a high risk to develop multiple primary tumors, since its occurrence has a negative impact on the survival of HNSCC patients [Snow *et al.*, 1987]. Several exogenous factors have already been shown to be risk factors for HNSCC [Schottenfeld, 1985]. However since these factors cannot account for all cancer cases it was hypothesized that endogenous factors also may play a role [De Vries *et al.*, 1987; Knudson, 1986].

The utilization of mutagen sensitivity as an independent risk factor for HNSCC will depend on a low intra-individual variation and lack of influence by the established exogenous risk factors for HNSCC. Our results indicate that the bleomycin clastogenicity score was highly consistent in time. In addition, the intra-individual variation was very low (0.08) compared to the inter-individual variation (0.35).

It is important to note that we did not find a correlation between the b/c values and environmental risk factors like age, smoking habits and alcohol abuse. These findings seem to be at variance with those of Schantz *et al.* [1990] who reported an inverse relationship between the b/c value and age or pack years [Schantz *et al.*, 1989a] as determined in HNSCC patients. However, it can be argued that this inverse relationship reflects selection towards the occurrence of HNSCC, since persons with a high sensitivity score may be more prone to develop HNSCC at an early age. In addition, persons with extreme tobacco exposure may still develop a tumor, while having a low clastogen sensitivity.

Our results indicate that the bleomycin-induced chromosomal aberration assay reported here primarily determines a constitutional factor, which would allow the measurements of an individual's constitutional sensitivity towards certain genotoxicants. Further study will be necessary to translate the data of the individual patients to the findings of Schantz *et al.* [1990], namely that a high bleomycin clastogenicity is correlated with the risk of the developing a second primary tumor and to determine the mechanisms underlying this clastogen sensitivity.

Acknowledgements

The authors would like to thank Prof. Dr. T.C. Hsu and his co-workers at the Department of Cell Biology at the M.D. Anderson Cancer Center, Houston, Texas, USA for their support, and Dr. J.J.P. Gille, Department of Human Genetics of the Free University, Amsterdam, for critically reading this manuscript.

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Chapter 3

INCREASED MUTAGEN SENSITIVITY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA PATIENTS, PARTICULARLY THOSE WITH MULTIPLE PRIMARY TUMORS

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Int. J. Cancer, 56, 816-819, 1994

Abstract

Mutagen sensitivity is a constitutional factor which may be used to identify head and neck squamous cell carcinoma (HNSCC) patients at high risk for the development of multiple primary tumors (MPT). In this retrospective study, mutagen sensitivity was measured in HNSCC patients with a single primary tumor (SPT), HNSCC patients who have already developed MPT and control persons with no tumor history. *In vitro*, lymphocytes were challenged with bleomycin and chromosomal damage was quantified by scoring chromatid breaks of 100 cells. A significant difference ($p<0.001$) in the mean number of breaks per cell (b/c) was found between SPT patients (0.96 ± 0.31; $n=50$) and control individuals (0.77 ± 0.19; $n=52$). Patients with MPT showed a mean b/c value of 1.20 ± 0.47 ($n=20$), which was significantly higher ($p<0.025$) than that of SPT patients. This increase in mutagen sensitivity in HNSCC patients was not related to well known cancer risk factors such as age, or life-style factors such as smoking and alcohol drinking habits. In addition, tumor site but not tumor stage was found to be related to mutagen sensitivity. On the basis of our findings we propose that mutagen sensitivity is not an independent risk factor but a constitutional factor which reflects the way genotoxic compounds are dealt with and is thereby directly related to cancer risk.

Introduction

Patients with early stages of head and neck squamous cell carcinoma (HNSCC) can be successfully treated with either surgery, radiotherapy or a combination of the two. Still, the overall survival hardly increased in the last two decades [Franceschi *et al.*, 1992], which can partly be attributed to the occurrence of multiple primary tumors (MPT) in the head and neck region or lungs [Snow *et al.*, 1987]. Development of HNSCC is to a large extent related to exogenous factors such as smoking and alcohol abuse [Schottenfeld, 1985]. Occupational and dietary factors play a role as well [Maier *et al.*, 1991; de Vries *et al.*, 1990], while the role of viral infections with HPV and Herpes simplex remains to be elucidated [Snijders *et al.*, 1992]. The fact that only a minority of heavy smokers and drinkers develop HNSCC points to an endogenous as well as an individual susceptibility to these carcinogenic agents. Data indicating that endogenous factors play a role in the etiology of HNSCC have been reported, including HLA antigens, immunoglobulin allotypes, and familial occurrence of HNSCC [de Vries *et al.*, 1987*a*; de Vries *et al.*, 1987*b*; Copper *et al.*, 1993].

Another endogenous risk factor, for cancer in general, is chromosomal instability, which is associated with a predisposition to cancer in individuals with syndromes such as Ataxia telangiectasia [McKinnon, 1987], Fanconi's anemia and Bloom's syndrome [German and Crippa, 1987]. This hereditary form of chromosomal

instability is revealed by a high frequency of both spontaneous and induced chromosomal aberrations. A milder form of chromosomal instability related to cancer is found in Xeroderma pigmentosum patients who show a very high number of chromosomal aberrations, compared to controls, after mutagenic induction by UV light [Robbins, 1978].

For HNSCC patients a mild chromosomal instability was found after induction with bleomycin [Schantz and Hsu, 1989]. To assess this "hidden" chromosomal instability an assay was adopted to quantify a constitutional sensitivity for exogenous genotoxic factors [Hsu, 1987]. Using an in vitro cytogenetic assay, chromatid breaks were scored in lymphocytes, induced by bleomycin in the late S-and G2-phase of the cell cycle. The number of breaks per cell after screening at least 50 metaphases was used as a measure for mutagen sensitivity. As a cell source, peripheral blood lymphocytes were used since they are easily available and can be cultured for a few days from whole blood samples. These cells could be used when it is considered that a constitutional factor can be measured in all somatic cells of the body.

In this paper we present a case-control study, in which mutagen sensitivity was determined of HNSCC patients, control persons without a tumor history, and HNSCC patients who already developed MPT. The relationship between mutagen sensitivity and age, smoking, alcohol consumption, tumor site and tumor stage has been analyzed. Moreover, a model is proposed in which mutagen sensitivity in combination with exogenous factors is correlated to cancer risk.

Materials and Methods

Subjects

Control persons consisted of healthy volunteers and hospitalized persons with no tumor history. The group of HNSCC patients consisted of patients with histologically proven squamous cell carcinoma of the mucosa of the upper aerodigestive tract. Blood samples were generally (in 90% of the cases) obtained before treatment, or shortly after treatment which was found not to interfere with mutagen sensitivity (data not shown). The characteristics of the groups are listed in Table 1. Smoking habits and alcohol consumption were assessed by an extensive questionnaire. The number of pack years was calculated for each subject as being the number of years during which was smoked, multiplied by the number of cigarette-packs smoked daily (assuming that one pack contains 25 cigarettes). A similar calculation was used for the estimation of alcohol drinking history, in which one unit is defined as one standard glass of alcoholic beverage consumed daily, assuming that one glass of beer or liquor contains a similar amount of alcohol.

TABLE 1 *Characteristics of the subject groups*

subjects	n	F/M[1]	pack years	unit years	age (years)
Controls	50	14/36	20 ± 19	66 ± 136	46 ± 14
SPT	52	16/36	35 ± 22	110 ± 131	61 ± 13
MPT	20	4/16	31 ± 23	191 ± 230	69 ± 8

[1]Number of female (F) and male (M) subjects. Data are shown as mean ± sd. Pack years and unit years are defined in the Material and Methods.

The average age of the control group was less than that of the cancer patients groups, making it necessary to correct for age when the cancer risk was evaluated. Since pack years and unit years reflect a cumulative tobacco and alcohol consumption, these values are related to age. Previously reported data show that these non-constitutional factors do not influence the b/c value in control persons [Cloos *et al.*, 1993]. To determine whether the increased b/c values in cancer patients could be attributed to these factors a linear regression was performed.

Cell culture and bleomycin treatment

Human peripheral blood lymphocytes were cultured at 37°C, 5% CO_2, for 72 hr. One half ml heparinized whole blood was diluted in 4.5 ml RPMI 1640 medium (Flow laboratories, Irvine, UK) with 2 mM L-glutamine (Gibco, Paisley, UK) supplemented with 15% fetal calf serum (Flow Laboratories, Irvine, UK), 1.5% phytohaemagglutinin (Wellcome Diagnostics, Dartford, UK), 100 IU/ml penicillin and streptomycin (ICN Biomedicals ltd, Irvine, UK). Culture of whole blood was either started the same day it was obtained, or stored at 4°C for two days maximum before culturing. Of each subject duplicate cultures were used. 30 mIU/ml bleomycin (Lundbeck, Copenhagen, Denmark) was added 5 hours before cell harvest, ensuring that damage induced in the late S- and G2-phase of the cell cycle could be evaluated at metaphase. A stock solution of bleomycin (1.5 IU/ml in sterile water) was stored in aliquots of 1 ml at -20°C. Cells were arrested in metaphase by adding 100 μl, 50 μg/ml Colcemid (Sigma, St Louis, USA) 1 hour before harvesting. The cells were collected by centrifugation (300*g*, 5 min) and treated with a hypotonic solution (0.06 M KCl) for 20 min. For fixation and washing (3x) of the cells, Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) was used. Fixed cells were stored at -20°C.

Slide preparation and scoring

Cells were washed with fresh fixative and dropped on wet slides. After air drying, slides were stained with 5% Giemsa solution (Merck, Darmstadt, Germany), coded and scored under a light microscope with a magnification of 1250 x. From each

slide, 50 metaphases were evaluated double blind for the presence of chromatid breaks. The mean number of breaks per cell, based on evaluation of 100 metaphases, was taken as a measure for mutagen sensitivity.

This mutagen sensitivity assay can be subjected to variability depending on definition of the scoring procedures. In this study the guidelines of Hsu *et al.* [1989] were used, which proved to generate reliable data [Cloos *et al.*, 1993]. The percentage of aberrant metaphases (am%) was determined as the percentage of cells which contained one or more chromatid breaks.

Statistical analysis

To compare breaks per cell values (b/c) between groups a one sided Student's t-test was used. The correlation between several factors and the b/c values was established using linear regression (Spearman Rank Correlation). To analyze whether b/c values in combination with smoking habits would increase cancer risk, multiple logistic regression was performed, using a microcomputer program for multiple logistic regression by unconditional and conditional maximum likelihood methods from the Ludwig Institute for Cancer Research, Epidemiology and Biostatistics Unit, Sao Paulo, Brazil.

Results

In Table 2 the mean breaks per cell (b/c) values ± standard deviation (sd) are summarized of 52 HNSCC patients with SPT, 50 control individuals and 20 HNSCC patients who already have developed MPT.

TABLE 2 *Several parameters for bleomycin-induced chromosomal damage (mean ± sd) of the different subject groups*

subjects	n	b/c	am%[1]	b/c >1[2]
Controls	50	0.77 ± 0.19	42 ± 7	14%
SPT	52	0.96 ± 0.31[3]	48 ± 9[3]	44%
MPT	20	1.20 ± 0.47[4]	54 ± 12[4]	75%

[1]am% is the percentage of metaphases containing one or more breaks; [2]patients with a b/c>1 were defined to have a hypersensitive phenotype; [3]significantly different (Student's t-test) from controls $p<0.001$; [4]significantly different (Student's t-test) from SPT HNSCC ($p<0.025$).

A significant difference (Student's t-test) in the mean number of b/c was found between SPT HNSCC patients (0.96 ± 0.31) and control individuals (0.77 ± 0.19; $p<0.001$). Moreover, patients with MPT showed a b/c value of 1.20 ± 0.47 which was significantly higher ($p<0.025$) than that of the SPT patients.The percentage of aberrant metaphases (am%), another parameter of chromosomal damage, showed similar differences between the groups as observed with the b/c values.

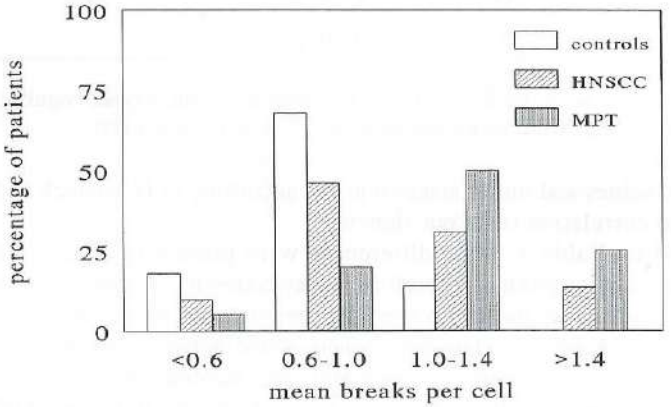

FIGURE 1 Frequency analysis. Subjects were divided into four groups after measuring the b/c values as described in Materials and Methods

Judged by the standard deviations, a considerable overlap in the b/c score among the three groups is present; however, frequency analysis (Figure 1) shows pronounced differences between the populations. There were no control persons with a b/c score of >1.4, and only 6 cancer patients had a score of < 0.6 b/c. Of the SPT HNSCC patients 44% were in the hypersensitive region of >1.0 b/c, compared to only 14% of the controls, whereas 75% of the patients with MPT revealed the hypersensitive phenotype.

Differences in b/c values of patients with tumors at several sites are summarized in Table 3. Patients with larynx or pharynx tumors scored mean b/c values (1.04 ± 0.29 and 1.05 ± 0.31 respectively) which were significantly different (Student's t-test; $p<0.001$) from control persons (0.77 ± 0.19), whereas the b/c values in patients with tumors in oral cavity (other than tongue) and the tongue were not (0.89 ± 0.36; $0.3<p<0.4$ and 0.81 ± 0.26; $p>0.4$ respectively). There also was a statistically significant difference ($p<0.05$) between the patient groups with pharynx or larynx tumors versus oral cavity including tongue.

TABLE 3 *Relation between b/c score and tumor site*

Tumor site	n	mean b/c ± sd	b/c >1[1]
Tongue	7	0.81 ± 0.26	29%
Oral cavity	15	0.89 ± 0.36	33%
Larynx	17	1.04 ± 0.29[2]	47%
Pharynx	12	1.05 ± 0.31[2]	67%
Oesophagus	1	0.69	

[1]patients with a b/c >1 were defined to have a hypersensitive phenotype; [2]significantly different (Student's t-test) from control persons (0.77 ± 0.19) $p<0.001$.

Analysis of the b/c values and tumor stages 0 to IV according to Hermanek and Sobin [1987] revealed no correlation (data not shown).

As illustrated by Table 1, large differences were present in nonconstitutional factors between the subject groups. Therefore it was necessary to ascertain that these factors would not influence the b/c values by performing linear regression. No correlation (Spearman Rank correlation test) was found between the b/c value and pack years ($r=6.0\cdot10^{-3}$, slope$=8.6\cdot10^{-5}$, $p=0.90$), and alcohol unit years ($r=-0.07$, slope$=-1.7$, $p=0.6$). The correlation between b/c and age ($r=0.34$, $p=0.02$), was meaningless since the slope of the regression line was almost zero ($8.0\cdot10^{-3}$). These findings are premisses for the hypothesis that mutagen sensitivity is a constitutional factor which is involved in carcinogenesis.

Discussion

Retrospectively, sensitivity for bleomycin induced chromosomal damage of SPT HNSCC patients was measured in comparison with control persons without a tumor history and MPT patients. In the bleomycin treated cultures a significant difference was found in b/c values between SPT patients and control persons. In addition, mutagen sensitivity of HNSCC patients with MPT was significantly higher than SPT HNSCC patients. Apparently a selection occurred towards patients who were hypothesized to be hypersensitive. 75% of the patients with MPT were hypersensitive, whereas of SPT patients 44% showed the hypersensitive phenotype. Of control persons without a tumor history only 14% showed a b/c value of >1. These retrospective results are in agreement with the findings of Schantz *et al.* [1990], that hypersensitive HNSCC patients are at high risk of developing a second primary tumor. The mutagen sensitivity assay can probably only be used for risk assessment for MPT but

presumably not for cancer in general, due to a broad spectrum of b/c values in the control group (0.5 - 1.24 b/c). A prospective study of control persons would require a very large number of persons and a very long follow-up time in order to estimate the risk of developing cancer [Brøgger *et al.*, 1990]. This is also indicated by a region of b/c values in which much overlap was shown between the three subject groups. Frequency analysis (Figure 1), however, showed that there was a large difference in distribution of subjects over several b/c classes, indicating three distinct populations.

Analysis of the tumor sites in primary HNSCC patients revealed a clear difference between oral cavity (including tongue) cancer patients and patients with a tumor in the larynx or pharynx. This phenomenon cannot be easily explained, but it is in agreement with the data of others [Schantz and Hsu, 1989; Spitz *et al.*, 1989], who argued that the difference might be due to different embryogenetic development or different exposure to both alcohol and tobacco for these tissues. The latter argument might be appropriate, since influence of tobacco and alcohol may differ for several anatomical sites [Maier *et al.*, 1992; Brugere *et al.*, 1986; Jovanovic *et al.*, 1993]. No significant differences were found between several tumor stages either in all HNSCC patients or in patients adjusted for the tumor site. Schantz and Hsu [1989] have shown a small increase in b/c value with advanced stage disease. However, it was argued that larger epidemiologic studies would be necessary to confirm this.

HNSCC patients had a different profile compared to the control persons with respect to age, pack years and alcohol consumption. Therefore it was necessary to exclude the possibility that b/c values would be dependent on these nonconstitutional factors. No correlation was found between b/c values and age, pack years and unit years in SPT patients. Moreover our previously reported data indicated that these factors do not influence the mutagen sensitivity score in control persons [Cloos *et al.*, 1993]. In that study it was also found that there was no difference in b/c values between males and females. Neither did current smoking and/or drinking influence the mutagen sensitivity. Thus it is suggested that mutagen sensitivity is a constitutional factor which is significantly different in HNSCC patients compared to control persons.

Since mutagen sensitivity is not influenced by environmental factors and only relevant in tissues in contact with exogenous factors [Hsu *et al.*, 1989], we propose a model in which b/c value is not an independent risk factor for cancer but a factor that modifies the extent to which exogenous factors cause DNA damage. This is in line with the assumption that inter-individual variation in carcinogen metabolism is an important determinant of cancer susceptibility [Harris, 1991; Nowak *et al.*, 1988]. Theoretically, some arguments in favor of this model can be given, for example, some people drink and smoke excessively but never develop a tumor while others who drink and smoke less develop a tumor at an early age. Most convincing is the fact that mutagen sensitivity is only related to tumors in tissues exposed to environmental factors; if mutagen sensitivity were an independent risk factor one would expect that in patients with high b/c values tumors would also occur in other tissues of the body.

Based upon our data we evaluated a logistic regression model in which b/c values in combination with smoking habits were related to cancer risk. Therefore the control persons and SPT patients were divided over three groups with different smoking histories: non-smokers, <30 pack years, and ≥30 pack years. Since age is a factor known to increase cancer risk this model was corrected for age. Smoking history was determined to be an effect modifier, so, in this model an increase in tobacco exposure should correspond with an increase in the slope of the correlation between b/c and cancer risk, as is visualized in Figure 2. The presumption was made that the regression lines would meet at the intercept of the Y-axis. This intersection corresponds to persons with very low b/c values who can smoke extensively but still have a rather low risk for developing cancer. This phenomenon is described by Hsu *et al.* [1989], who showed that elderly men who never developed a tumor, but who had an extensive exposure to alcohol and tobacco, were very insensitive.

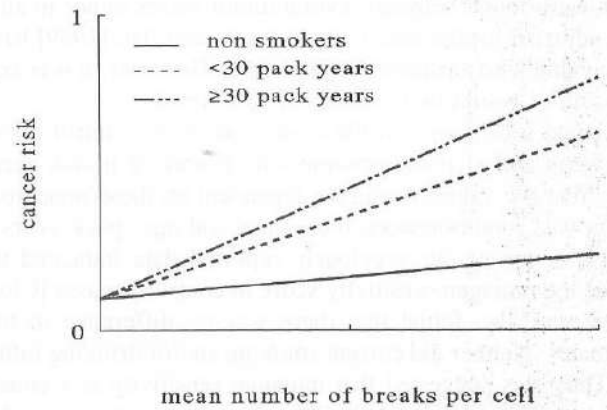

FIGURE 2 This figure gives a schematic representation of the hypothesized modifying effect by tobacco exposure. With our limiting data it is not yet possible to relate certain b/c values and smoking habits with a defined cancer risk. This model was analyzed using multiple logistic regression in which cancer risk is the probability to develop HNSCC.

Our data show that b/c value alone (in non-smoking subjects) revealed no increase in cancer risk. This may explain the fact that HNSCC patients without a smoking history did not all show a high sensitivity but varied from 0.66-1.24. It may be argued that in the patients with no smoking history other risk factors may have played a dominant role in carcinogenesis, for example a virus infection. When subjects had a moderate

smoking history (<30 pack years) the slope increased towards a higher risk for cancer with an increased b/c value (Figure 2). This was even more pronounced in subjects with heavy smoking habits (≥30 pack years). The major principle of the model, namely that the slope of the regression lines would increase with tobacco exposure, was demonstrated using our data. However, differences between the lines of the three tobacco exposure groups were not statistically significant ($p=0.19$). This may be due to the limited number of subjects and/or the variation in tumor sites. A study with more subjects is needed to provide definite evidence for this hypothesis.

In conclusion, it was found that HNSCC patients are more sensitive for bleomycin-induced chromosomal damage than control persons. The hypothesis that HNSCC patients with a hypersensitive phenotype are more prone to develop MPT was supported by a significantly higher mutagen sensitivity score in HNSCC patients who already have developed MPT. To assess the prognostic value of this constitutional factor for the risk of MPT a prospective study will be conducted. Identification of patients at high risk of developing MPT may improve their prognosis, as they could be subjected to a more intense follow-up, or be eligible for chemoprevention [Byar and Freedman, 1990]. To determine the precise role of hypersensitivity in the process of carcinogenesis, more research has to be performed on the mechanisms underlying mutagen sensitivity.

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Chapter 4

GENETIC SUSCEPTIBILITY TO HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Submitted

Abstract

Background: Besides exposure to carcinogenic compounds, the development of cancer is also influenced by inter-individual differences in susceptibility. Biomarkers for individual cancer susceptibility can be a powerful addition to epidemiological analyses of cancer risk.

Purpose: This multicenter case-control approach was performed to substantiate the value of mutagen sensitivity as a biomarker of susceptibility to head and neck cancer, and more importantly, to gain insight into the interaction between susceptibility and exposure to carcinogens.

Methods: Mutagen sensitivity (sensitivity to bleomycin in the G2-phase of the cell cycle) was determined in lymphocytes of 313 head and neck cancer patients and 334 control persons at two major American Institutes and one European Institute yielding a unique study population. Age, tobacco and alcohol use of the subjects were also recorded. The relation between variables was analyzed using Student's t-tests, Spearman rank correlations and multiple linear regression. For estimation of cancer risk crude odds ratios were measured and multiple logistic regression was performed. All p-values are based on two-sided tests.

Results: There were no differences across institutions in the distribution of mutagen sensitivity (Kruskall-Wallis test) for both cases and controls. Case values were consistently and significantly ($p < 0.0001$) higher compared to control values in the overall analyses. Neither in the control nor in the case subjects did age, tobacco and alcohol use influence the outcome of the mutagen sensitivity value. There was a dramatic increase in odds ratios from 10.6 (95% confidence interval; 4.5-25.8) to 45.1 (95% confidence interval; 17.0-120) for head and neck cancer in mutagen hypersensitive people who were also heavy smokers. The use of alcohol potentiated the effects of smoking, resulting in an odds ratio of 57.5 (95% confidence interval;17.5-188) in hypersensitive persons. Multiple logistic regression analysis showed that a hypersensitive phenotype did not appear to result in an increased cancer risk in non-smokers (relative risk 0.63, $p=0.25$), whereas there was a dose dependent association in individuals exposed to tobacco (relative risks ranging from 1.75 to 17.4; likelihood ratio test $p < 0.0001$).

Conclusions: Mutagen sensitivity was found to be a constitutional factor that can be used as a biomarker of susceptibility. This study underscores the importance of utilizing both susceptibility markers and exposure data for the identification of persons at high risk for cancer.

Implications: More accurate risk estimation can define susceptible subgroups which can be targeted for intensive behavioral interventions, surveillance through screening, and enrolment in chemopreventive programs.

Introduction

Tobacco and alcohol exposure are the major determinants of head and neck squamous cell carcinoma (HNSCC) [Baron, 1993]. However, since only a fraction of exposed individuals develops cancer, an intrinsic susceptibility to environmental genotoxic exposures has also been suggested as playing a role in carcinogenesis [Li and Montesano, 1994]. Within the general population, there may exist varying degrees of DNA repair and maintenance capability. To investigate this hypothesis, Hsu has developed an in vitro assay in which the number of chromatid breaks is scored in metaphase spreads of cultured lymphocytes challenged with bleomycin (BLM) in the G2-phase of the cell cycle [Hsu, 1983]. It has now been shown that mutagen sensitivity (defined as the mean number of breaks/cell (b/c)) determines a susceptible phenotype [Spitz and Bondy, 1993] and is a risk factor for the development of HNSCC, as well as lung cancer [Spitz *et al.*, 1995]. Consideration of markers of individual susceptibility helps to refine the risk assessment process [Harris, 1991]. However, these types of molecular epidemiologic studies require large numbers of subjects. We have thus combined data from three similar studies conducted at the University of Texas M.D. Anderson Cancer Center, Houston, the Memorial Sloan-Kettering Cancer Center, New York and the Free University Hospital, Amsterdam into a unique set of data with which to test the most optimal model to explain the relationship between carcinogen exposure and mutagen sensitivity as a risk for HNSCC.

Materials and methods

Subjects

The three groups of HNSCC patients consisted of previously untreated patients with histologically confirmed tumors in the mucosa of the upper aerodigestive tract (oral cavity, larynx, oro-and hypopharynx). From Houston the cases were white patients registered at The University of Texas M.D. Anderson Cancer Center. The controls were recruited from the blood bank of that institute, and were frequency matched by age, sex, and ethnicity to the cases. The patient characteristics regarding cigarette smoking and alcohol drinking habits were collected using extensive self-administered questionnaires. The same questionnaire was used for collection of subject data in New York, where the cases were recruited from the Memorial Sloan-Kettering Cancer Center. The control persons were healthy volunteers identified from the Blood Bank Center of that institute and were matched by age and gender to the cases. The cancer patient group of Amsterdam was registered at the Free University Hospital. The group of control persons were not individually matched to the cases and consisted of either cancer-free patients who registered at the hospital or healthy volunteers. The

lifestyle data were obtained through direct inquiry by the treating doctor.

The number of "pack years", a measure for cumulative smoking, was calculated as the number of years smoked, multiplied by the number of cigarette-packs smoked daily (assuming that one pack contains 25 cigarettes). Units per day was taken as a measure for daily alcohol use in which one unit is defined as one alcoholic beverage per day.

Mutagen sensitivity

The assay to determine the mutagen sensitivity has been described in detail previously [Hsu, 1987]. In short, phytohaemagglutinin stimulated lymphocytes were incubated for 5 hr with 30 mU/ml bleomycin (Lundbeck, Amsterdam and Brystol-Laboratories, Syracuse, New York). To arrest the cells at metaphase, 0.04 μg /ml colcemid was added to the cultures one hour before harvesting. The cells were swollen in hypotonic solution and fixed in Carnoy's fixative. After dropping the cells on wet slides the metaphase spreads were air dried and stained with Giemsa. Before scoring at least 50 metaphase spreads for the presence of chromatid breaks, the slides were coded to assure objective "blinded" screening. All U.S. data were scored in Houston and the investigator from Amsterdam was trained in Dr. Hsu's laboratory in Houston.

Statistics

The Mann Whitney U test and the Student's t-test were used for comparison of means between the groups. Both Spearman Rank correlations and linear regression were performed to study the correlation between relevant variables. Variation in the data between the three institutes was calculated using the Kruskall-Wallis test. For estimation of cancer risk, crude odds ratios (OR) were calculated. To estimate the joint influence of the parameters on the mutagen sensitivity, multiple linear regression was performed. In order to analyze the precondition of a hypersensitive phenotype for the influence of exposure to carcinogens on cancer risk, multiple logistic regression was used. All p-values were determined based on two-sided tests. All analyses were performed using both Statistical Package for the Social Sciences (SPSS, version 4.0) and BMDP (Stat. Soft Inc, Los Angeles, US) computer programs.

Results

Table 1 summarizes select demographic characteristics of the cases and controls by study site. There was a significant difference across the study sites by age of the cases and controls; the mean age of the Amsterdam cases was higher and the controls lower than the other two groups.

Table 1 *Distribution of select characteristics by study site and case-control status*

	Houston		New York		Amsterdam		Overall	
	cases	controls	cases	controls	cases	controls	cases	controls
No	108	108	153	176	52	50	313	334
age (years)	53.8 ± 15.0	51.0 ± 13.4	59.5 ± 11.6	56.8 ± 13.6	60.6 ± 13.0	45.7 ± 14.5	57.7*# ± 13.4	53.3* ± 14.3
pack years	35 ± 29	14 ± 21	36 ± 26	14 ± 19	35 ± 21	21 ± 19	35# ± 26	14* ± 20
alcohol u/d†	4.3 ± 6.7	1.1 ± 2.2	5.6 ± 7.7	1.9 ± 4.7	3.4 ± 4.6	3.0 ± 5.4	4.8*# ± 7.0	1.8* ± 4.2
breaks per cell	1.00 ± 0.44	0.87 ± 0.43	1.04 ± 0.40	0.79 ± 0.36	0.97 ± 0.32	0.78 ± 0.20	1.01# ± 0.40	0.82 ± 0.37

* significantly different ($p<0.01$) distribution across the institutions # statistically higher compared to controls ($p<0.0001$) † units alcohol per day. Mean values are given ± standard deviation.

All three case groups were heavy smokers (mean of 35-36 pack years) compared to a mean of 14 pack years for the American controls and 21 for the Dutch controls. Similarly, alcohol consumption was higher in that latter group.

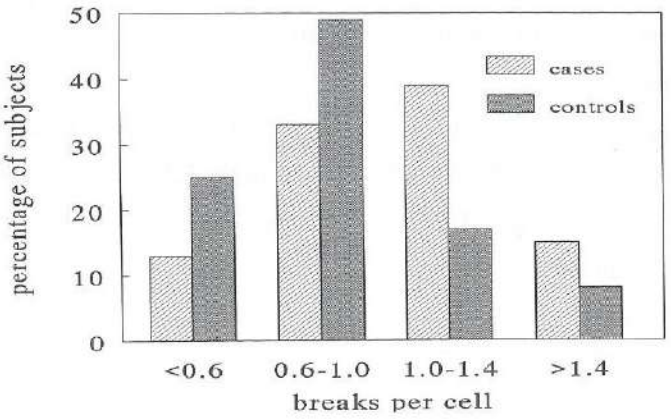

FIGURE 1 Frequency distribution in which the patients and control persons were subdivided in several b/c score classes

There were no differences across institutions in the distribution of mutagen sensitivity (Kruskall-Wallis test) for both cases and controls. HNSCC case values were consistently and significantly ($p<0.0001$) higher compared to control values in the overall analyses. In controls, no correlations were found in the combined data between b/c values and pack years, daily tobacco consumption, unit years, daily alcohol intake or age. In the HNSCC patient group, however, a very small ($r=0.18$; $p< 0.01$) correlation was found between b/c values and age. The means of the patients and controls were significantly different (Mann Whitney U test) for age, pack years, daily alcohol intake and mutagen sensitivity.

Although the absolute difference between the average b/c levels of patients and control persons was not large, frequency analysis clearly shows a difference in the distribution of b/c values of the two populations (Figure 1). Moreover, 53.8% of the HNSCC patients had a b/c score ≥ 1, defined as a hypersensitive phenotype [Hsu *et al.*, 1989]. Of the control persons only 25.7% exhibited the hypersensitive phenotype.

TABLE 2 *Risk estimates (crude odd ratios (95% confidence intervals)) for mutagen sensitivity by tobacco and alcohol exposure*

stratification		all	b/c <1	b/c ≥1
Tobacco use only				
never smoked		1	1	2.64 (0.92-7.6)
< 30 pack years		3.54 (1.96-6.4)	3.13 (1.37-7.19)	12.1 (5.0-29.4)
≥ 30 pack years		12.8 (6.9-23.9)	10.6 (4.5-25.8)	45.1 (17.0-120)
Alcohol use only				
0-2 u/day		1	1	3.45 (2.04-5.83)
2-5 u/day		1.83 (1.2-2.9)	1.76 (0.96-3.24)	7.09 (3.33-15.1)
≥ 5 u/day		7.18 (3.8-13.6)	8.0 (3.7-17.3)	25.4 (7.3-88)
Tobacco and alcohol use				
non-smoker	< 2 u/day	1	1	2.25 (0.5-9.4)
	≥ 2 u/day	-*	-*	-*
smoker	< 2 u/day	4.6 (2.1-9.9)	3.7 (1.3-10.1)	13.2 (4.6-37.6)
	≥ 2 u/day	14.2 (6.5-31)	11.0 (3.9-30)	57.5 (17.5-188)

* The number of subjects in the group of non-smokers who drink more than two units of alcohol per day was too low for the accurate estimation of odds ratios.

No difference was found in the b/c levels between males and females for both the patient (1.0 and 1.02 respectively) and control group (0.81 and 0.84 respectively). There were no differences in b/c values by tumor site.

Multiple linear regression was performed to estimate the joint influence of age, tobacco and alcohol use on the b/c values. Neither in the controls nor in the HNSCC patients did any of these variables influence the outcome of the mutagen sensitivity value. We also performed stratified analysis to assess the interaction of mutagen sensitivity with several smoking and alcohol categories (Table 2). There was an interaction between mutagen sensitivity and heavy smoking or alcohol that appeared to be greater than multiplicative. This pattern appeared to persist in the site-specific analyses; however the numbers in several groups were too small for precise estimation of risks. A strong increase in risk was also noted for those persons who were mutagen sensitive and were also exposed to both tobacco and alcohol. However, there was only one non-smoking patient who drank more than 2 units of alcohol per day. Thus, the influence of drinking in non-smoking subjects could not be evaluated.

TABLE 3 *Multiple logistic regression data after stratification for cumulative smoking*

Variable	Coefficient	P-value	Relative risk	95% confidence interval
age	0.0067	0.38	1.01	0.99 - 1.02
b/c (non smoker)	-0.47	0.25	0.63	0.28 - 1.38
b/c x 0>py>10*	0.56	0.39	1.75	0.49 - 6.28
b/c x 10≥py>20	1.68	0.002	5.39	1.88 - 15.5
b/c x 20≥py>30	2.09	0.0002	8.07	2.65 - 24.5
b/c x ≥30py	2.85	0.0000	17.4	6.4 - 47.1
constant	-1.03	0.0149		

*py: pack years

On multiple logistic regression, corrected for age, the b/c level did not influence cancer risk significantly in non-smokers. However, mutagen sensitivity had a large impact on cancer risk for those subjects exposed to tobacco in a dose dependent relation (Table 3). The model on which the multiple logistic regression analysis was based [Cloos *et al.*, 1994] fits the data statistically significantly (likelihood ratio test: $p<0.0001$). An important observation was that addition of "hospital" as a variable did not change the outcome, indicating that the analyses were not influenced by the study site.

Discussion

Intrinsic susceptibility and exposure to carcinogens can act in concert to modulate cancer risk [Li and Montesano, 1994; Harris, 1991; Ponder, 1990]. At the extreme end of the susceptibility spectrum are patients with Ataxia telangiectasia (AT). Even heterozygote carriers of the AT-gene have been shown to be sensitive to *in vitro* radiation in the G2-phase [Sandford *et al.*, 1990, Scott, 1994] and are thought to be at increased risk for the development of breast cancer after exposure to low amounts of radiation [Swift *et al.*, 1991]. An approach in detecting cancer susceptibility is the use of the "mutagen sensitivity assay" in which chromosomal instability is measured in peripheral blood lymphocytes after inflicting damage *in vitro* with bleomycin in the S-G2 phase of the cell cycle. A hypersensitivity phenotype has been found to be a risk factor for common cancers, such as HNSCC, lung, and colon cancer [Hsu *et al.* 1989].

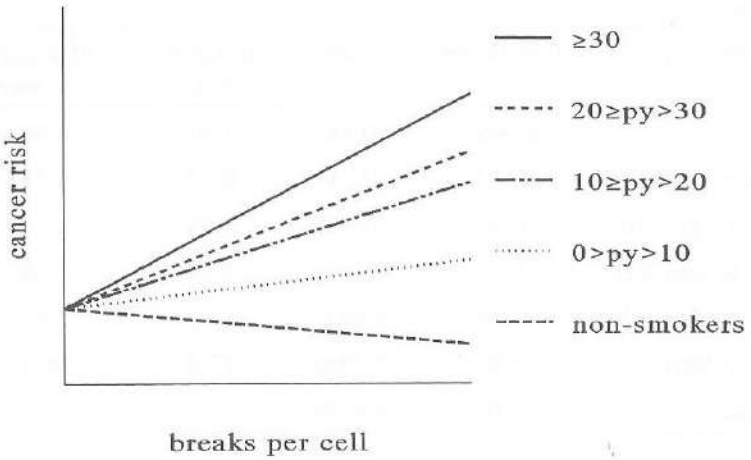

FIGURE 2 Representation of the model which was used to perform multiple logistic regression Increasing values of b/c (x-axis) influences cancer risk (y-axis) only in tobacco exposed individuals.

For the investigation of cancer susceptibility, a multi-disciplinary approach [Hecht, 1994], and large numbers of subjects are required. The grouping of data from three different institutions resulted in a unique population of subjects for whom mutagen sensitivity was assessed.

Analysis of the combined data confirmed a significant difference in b/c value between patients and controls, as previously reported [Cloos *et al.*, 1993]. Age, cigarette pack years or daily alcohol intake did not influence the b/c score. The degree of concordance of the mutagen sensitivity data across the three studies is also highlighted.

The most important finding of this study is that mutagen sensitivity greatly improved the risk estimation when exposure characteristics were also considered. In smokers, mutagen sensitivity increased cancer risk in a dose dependent and interactive pattern. The multiple logistic regression analysis as visualized in Figure 2 shows that at a low mutagen sensitivity level (intersection with the y-axis), exposure to carcinogens does not greatly increase cancer risk. This phenomenon was already observed in a study in which the mutagen sensitivity was measured of elderly men who never developed a tumor, but who had an extensive exposure to alcohol and tobacco [Hsu *et al.*, 1991]. Interestingly, 98% of the subjects in this latter population had a non-sensitive phenotype. The low impact of mutagen sensitivity in non-exposed subjects may indicate that endogenous DNA damage does not play a large role in

carcinogenesis for these persons.

This figure shows a schematic representation of the multiple logistic regression model in which the angle of the lines represent the coefficients mentioned in table 3. Therefore, no values can be given on the coordinates. The data fitted statistically significant ($p < 0.0001$) into this model.

The potentiating effect of alcohol on the effects of tobacco smoking which we find in this population has been described by others as well [Baron, 1993; Maier *et al.*, 1992], and can be explained by the assumption that alcohol, although not a carcinogen, can temporarily inhibit DNA repair capacity [Hsu and Furlong, 1991].

This multi-institutional and interdisciplinary study emphasizes the importance of intrinsic susceptibility in estimating carcinogenic risk (Figure 3). Mutagen sensitivity is a model system which measures response to genotoxic assaults, a phenotype which may reflect susceptibility to cancer in exposed individuals. One important implication of this mutagen sensitivity assay for HNSCC patients is that it may serve to predict which patients have the highest risk of developing second primary tumors [Spitz *et al.*, 1994] and who can be targeted for more intense follow-up [Snow, 1992], behavioral interventions and chemoprevention studies [Lippman *et al.*, 1994]. In addition to a high number of chromatid breaks it was recently found that the induced chromatid breaks are not random, but occur at specific targeted sites [Dave *et al.*, 1994; Wu *et al.*, 1995]. This stimulates further research to be conducted to study the molecular mechanisms underlying mutagen sensitivity.

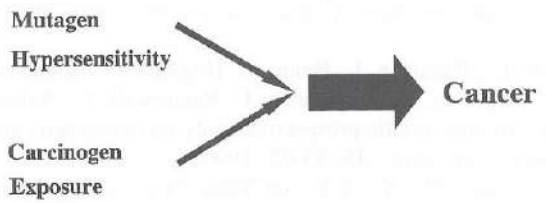

FIGURE 3 Mutagen sensitivity as a dependent risk factor for the development of HNSCC due to carcinogenic exposure The interaction of a hypersensitive phenotype with the exposure to environmental carcinogens amplifies the risk for cancer as indicated by the bold arrow.

We would like to encourage other investigators in this field of research to include the mutagen sensitivity assay in their investigations of susceptibility markers. Although

controversy exits whether the scoring of chromatid breaks may be subject to inter-observer variation [Brøgger *et al.*, 1990], the assay can easily be performed in all kinds of laboratories, when standardized scoring procedures are used. Moreover, we would like to emphasize the importance of including any relevant exposure factors in the risk estimations of intrinsic susceptibility markers for cancer.

The profound importance of mutagen sensitivity for overall concepts of cancer susceptibility for environmentally related cancers is underscored by this study. Mutagen sensitivity is a model system which measures response to genotoxic assaults (Figure 3), a phenotype which may reflect susceptibility to cancer in exposed individuals.

Acknowledgements

This study was supported by the Scientific Counsel of Smoking and Health, The Netherlands. Grant NCI RO1 CA 51845 provided support for the study of the Memorial Sloan-Kettering Cancer Center, and RO3 CA 50945 for the MD Anderson Cancer Center.

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Chapter 5

DNA DAMAGE PROCESSING IN BLOOD LYMPHOCYTES OF HEAD AND NECK SQUAMOUS CELL CARCINOMA PATIENTS IS DEPENDENT ON TUMOR SITE

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Submitted

Abstract

In this study two assays were performed to measure DNA damage in human peripheral blood lymphocytes. A chromosomal aberration assay which determines sensitivity to chromatid breaks induced by bleomycin was used and an elegant immunochemical assay which measures induction of radiation induced strand breaks as well as subsequent repair. The aim of this study was to evaluate whether besides bleomycin induced chromosomal instability, radiation induced initial DNA damage and subsequent repair may be associated with developing HNSCC.

Age, smoking and alcohol drinking behavior did not influence the number of chromatid breaks, initial DNA damage or repair capacity. The mean number of chromatid breaks per cell induced by bleomycin was significantly different between patients and control persons, whereas, the amount of initial DNA damage induced by radiation was not. No correlation was found between the outcome of the two assays neither in head and neck squamous cell carcinoma patients nor in control persons. In contrast to laryngeal carcinoma patients, oral cavity carcinoma patients showed a significantly slower repair capacity compared to control persons. Therefore we hypothesize that the way DNA damage is processed by the patients determines at which site the cancer develops in in the head and neck area.

Introduction

It is becoming increasingly accepted that the risk for the development of cancer depends not only on exposure to carcinogens, but also upon individual cancer susceptibility [Markham *et al.*, 1994]. Suitable assays to measure an intrinsic sensitivity for DNA damage are based on determination of chromosomal damage in peripheral blood lymphocytes after induction of strand breaks in the G2-phase of the cell cycle [Hsu, 1987; Sanford *et al.*, 1990; Scott, 1994]. In the assay of Hsu, chromosomal damage (also referred to as mutagen sensitivity) is measured as the mean number of chromatid breaks per cell (b/c) in mitogen stimulated peripheral blood lymphocytes (PBL) which are challenged *in vitro* with bleomycin (BLM). Using this assay in a retrospective case-control study it was demonstrated that the PBL of head and neck squamous cell carcinoma (HNSCC) patients exhibited a significantly higher b/c level compared to control persons. Values were highest in patients that had developed multiple primary tumors [Cloos *et al.*, 1994]. This test may therefore be used as an important tool to identify those patients at high risk for multiple primary tumors. Each year about 3% of the curatively treated HNSCC patients develop a second primary tumor. Identification of high risk individuals is important since they can be followed up more frequently so that early detection of further primary tumors may result in increased patient survival [Snow, 1992].

The chromosomal aberration assay, either with bleomycin or with radiation, has shown to be valuable for the identification of patients who are hypersensitive to DNA damaging agents. For example, both homo- and heterozygote Ataxia telangiectasia patients who are at increased risk of cancers can be identified using such a procedure [Sanford *et al.*, 1990]. Recently an assay was designed to determine the DNA breaks in cells [Timmerman *et al.*, 1995] which is based on measurement of single stranded DNA after partial alkaline unwinding. The percentage of single-strandedness is measured using a sandwich-ELISA system with an antibody against single stranded DNA.

The aim of this study was to investigate whether besides the induced chromosomal instability, as measured with the mutagen sensitivity assay, a high level of initial DNA damage or DNA repair, as measured with the immunochemical assay, may also be related to the development of HNSCC. Moreover, when this fast test system could detect sensitive persons it may facilitate the determination of cancer susceptibility in a diagnostic setting.

Materials and methods

Subjects

The characteristics of the subjects are summarized in table 1. The control persons (n=19) in this study were healthy laboratory personnel or patients from the clinic without a tumor history. The HNSCC patients (n=18) consisted of previously untreated patients with tumors in the oral cavity (including tongue) or larynx.

TABLE 1 *Subject characteristics*

variable	control persons	HNSCC patients
age	38.9 ± 12.4 (18)	60.9 ± 7.2 (16)[1]
cigarettes per day	3.5 ± 7.2 (18)	18.4 ± 13.3 (16)[1]
alcohol units per day	1.3 ± 1.2 (17)	3.8 ± 3.0 (16)[1]

[1] significantly different ($p<0.01$) from control persons. Data are given as means ± standard deviation (number of subjects).

Detection of gamma-radiation induced DNA breaks

In whole blood of the subjects the amount of DNA damage was determined using an immunochemical method [Timmerman et al., 1995]. This assay measures DNA damage which gives rise to single-strandedness in DNA during partial unwinding under alkaline conditions. The blood had to be used within 2 hours after collection to obtain the most reproducible results (data not shown). In short, 200 µl whole blood was chilled on ice for 10 min and irradiated still on ice using a ^{60}Co-γ-source (Gamma Cell 200 Atomic energy of Canada, Ltd.) with a rate of 3 Gy/min. From the irradiated blood the DNA was either unwound immediately or allowed to repair at 37°C for several time periods. Protected from UV light, 30 µl of the irradiated blood was transferred to a round-bottom polystyrene-tube (Costar Europe Ltd., Badhoevedorp, The Netherlands) and incubated for 6 min with 800 µl 1.3 M NaCl adjusted for pH 12.3 with 1.0 M NaOH for the alkaline unwinding of the DNA. The unwinding was terminated by addition of 145 µl 250 mM NaH_2PO_4, and the solution was immediately sonicated to fragment the DNA and to prevent rewinding. At this stage the samples could be stored at -20°C before further processing. To measure the amount of single-strandedness in the DNA, a sandwich ELISA was performed. A 96-wells high binding microtiter plate (Costar) was coated with an antibody directed against single stranded DNA (D1B; developed and produced by TNO Rijswijk, The Netherlands). The thawed unwound blood samples were diluted in phosphate buffered saline (PBS) depending on the expected amount of single strandedness: 0 Gy samples 2.5 times and the 2 and 5 Gy samples 5 times. The amount of single strandedness in the samples was calculated as a percentage of their completely unwound controls (100% single stranded).
In duplicate, the samples (20 µl in 120 µl PBS) and their 100% controls were 6 times serialy diluted 1:1 in PBS and incubated for 30 min at room temperature while shaking. After washing the plates 3 times with PBS containing 0.05% Tween 20, the same antibody which was labelled with alkaline phosphatase (1:15,000 D1B-AP in PBS containing 5% FCS and 0.05% sodium dodecyl sulphate) was incubated for 30 min at room temperature. Then the plates were washed three times with PBS containing 0.05% Tween 20, and 2 times with 0.1 M diethanolamine pH 9.8. As a substrate for the alkaline phosphatase, 4-methylumbellipheryl phosphate (MUP) was used (200 µM in reaction buffer: 10 mM diethanolamine and 1 mM $MgCl_2$). After an incubation of about 15 min at room temperature fluorescence was measured using a Fluorocount 96 platereader (Pharmacia, Uppsala, Sweden). The percentage of single-strandedness was calculated using the fluorescence signal of the 100% single stranded control of the sample with the corresponding signal of the sample. These calculations were carried out only for those dilutions for which a linear relationship was observed between the amount of DNA in the wells and the level of fluorescence after being corrected for background fluorescence levels, and dilution.

Mutagen sensitivity assay

Human peripheral blood lymphocytes were cultured in duplicate at 37°C, 5% CO_2, for 72 hr. One half ml heparinized whole blood was diluted in 4.5 ml RPMI 1640 medium (Flow laboratories, Irvine, UK) with 2 mM L-glutamine (Gibco, Paisley, UK) supplemented with 15% fetal calf serum (Flow Laboratories, Irvine, UK), 1.5% phytohaemagglutinin (Wellcome Diagnostics, Dartford, UK), 100 IU/ml penicillin and streptomycin (ICN biomedicals ltd, Irvine, UK). 30 mIU/ml bleomycin (Lundbeck, Copenhagen, Denmark) was added 5 hours before cell harvest, ensuring that damage induced in the late S- and G2-phase of the cell cycle could be evaluated at metaphase. Cells were arrested in metaphase by adding 100 μl, 50 μg/ml Colcemid (Sigma, St Louis, USA) 1 hour before harvesting. The cells were collected by centrifugation (300 *g*, 5 min) and treated with a hypotonic solution (0.06 M KCl) for 20 min. For fixation and washing (3x) of the cells, Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) was used. Cells were dropped on wet slides and after air drying, were stained with 5% Giemsa solution (Merck, Darmstadt, Germany), coded and scored under a light microscope with a magnification of 1250 x. From each slide, 50 metaphases were evaluated for the presence of chromatid breaks. The mean number of breaks per cell (b/c), based on evaluation of two slides of one person (100 metaphases), was taken as a measure for mutagen sensitivity.

Statistics

Unless stated otherwise, the significance level of differences between parameters was calculated using a two-sided Student's t-test. Correlations were determined using the Pearson correlation coefficient.

Results

The patients differed significantly ($p<0.01$) from control persons in age, cigarette smoking and daily alcohol intake (Table 1). The results of the mutagen sensitivity assay and the immunochemical assay are listed in table 2. There was no correlation in neither the patient nor the control person group between the outcome of both assays and the variables age, smoking and alcohol intake.

TABLE 2 *Chromosomal and DNA damage as measured using the mutagen sensitivity assay and the immunochemical method, respectively*

		controls n=19	HNSCC n=18	oral cavity n=9	larynx n=9
Mutagen sensitivity (b/c)		0.68 ± 0.23	0.85 ± 0.27[1]	0.82 ± 0.24	0.88 ± 0.31
Single strandedness (%)	0 Gy	3.7 ± 1.2	4.6 ± 1.5	4.4 ± 1.6	4.8 ± 1.4
	2 Gy	13.7 ± 4.3	12.8 ± 4.7	10.7 ± 1.2	13.6 ± 5.4
	5 Gy	23.5 ± 5.2	25.6 ± 5.2	25.2 ± 5.8	25.9 ± 4.8
	10'[2]	15.1 ± 2.7	18.3 ± 5.2[1]	20.4 ± 5.7[1]	16.1 ± 3.9
	30'[2]	10.8 ± 2.8	13.9 ± 4.3[1]	14.9 ± 3.8[1]	13.0 ± 4.7
	60'[2]	9.7 ± 2.6	11.7 ± 4.0	13.7 ± 3.4[1]	9.7 ± 3.5

[1] significantly different ($p<0.05$) from control persons. Data are given as means ± standard deviation. [2] repair time (min) at 37°C after 5 Gy irradiation.

There was no correlation with any of the parameters measured with the immunochemical method and the b/c value according to the mutagen sensitivity assay, either in the patients or in the control persons. In line with earlier studies, a significant difference ($p<0.025$, one sided Student's t-test) was found in b/c value between patients (0.85 ± 0.27; n=17) and control persons (0.68 ± 0.23; n=18). This difference was not found for the initial percentage of single strandedness after 0, 2 or 5 Gy radiation using the immunochemical assay. Only after 10 to 60 min of repair at 37°C there was a difference between the single strandedness of patients and control persons. As a control for background damage the non-irradiated samples were also measured after several incubations at 37°C and no increase in damage up to 60 min was observed either in the patients group nor in the control persons. The percentage of single strandedness of non-irradiated samples was low

and did not correlate with the initial DNA damage after 2 or 5 Gy.

When the HNSCC patient group was separated on tumor site (Figure 1) a trend was found in which the larynx tumor patients differed from the oral cavity tumor patients. However, this was only statistically significant ($p=0.04$) after 60 min of repair at 37°C.

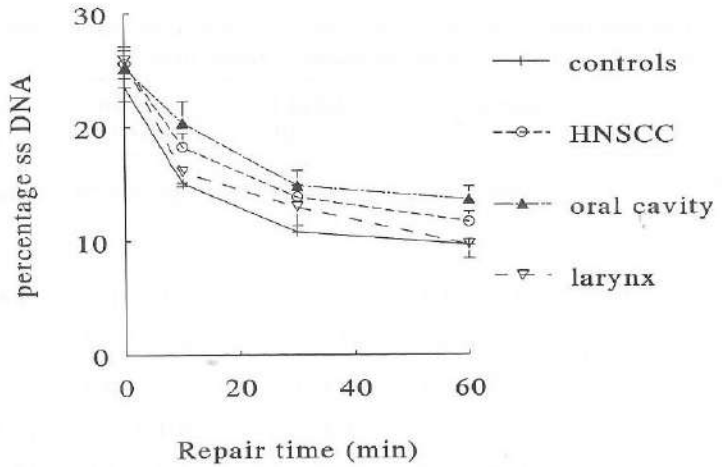

FIGURE 1 The percentage of single-stranded DNA was measured using the immunochemical method after induction of damage at 5 Gy gamma-radiation. The cells were allowed to repair for several time periods at 37°C. Means are given with their standard error of the mean (SEM). HNSCC refers to both larynx and oral cavity patients.

There was no difference in any of the variables measured with the ELISA system between control persons and larynx cancer patients. On the other hand, the oral cavity cancer patients showed a significant difference with control persons at all the time points after repair at 37°C with the exception of the initial amount of single strandedness induced by 5 Gy radiation. Figure 2 shows the relative repair capacity towards the initial damage at 5 Gy which was set at 100% for each individual.

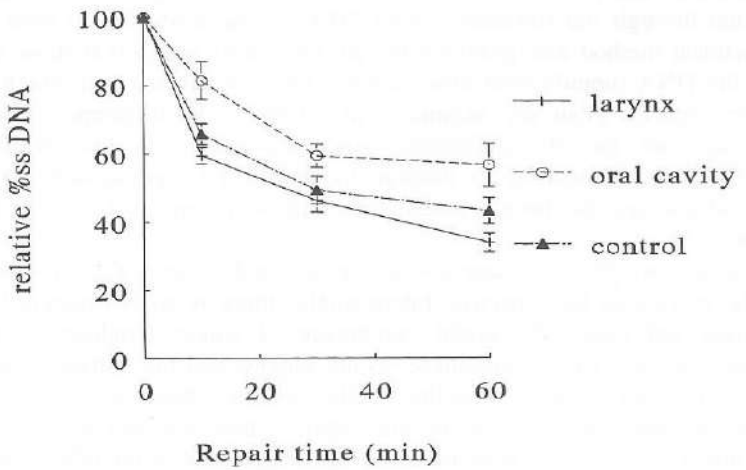

FIGURE 2 For each person the damage at 5 Gy was taken as 100% and the relative repair rate was calculated. Means are given ± SEM.

This figure clearly demonstrates the slow repair kinetics in the cells of oral cavity tumor patients compared to cells of larynx tumor patients or control persons. Age, smoking, or alcohol drinking behavior did not differ between larynx and oral cavity tumor patients.

Discussion

The immunochemical assay used in this study offers a very elegant and fast assay to detect DNA damage [Timmerman *et al.*, 1995] in human blood cells. The assay is not suited for cultured lymphocytes since the detection of breaks is very sensitive resulting in a high level of background damage in non-treated cells (possibly due to okasaki fragments of S-phase cells or apoptotic cells). In whole (uncultured) blood, all the cells are in the G0-phase and strand breaks can therefore be very accurately measured. The bleomycin induced chromosomal aberration assay, however, is performed in cultured cells with special emphasis on damage induced in the G2-phase of the cell cycle. This difference in cell cycle may explain the lack of correlation we have found. Moreover, the chromosomal aberration

assay only detects double strand breaks whereas the immunochemical assay measures both single- and double strand breaks. In addition, the cultured blood in the chromosomal aberration assay mainly consists of T-lymphocytes which have been selected through the stimulation with PHA. In the whole blood used for the immunochemical method also granulocytes are present of which it is more difficult to release the DNA (unpublished observations, Van der Schans) and which have a slow repair capacity [Van der Schans *et al.*, 1989]. The difference in damage inducers may not be of importance, since bleomycin is recognized as a radiomimetic agent. Sensitivity to damage induction in G2-phase cells of cancer prone individuals has also been reported for radiation [Scott *et al.*, 1994; Sanford *et al.*, 1990].

Although the two DNA damage assays are not easily comparable an association between the two could be expected. Interestingly, there is no correlation between the b/c level and either the initial percentage of single-strandedness or after allowing the cells to repair. Thus, these results suggest that the mutagen sensitivity measured with bleomycin in cycling cells reflects other aspects of DNA maintenance besides damage induction and repair. These findings are in line with an earlier report [Pandita and Hittelman, 1995] using neutral filter DNA elution, in which mutagen sensitive lymphoblastoid cell lines showed, in contrast to the high level of chromosomal damage, a similar initial DNA damage and repair compared to control cell lines in all phases of the cell cycle. These authors concluded that among other factors, mutagen sensitive persons may have an increased efficiency for translating DNA damage into chromosome damage depending on the chromatin structure.

Comparing the initial percentage of single strandedness of the patients with the control persons no difference could be detected. However, after repair the patients showed a slightly higher level of damage. Separating the patients on basis of the site of the tumor revealed that the difference was entirely due to the oral cavity tumor patients, who had a poorer repair capacity. The difference between oral cavity and larynx cancer patients can not be explained by age, smoking or daily alcohol intake, which were the same in both groups. Oral cavity cancer patients may have a relatively higher amount of granulocytes in their blood explaining the slow repair capacity found in our assay, although no data are available to substantiate this hypothesis which however, remains unlikely. A difference in the carcinogenic process between larynx and oral cavity tumor patients is more feasible since it has already been indicated by differences in etiology [Schottenfeld, 1985] based on differences in the impact of risk factors such as tobacco and alcohol. Moreover, a variable susceptibility to the carcinogenic action of alcohol and tobacco by tumor site was suggested after a large case comparison analysis [Spitz *et al.*, 1988].

In the present study, the larynx cancer patients had the capability to repair the breaks very quickly. However, it is possible that this "fast" repair may be error prone. When an erroneously filled break is not corrected, persistent mutations may occur, initiating carcinogenesis and thus explaining cancer susceptibility. Similar results have been found in Ataxia telangiectasia cells [Powell *et al.*, 1993] of which it is known that they do not take the time to repair correctly due to a lack of a cell cycle block after damage induction [Kastan *et al.* 1992]. Indications of this impaired repair fidelity have not only been found for Ataxia telangiectasia patients [Luo *et al.*, 1995] but also for mutagen sensitive persons [Wei *et al.*, 1995]. Further studies will be conducted to measure repairing ability in cells of larynx cancer patients using a plasmid reporter gene reactivation assay, to substantiate the error-proneness of the "fast" repair which we found for larynx tumor patients.

This study indicates that the radiation induced damage and repair are not associated with bleomycin induced chromosomal instability. The fact that no difference is found in initial DNA damage and repair between patients and control persons indicates that the immunochemical assay is not suitable to assess an individual's susceptibility to cancer. However, the immunochemical assay may give information about the amount of initial DNA damage and repair capacity that may be of importance in explaining the process of head and neck carcinogenesis. It is suggested that this process differs between the development of oral cavity versus laryngeal cancer.

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Chapter 6

INFLUENCE OF THE ANTIOXIDANT N-ACETYLCYSTEINE AND ITS METABOLITES ON DAMAGE INDUCED BY BLEOMYCIN IN PM2 BACTERIOPHAGE DNA

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Carcinogenesis, In Press

Abstract

Bleomycin is considered to be a useful model compound for studying environmental carcinogenesis, due to its broad spectrum of DNA-damaging properties. In addition, bleomycin is a useful antitumor drug because of its cytotoxic properties. To investigate the influence of the antioxidant N-acetylcysteine and its metabolites glutathione and cysteine on the bleomycin-induced DNA damage and more importantly to get insight into the biological relevance of such damage, PM2 DNA was exposed to Cu^{2+}-bleomycin in the presence and absence of the thiols N-acetylcysteine, glutathione, and cysteine. It was found that the presence of these thiols lead to a considerable enhancement of bleomycin-induced single- and double-strand breaks and a concomitant decrease of the biological activity of PM2 DNA in a dose dependent way. A similar observation was made when ascorbic acid was used. Bleomycin showed no DNA-damaging activity when PM2 DNA was pretreated with the strong Fe-ion chelator desferal and its activity was strongly inhibited by the addition of Cu^{2+}-ions or under hypoxic (N_2) conditions. Cu^{2+}-bleomycin under our conditions is not active by itself, but most probably after binding to DNA exchanges Cu^{2+} against Fe^{3+} bound to DNA. Fe^{3+}-bleomycin is then reduced to Fe^{2+}-bleomycin, a process which is potentiated by the added antioxidants, and subsequentially activated by O_2. The contribution to biological inactivation by bleomycin alone or in the presence of ascorbic acid is only about 15%. The contribution to lethality in the presence of thiols is higher. These results indicate that ascorbic acid just enhances the DNA-damaging properties of bleomycin, whereas the thiol compounds in addition influence the type of DNA damage. The remainder part of the biological inactivation is probably caused by double-damages such as single strand breaks with closely opposed alkali-labile sites or base-damages.

Introduction

A new goal in cancer research is to protect persons against the introduction of DNA damage in order to prevent mutational events that may induce carcinogenesis. To investigate this, population- based studies are currently conducted to delay or inhibit the process of carcinogenesis through the supplementation of protective agents [Boone and Wattenberg, 1994]. These so-called chemoprevention strategies are not readily feasible for the whole population. However, for selected groups of people with an increased risk to develop cancer, chemoprevention has much better opportunities. One such specific group that could possibly benefit from chemoprevention are curatively treated head and neck squamous cell carcinoma (HNSCC) patients who are at high risk for the development of second primary tumors. For this latter group, chemoprevention trials are performed by

supplementation of free radical scavengers [De Vries *et al.*, 1992] with the aim to protect against free-radical-mediated DNA damage. An important and presently used chemopreventive drug is the thiol N-acetylcysteine (NAC), a potent antioxidant, which is often used as a mucolytic drug in lung diseases and which has only minor toxic side effects [Ventresce *et al.*, 1989]. The selection of NAC for clinical trials was based on its antimutagenic [De Flora *et al.*, 1991] and anticarcinogenic [De Flora *et al.*, 1986] activity in bacteria and murine model systems.

It has been well established recently, that people differ in their susceptibility for cancer. In an attempt to measure this phenomenon it has been found that peripheral blood lymphocytes of HNSCC patients are very sensitive for the clastogenic effects of bleomycin (BLM) [Hsu *et al.*, 1989; Cloos *et al.*, 1994]. This finding was based on *in vitro* measurements of chromosomal aberrations in mitogen-stimulated lymphocytes which were challenged with BLM. This antibiotic and cytotoxic drug may be considered as a very useful test compound, since it has a broad spectrum of DNA-damaging properties which are comparable to carcinogens to which the patients are exposed [Pryar, 1982]. An important mechanism for the genotoxic effects of BLM is the formation of single-strand breaks (SSB), double-strand breaks (DSB) and apurinic/apyrimidimic (AP) sites containing oxidized deoxyribose moieties by the activated BLM and possibly in part by hydroxyl radicals generated via free-radical-mediated reactions [An and Hsie, 1993]. Theoretically, antioxidants, such as thiols, may be able to protect against BLM-induced DNA damage through scavenging of the free radicals. On the other hand, instead of a protective effect, a potentiation of DNA damage by thiols has also been reported [Hofmann *et al.*, 1994; Lafleur and Retèl, 1993]. This latter effect is interesting for the cytotoxic properties of BLM which may imply an increased efficacy for the treatment of cancer.

The purpose of the study presented in this paper was to investigate whether the thiol NAC can interfere with the introduction of DNA damage by BLM and whether this interference leads to protection or potentiation. The influence on DNA damage was studied in a cell-free system, in which supercoiled PM2 DNA was exposed to BLM in combination with NAC. It has been reported that NAC is very easily deacetylated even before it enters the cell and is converted into cysteine (Cys) [Sjodin *et al.*, 1989]. Moreover, a significant increase in glutathione (GSH) concentration has been found in the plasma of rats after oral administration of 100 mg NAC [De Flora *et al.*, 1991] and in humans after daily intake of 600 mg [Bongers *et al.*, 1995]. Since in our cell free system no enzymes were present to convert NAC, the experiments were also performed by adding these two active metabolites to our system. To compare the results obtained with these thiols, with another well known potent antioxidant, experiments were also performed with ascorbic acid (Asc) [Henson *et al.*, 1991]. The PM2-system not only allows us to determine the kind and number of certain types of DNA lesions such as SSB and

DSB by agarose gel electrophoresis, but in addition, the influence of these lesions on the biological activity of the DNA could be measured. This latter measurement was performed by transformation of the exposed PM2 bacteriophage DNA to its host bacteria (*Alteromonas espejiana*).

Materials and methods

Bacteriophage DNA and bacterial strains

PM2 DNA was obtained from Boehringer Mannheim, Germany. For biological activity measurements, the bacteria strain *Alteromonas espejiana (Pseudomonas BAL-31)* was used from the National Collection of Industrial and Marine Bacteria (NCIMB (cat. no.1879)), Aberdeen, Scotland. From this strain we isolated a variant, *Alteromonas espejiana/PM2^R*, which can not be infected by PM2 bacteriophages [Espejo *et al.*, 1969; Van der Schans *et al.*, 1971].

Exposure conditions

Ten μl of PM2 DNA (30 ng/μl) was dissolved in 10 μl of 250 mM phosphate-buffer (KH_2PO_4:K_2HPO_4 1:4 at pH 7.4) and exposed to 10 μl of several concentrations of Cu-BLM (a gift of Lundbeck, Amsterdam, The Netherlands) and 20 μl of H_2O (or several concentrations of antioxidants) in a 0.5 ml Eppendorf vial for 1 hr at 37°C. The reaction was stopped by adding 10 μl of 30 mM EDTA. Stock solutions of antioxidants (1 mM) were prepared in 250 mM phosphate buffer and checked for pH 7.4. Asc and Cys were obtained from Sigma, St Louis, MO, USA. GSH was purchased from Boehringer Mannheim, Germany. NAC was obtained from BUFA BV., Uitgeest, The Netherlands. All solutions were freshly prepared before performing the incubations.

In a single experiment the iron concentration was reduced by dialyzing the DNA against 0.3 mM desferal (in tridest) for 24 hr followed by 1 week dialyzation against tridest. To determine the influence of copper, the BLM activity was also determined at 10 and 50 μM $CuCl_2$.

Measurements of strand breaks

Strand breaks were measured using gel electrophoresis (0.8% agarose (Pronarose Hispanic, SphaeroQ, Leiden, The Netherlands)) in TAE buffer (40 mM Tris, 20 mM Na-Acetate, 2 mM EDTA, pH 7.6 with acetic acid) containing 0.5 μg/ml ethidium bromide. To 10 μl of the exposed DNA (50 ng) 2 μl sample buffer (15% Ficoll, 5 mM EDTA, 0.25% bromophenol blue) was added and the whole mixture was loaded on the gel. After electrophoresis at 50 V, 100 mA for 2 hr, the gel was illuminated with UV and the image was stored using a Charge Coupled

Device videocamera coupled to a Image Processing system (Cybertech, Berlin, Germany). DNA in the covalently-closed circular (CCC), open-circular (OC) and linear form was quantified using two-dimensional densitometry. A correction factor of 1.2 was used to compensate for the reduced binding of ethidium bromide to CCC DNA. The probability of a DNA molecule to contain single-strand breaks (Pss) or double-strand breaks (Pds) was calculated from the exponential decrease of unbroken (CCC) DNA molecules. According to the Poisson statistics, the probability that a DNA molecule will be broken is $e^{-\lambda}$. In practice, this is denoted as Pss = -ln [(intensity measured in the CCC band x 1.2) / intensity of all the bands (total DNA)] and Pds = -ln [((intensity of the CCC band x 1.2) + intensity of the OC band) / intensity of all the bands (total DNA)]. The amount of heat-labile lesions was measured by the decrease in CCC DNA after an additional incubation of 2 hr at 45°C. The alkali-labile lesions were measured by addition of 7.2 μl of 0.96 M NaOH (final pH 12.5) at 45°C to the 60 μl exposed DNA sample. After 75 min this was neutralized by adding 23 μl of 0.3 M HCl (in 0.2 M Tris pH 7). The number of alkali-labile lesions was calculated as the increase in SSB by alkali-treatment.

Determination of biological activity of the exposed PM2 DNA

The bacteria were cultured till an OD of 0.5 was reached (measured at A_{610}) at 25°C in BAL-broth medium which contains 8 g Nutrient Broth (Oxoid Ltd.,Basingstoke, Hants., UK) in AMS solution (10 mM KCl, 0.5 M NaCl, 50 mM $MgSO_4$ and 10 mM $CaCl_2$ in MilliQ water). Spheroplasts were prepared by resuspending the pellet of 50 ml *Alteromonas espejiana/PM2*^{R} in 1 ml NaCl-citrate (1 M NaCl and 20 mM tri-sodium citrate, pH 7.0) and 0.3 ml 30% (w/v) bovine serum albumin (Sigma, St. Louis, MO, USA). During this whole procedure the solutions were kept on ice. Lysozyme solution (100μl of 2 mg/ml freshly prepared in 10 mM phosphate buffer, pH 7.0 (HMP)) was added and after 5 min 4.9 ml EDTA-broth (BAL-broth without $MgSO_4$ but with 3 mM Na_2-EDTA) was incubated for 6 min. Finally, a mixture of 260 μl 1 M $MgSO_4$ and 40 μl 1% (w/v) protamine sulphate (Sigma, freshly prepared in HMP) was incubated for 10 min, after which the spheroplasts remained competent on ice for several hours. Transformation was performed by incubating 100 μl spheroplasts with 150 μl PM2 DNA (12 μl sample (60 ng DNA) plus 138 μl HMP) for 10 min on ice. The mixtures were cultured in 800 μl of BAL-broth for 4 hr at 25°C while gently shaking. After centrifugation the supernatant containing the phage particles was serially diluted (steps of 10-fold) in Tris-HCl (1 M NaCl, 20 mM Tris, pH 8.1) and mixed with 6 ml BAL-agar containing 8 g Bacto-Tryptone (Oxoid) and 5 g Bacto-Agar (Difco Laboratories, Detroit, MI, USA) per liter AMS solution (maintained at 42°C) and 100 μl of *Alteromonas espejiana* (overnight culture 1:2 diluted in BAL-broth). The mixture was poured out on a Petri dish and incubated O/N at 25°C. Biological activity was measured as plaque forming units (PFU) per ml. The induction of lethal hits was calculated from the exponential decrease in biological activity using the Poisson statistics: (-ln{[PFU from exposed DNA]/[PFU from unexposed DNA]}). Double strand breaks (DSB) are considered to be lethal for almost 100%, so the contribution of DSB to the biological inactivation could also be calculated. To achieve this the mean number of DSB of the exposed DNA was divided by the number of lethal hits calculated from the biological activity assay (x100%). As controls we used fresh PM2 DNA and DNA that had undergone the whole procedure except for the addition of any damaging compound.

Results

The influence of NAC, Cys, GSH and Asc on DNA strand break induction by BLM

Before screening for a possible influence of NAC on BLM-induced DNA damage, a dose-response experiment was performed in which PM2 DNA was exposed to increasing concentrations of BLM (Figure 1).

Detectable induction of both SSB and DSB occurred above a BLM concentration of about 1 μM. From 1 to 50 μM BLM a linear (r=0.98) dose response effect was noted.

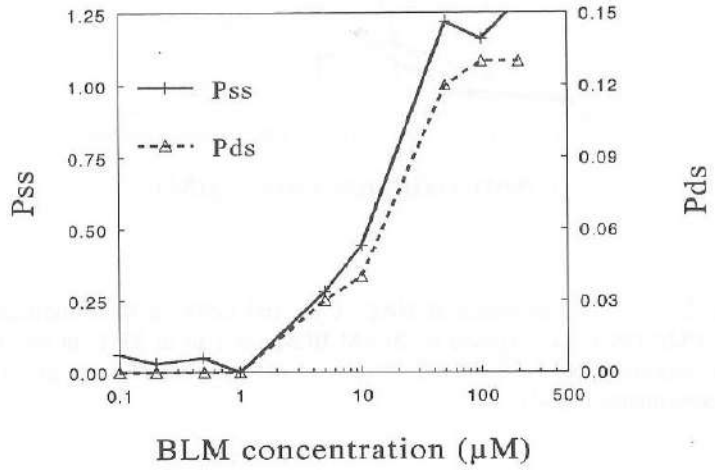

FIGURE 1 Typical example of a dose-response experiment in which several concentrations of BLM were incubated for 1 hr at 37°C with PM2 DNA. The mean number of SSB and DSB in one molecule, determined after gelelectrophoresis, are indicated as Pss and Pds which are calculated as described in Materials & Methods.

The induction of SSB and DSB appeared to proceed in a parallel fashion in which the DSB:SSB ratio was about 1:8. At about 50 μM a plateau was reached. For further study of possible influences of antioxidants, two concentrations of BLM were taken, *viz.* 2μM and 20μM. At 2 μM a potentiation of the induction of breaks can be accurately assessed, whereas at 20 μM BLM a possible protective effect of the antioxidants can be measured.

Figure 2 shows the induction of DSB at a fixed concentration of 20 μM BLM with increasing concentrations of the antioxidants. A clear potentiation of DNA damage was observed. For SSB comparable results were obtained and this was also found when the BLM concentration was fixed at 2 μM (data not shown). Especially at higher concentrations the potentiation by Cys tends to be stronger than by NAC and GSH.

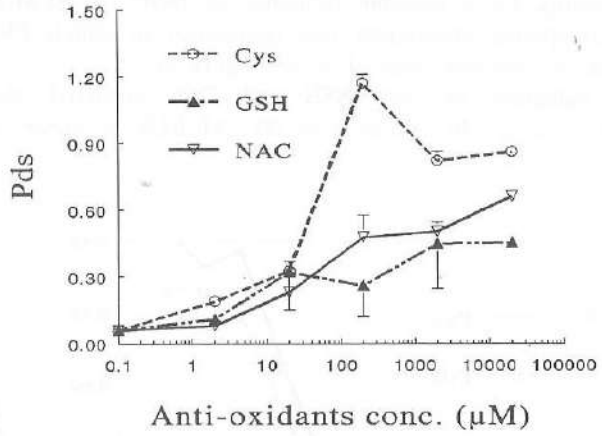

FIGURE 2 The influence of NAC, Cys, and GSH on BLM-induced strand breakage. PM2 DNA was exposed to 20 μM BLM for 1 hr at 37°C in the presence of various concentrations of the thiols. Means of the induced DSB are given from 2 separate experiments ($\pm$ sd).

The decrease in DSB at high Cys concentrations, shown in Figure 2, is due to considerable loss of material by excessive breakdown of the DNA and therefore Pds is underestimated. Exposure of the DNA to antioxidants without BLM showed no DSB and only a limited number of SSB (2 mM Cys: Pss=0.5).

To investigate whether this potentiation by thiol compounds is a common

feature of antioxidants, Asc was also tested. A broad range of Asc concentrations was used (0.2 μM to 10 mM) with 0.2 μM to 20 μM BLM. Figure 3 shows a representative example of the influence of Asc on DSB induction by BLM. Asc was very effective in enhancing the BLM-induced damage. Virtually no DSB were induced by Asc alone (10mM Asc: Pds=0.31; Pds:Pss= 1:100). The potentiation of BLM-induced DSB after preincubation of BLM with Asc for 1 hr at 37°C, was slightly less (Figure 3).

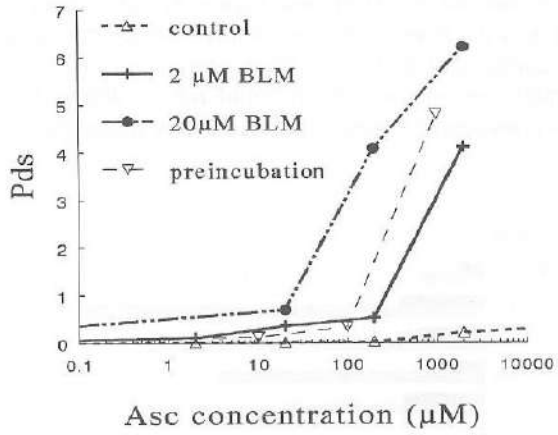

FIGURE 3 A representative experiment of the induction of PM2 DNA strand breakage by Asc. The PM2 DNA was incubated for 1 hr at 37°C with several concentrations of Asc. This was either performed without BLM (control) or with a fixed BLM concentration of 2 and 20 μM. The preincubation with Asc was performed at 10 μM BLM for 1 hr before addition of the DNA. Results are expressed as the probability of a DNA molecule to contain DSB (Pds).

The presence of iron-ions appeared to be essential for BLM to become active. When all iron ions were removed from the DNA by pretreatment with desferal no BLM-induced DNA damage could be detected anymore. A similar observation was made when copper-ions were added to the reaction mixture. Under these conditions with high copper concentrations the BLM activity was inhibited. Besides iron-ions, O_2 is also necessary for BLM activation. When the O_2 concentration was considerably reduced by replacing air in the tubes and solutions by nitrogen (N_2), the formation of BLM-induced SSB is strongly and that of DSB completely

inhibited, as shown in Figure 4. This was found both in the presence and the absence of antioxidants.

In addition to SSB and DSB, heat-labile and alkali-labile sites, i.e. lesions which can be converted into strand breaks by a heat- or alkali-treatment (17) may be expected to be introduced by exposure of DNA to BLM. No heat-labile sites appeared to be formed in PM2 DNA, 1 hr exposed to 2- or 20μM BLM, since no extra breaks could be detected upon an additional incubation of the exposed DNA at 45°C for 2 hr. On the other hand, a small amount of alkali-labile sites, detected by treatment of the exposed DNA with an additional incubation at pH 12.5 for 75 min at 45°C, were induced by BLM (see Figure 5). The alkali-labile lesions could only be measured as a decrease in the covalently-closed circular (CCC) form DNA. The alkali-labile lesions that may have been present in the OC-form DNA (which per definition contain one or more SSB) could not be detected in this assay, since this DNA form is converted to single stranded DNA fragments by alkali treatment.

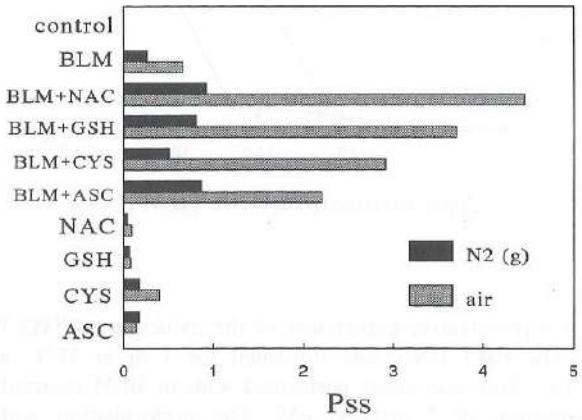

FIGURE 4 The influence on the induction of SSB in PM2 DNA by BLM and thiols is shown both under normal (aerobic) conditions and under N_2(g). No DSB were induced under the anaerobic conditions.

Unexposed DNA also appeared to contain alkali-labile sites, *viz.* on the average about 2 per PM2 DNA molecule. In the presence of GSH and Asc the number of these lesions were considerably reduced both in BLM-exposed and unexposed DNA. In the presence of Cys, only without BLM this reduction was less. However, the amount of alkali-labile lesions was not influenced by the presence of NAC.

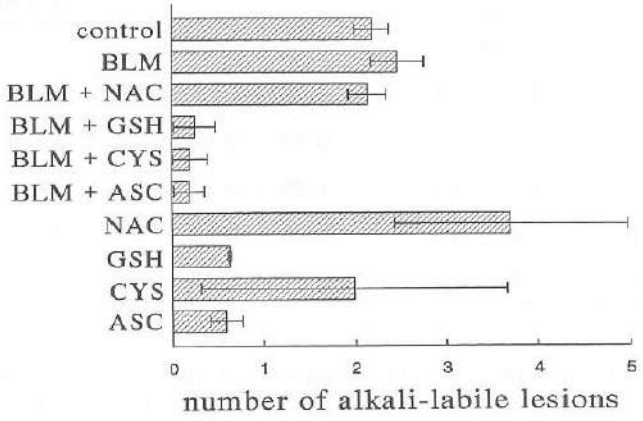

FIGURE 5 Exposed DNA samples (1 hr at 37°C to 20 μM BLM with and without 200 μM thiols or 20 μM Asc) were additionally incubated at pH 12.5 for 75 min at 45°C. The number of alkali-labile-lesions were calculated as: -ln(fraction of the DNA remaining in the CCC-form after alkaline treatment). Results are given as means of 3 experiments ± SD.

Biological activity of the exposed bacteriophage DNA

The extent to which the PM2 DNA is still capable of forming mature virus particles (plaque forming units or PFU's) is a measure for the biological activity of the DNA. From the exponential decrease in PFU's the mean number of lethal hits was calculated and summarized in Table 1. Treatment of PM2 DNA with 20μM BLM resulted into a clear biological inactivation, which considerably increased when the exposure was performed in the presence of the thiol compounds or Asc. As is also shown in Table 1, the biological inactivation can only partly be explained by the introduced DSB, which are known to be lethal for almost 100% in PM2 DNA. In case of the exposure of PM2 DNA to BLM alone or BLM plus Asc the contribution of DSB to the total inactivation is only about 15%.

TABLE 1 *Effect of BLM, in combination with antioxidants, on the inactivation of PM2 DNA*

DNA exposure	Lethal Hits	Pds	DSB/Lethal Hits (x 100%)
BLM 0	0	0	
BLM 20 μM	0.5 ± 0.1	0.07 ± 0.01	15 ± 6
+ 20 μM Asc	4.5 ± 0.6	0.57 ± 0.18	13 ± 6
+ 200 μM Cys	4.2 ± 0.9	1.26 ± 0.17	31 ± 2
+ 2000 μM GSH	1.4 ± 0.4	0.38 ± 0.10	30 ± 16
+ 2000 μM NAC	1.4 ± 0.4	0.65 ± 0.17	51 ± 28

In the case of incubations in the presence of the thiol compounds this contribution varies from about 30% (Cys, GSH) to 50% (NAC). These results indicate that an important part of the DNA inactivation must be due to types of DNA damage other than DSB.

Discussion

The broad spectrum of the DNA damaging effects, makes BLM a rather good model compound for studies of environmentally-related DNA damage [Pryar, 1982]. In order to induce DNA damage the metal-BLM complex needs to be activated. For this activation process reducing agents, the presence of DNA, and O_2 are required. Besides the direct genotoxic effects of BLM also the generation of hydroxyl radicals are important. The interaction between BLM and antioxidants is interesting from two different points of view: the combination of BLM with antioxidants may give 1) potentiation of DNA damage through their reducing properties which may result in a more effective treatment of cancer, or 2) inhibition of DNA damage through scavenging properties and thereby protect the cells against environmental carcinogens or decrease side effects in a therapeutic approach in case of antitumor treatment.

In the cell-free system used in this study, the thiols NAC, Cys and GSH potentiated the BLM-induced DNA damage. Moreover, the very potent antioxidant Asc, which was used for comparison, also increased the BLM-induced DNA damage. The potentiating effect of the various compounds can be explained by the following sequence of reaction events: Cu^{2+}-BLM, the starting compound in our experiments (see Materials and Methods) will probably bind to DNA followed by

exchange of Cu-ions chelated to BLM against Fe-ions bound or chelated to DNA. That such an exchange takes place and that Cu^{2+}/Cu^{+}-BLM by itself is not able to damage DNA under our conditions was supported by the fact that when DNA was pretreated with the strong Fe-ion-chelator desferal, BLM did not show DNA damaging activity anymore. Moreover, the activity of BLM was increasingly inhibited by increasing concentrations of Cu^{2+}, added to the reaction solution. After metal-ion exchange most of the BLM bound to the DNA will be expected to be Fe^{3+}. To become activated, it is generally assumed that in the first step Fe^{3+}-BLM has to be reduced to Fe^{2+}-BLM by a reducing agent [Burger *et al.*, 1981]. Both the thiols used in our experiments and Asc can serve as reductants in this step. However, BLM appeared to have also DNA damaging properties in the absence of a reducing agent (Figure 1). This can be explained by the assumption that also Fe^{3+}-BLM to a low extent can be activated by O_2 (the next activation step, see below) or otherwise that a small amount of iron chelated to DNA is Fe^{2+}. In the next step of the activation process Fe^{2+}-BLM is converted by O_2 into the "activated BLM", which is probably a Ferric peroxide: Fe^{3+}-$HO_2^{\cdot}$-BLM. This latter species is highly oxidizing and able to abstract a H-radical (H) from the DNA, in particular from the C-4' of the deoxyribose moieties, which in turn in subsequent reactions lead to AP-sites with an oxidized deoxyribose (alkali-labile sites) or to strand breaks with accompanying release of base propanals [Hecht, 1986]. That the presence of O_2 is a prerequisite for BLM to become activated and DNA-damaging, can be derived from our finding, that the introduction of DNA damage is strongly inhibited when air was replaced by N_2 (Figure 4). In addition to H-abstraction from deoxyriboses Fe^{3+}-$HO_2^{\cdot}$-BLM can probably also decompose and eventually form OH-radicals ($^{\cdot}$OH), which are supposed to be mainly responsible for oxidative damages [An and Hsie, 1993; Gajewski *et al.*, 1992]. Finally, Fe^{3+}-BLM, which is formed after reaction of Fe^{3+}-$HO_2^{\cdot}$-BLM with DNA or decomposition can be activated again by reduction by the reducing compounds and O_2, explaining the strong potentiating effects of the thiols and Asc we have found in our experiments [Buettner and Mosely, 1992].

The advantage of the use of biologically active PM2 DNA is that the contribution of the various types of DNA damage to the total biological inactivation can be determined. Our experiments show, that in the presence of the various antioxidants not only DNA damage but also the degree of biological inactivation is strongly enhanced. As is shown in Table 1, the contribution of DSB, which in PM2 DNA are lethal, to the biological inactivation is rather low. In the absence of antioxidants this contribution is about 15%. A similar contribution is found in the presence of Asc, indicating that in this case the redox-cycle reaction model described above fully applies. On the other hand, the contribution of DSB to lethality is higher in the case of the thiol compounds, ranging from on the average about 30% in the presence of Cys and GSH to about 50% in the presence of NAC (Table 1). This finding strongly suggests, that the thiol compounds not only act as

Fe^{3+}-BLM-reducing agents but in addition are somehow active in DNA damage formation leading to a shift from one type (or types) of lethal damage to another (DSB).

We can only speculate on the other lesions, which might be responsible for DNA inactivation. Important lesions, which are formed as consequence of reaction of activated BLM with DNA are the alkali-labile (or "AP") sites. Their presence could be established by us in the CCC-form of PM2 DNA, but they probably represent only a small part in the total amount of these lesions, since it is known, that the BLM-induced AP-sites were found with a relatively high frequency in combination with closely opposed SSB and thus must be present in the OC form, which contains per definition at least one SSB [Steighner and Povirk, 1990; Porvik and Austin, 1991]. It is obvious that such double damages, *e.g.* SSB in combination with alkali-labile sites in the opposite strand or SSB or AP-sites in similar combination with base-damages, are very good candidates for lethal lesions other than DSB. After introduction of the exposed DNA into its bacterial host, it can be expected that such double damages are converted into (lethal) DSB as a consequence of enzymatic DNA repair, which in most cases involves a DNA-strand incision step.

The situation in our cell-free system may be different from that in cells which are exposed to BLM. It has been suggested that Cu^{2+}-BLM, which is quite stable to ligand-substitution reactions carries BLM to the nucleus, where Cu is replaced by Fe, possibly before BLM is bound to the DNA [Stubbe and Kozarich, 1987]. It is obvious, that when antioxidants are able to interfere with the action with Fe^{3+}-BLM before it becomes bound to the DNA by scavenging reactive oxygen species ($^{\cdot}OH$) or by inactivating Fe^{3+}-BLM itself, antioxidants will exert a protecting effect. Recently, Buettner and Mosely reported, that antioxidants possess such a protecting activity by showing that free Fe^{3+}-BLM in contrast to that bound to DNA is inactivated by Asc to a "redox-inactive" form [Buettner and Mosely, 1992]. The fact, that we did not find such a BLM inactivation in our experiments, in which BLM was pretreated with Asc before DNA was exposed, is probably due to the use of Cu^{2+}-BLM instead of Fe^{3+}-BLM in the Asc-pretreatment. Another important indication for protection (and potentiation!) by thiols of the clastogenic action of BLM in cells was obtained in studies in which a protective effect of Cys-pretreatment was found in human lymphocytes in vitro, whereas a posttreatment with Cys led to a potentiation [Chatterjee and Jacob-Raman, 1993].

In conclusion, according to our results, the effect of BLM-induced DNA damage by antioxidants is mainly potentiating. This may render an increase in the cytotoxicity of BLM as an anti-cancer drug. However, when we consider BLM as a model compound for various other carcinogens, the supplementation of antioxidants in the light of chemoprevention may not always provoke the desired protective effect [Cao and Cutler, 1993; Alpha-tocopherol, 1994] and may depend on the subtle redox-equilibrium within the cells.

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Chapter 7

LACK OF EFFECT OF DAILY N-ACETYLCYSTEINE SUPPLEMENTATION ON MUTAGEN SENSITIVITY

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Submitted

Abstract

The EORTC multicenter Euroscan trial was set up to prevent the occurrence of second primary tumors in the upper aerodigestive and respiratory tract in patients cured for early stage head and neck squamous cell carcinoma. One randomized group of patients receive daily N-acetylcysteine, an anti-oxidant which may be protective especially in the early steps of carcinogenesis. Mutagen sensitivity, measured as sensitivity to bleomycin in peripheral blood lymphocytes, has been found to be increased in head and neck squamous cell carcinoma and is hypothesized to reflect cancer susceptibility. The aim of this study was to investigate whether mutagen sensitivity is influenced by oral N-acetylcysteine supplementation and can therefore be used as intermediate endpoint in chemoprevention. Patients (n=19) were analyzed who had various periods of N-acetylcysteine supplementation (600 mg daily for 3 to 9 months). In addition, a patient group (n=14) who did not receive N-acetylcysteine supplementation was analyzed for comparison. Our results show that N-acetylcysteine did not influence the mutagen sensitivity. The only explanatory variable in the analysis of the difference between two samples of one person was the b/c value of the first measurement. Moreover, the variability in these repeated measurements (coefficient of variation of 14%) indicates that further studies should be performed to minimize this and to optimize the mutagen sensitivity to accurately identify individual patients at high risk for the development of multiple primary tumors.

Introduction

During the last decade the survival of head and neck squamous cell carcinoma (HNSCC) has only marginally increased, despite better treatment modalities [Francheschi *et al.*, 1992]. One of the reasons for this unchanged survival is the occurrence of multiple primary tumors (MPT) in the respiratory and upper digestive tract (RUDT). The development of MPT, which occur at a constant rate of about 3% per year for the first five years, is a major clinical concern [Jovanovic *et al.*, 1994].

To explain the development of MPT the concept of field cancerization has been suggested. The whole mucosa of the upper aerodigestive tract has been exposed to carcinogenic agents and when one tumor is diagnosed the whole area is at increased risk to develop more primary tumors [Slaughter, 1953; Hittelman *et al.*, 1991] To prevent or delay the process of carcinogenesis after the treatment of the first primary tumor synthetical and natural compounds are administered to the patients in so-called chemoprevention trials. One example is the Euroscan trial which is a cooperative multicenter EORTC trial [De Vries *et al.*, 1992]. One randomized group of early stage HNSCC patients who are curatively treated are

supplemented with micronutrients such as N-acetylcysteine (NAC), a precursor of glutathione (GSH). This compound mainly interferes at the level of detoxification in the early steps of carcinogenesis due to its detoxifying properties.

The identification of biological markers that predict or monitor the development of a tumor is important since they will shorten the length of a trial and thereby increase the efficacy of chemoprevention trials. The benefit of the drug can be monitored before the endpoint of the trial *e.g.* the occurrence of a second primary tumor, is reached. Another factor which will improve the efficacy of chemoprevention trials is the selection of patients at the highest risk, who will render a high number of cancer incidences over a relatively smaller time interval [Lippman *et al.*, 1994]. Besides these improvements of the trials, the fact that for these high risk subjects side effects of the agents are more easily justified is also important.

An endogenous risk factor [Cloos *et al.*, 1993] for HNSCC patients who are at high risk to develop multiple primary tumors is mutagen sensitivity [Cloos *et al.*, 1994; Spitz *et al.*, 1994]. This risk factor reflects chromosomal instability after the induction of damage with a clastogenic agent. A hypersensitive phenotype as measured with this assay in combination with exposure to carcinogenic agents such as cigarette smoke drastically increases the risk for HNSCC [Cloos *et al.*, 1995].

Although mutagen sensitivity has been proposed to be constitutional [Cloos *et al.*, 1993], some authors have suggested that it can be used to monitor the efficacy of chemopreventive agents [Trizna *et al.*, 1993; Küçük *et al.*, 1995]. When this latter finding holds true, then mutagen sensitivity may be utilized as intermediate endpoint of chemoprevention studies. NAC for instance, increases the concentration of GSH in the plasma of the patients [Bongers *et al.*, 1995]. If the patients would be mutagen hypersensitive because of an underlying deficiency in free radical detoxification the treatment of NAC would protect against this sensitivity.

On the other hand if NAC would not have an effect this would be in line with the hypothesis that mutagen sensitivity is constitutional. Aim of this study was to monitor whether the mutagen sensitivity is influenced by daily NAC supplementation. Subjects were recruited from patients randomized for the Euroscan trial of The University Hospital, Utrecht and The Free University Hospital, Amsterdam.

Materials and methods

Subjects. Blood samples were analyzed on mutagen sensitivity before and after NAC supplementation of 19 patients who were curatively treated for a primary tumor in the mucosa of the upper aerodigestive tract. These patients were treated with an oral dose of 600 mg NAC daily. For comparison a group of 14 patients

was analyzed who were treated for their tumor but who did not receive NAC supplementation. The number of smoked pack years was calculated for each subject as being the number of years during which was smoked, multiplied by the number of cigarette-packs smoked daily (assuming that one pack contains 25 cigarettes). A similar calculation was used for the estimation of alcohol drinking history, in which one unit is defined as one standard glass of alcoholic beverage consumed daily, assuming that one glass of beer or liquor contains a similar amount of alcohol (1,5 ml).

Mutagen sensitivity. The assay was performed as described previously [Hsu *et al.*, 1985]. In short: One half ml heparinized whole blood was cultured for 72 hr in 4.5 ml RPMI 1640 medium (Flow laboratories, Irvine, UK) with 2 mM L-glutamine (Gibco, Paisley, UK) supplemented with 15% fetal calf serum (Flow Laboratories), 1.5% phytohaemagglutinin (Welcome Diagnostics, Dartford, UK), 100 IU/ml penicillin and streptomycin (ICN biomedicals ltd, Irvine, UK). For each subject duplicate cultures were used. Bleomycin (Lundbeck, Copenhagen, Denmark) was added 5 hours before cell harvest, ensuring that damage induced in the late S- and G2-phase of the cell cycle could be evaluated at metaphase. Cells were arrested in metaphase by adding 100 μl, 50 μg/ml Colcemid (Sigma, St Louis, USA) 1 hour before harvesting. The cells were collected by centrifugation (300 *g*, 5 min) and treated with a hypotonic solution (0.06 M KCl) for 20 min. For fixation and washing (3x) of the cells, Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) was used. Fixed cells were dropped on wet slides. After air drying, slides were stained with 5% Giemsa solution (Merck, Darmstadt, Germany), coded and scored under a light microscope with a magnification of 1250 x. From each coded slide, 50 metaphases were evaluated for the presence of chromatid breaks. So, the breaks per cell (b/c) of 100 metaphases scored for each subject was taken as a measure for mutagen sensitivity.

Statistical analysis. Differences between repeated measurements of b/c values were calculated using paired Student's t-test. Differences between patient groups were estimated using two-sample Student's t-test. The relation between variables was analyzed with the Pearson correlation coefficient. Analysis of variance was performed to estimate the influence of variables on the difference between the first and the second measurement. The coefficient of variation was calculated for each person as the standard deviation divided by the mean b/c value of the two measurements x100%. Reported here are the means of the coefficients of variation for both patient populations.

Results

The two patient populations did not differ in mean b/c levels and age (Table 1). The interval time between the two measurements was significantly longer in the non-treated group compared to the NAC treated group ($p<0.01$) even when the interval for data censured at 12 months were fixed at 12.

No correlations were found between the difference between repeated measurement and age, interval time, cumulative smoking and alcohol use.

There was no difference between repeated measurements in both patient groups as measured with a paired Student's t-test. The mean difference between the two measurements was 0.04 for the NAC supplemented patients and 0.06 for the control patients. This indicates that NAC administration did not influence mutagen sensitivity.

A view of the variation in b/c values of the individual patients is visualized in Figure 1. A coefficient of variation of 14.4% was calculated for the NAC supplemented group and 14% for the control patient group. This variation in b/c level between the two measurements could not be explained by the NAC intake as tested with the Student's two samples t-test. There was a significant correlation between first and second measurement.

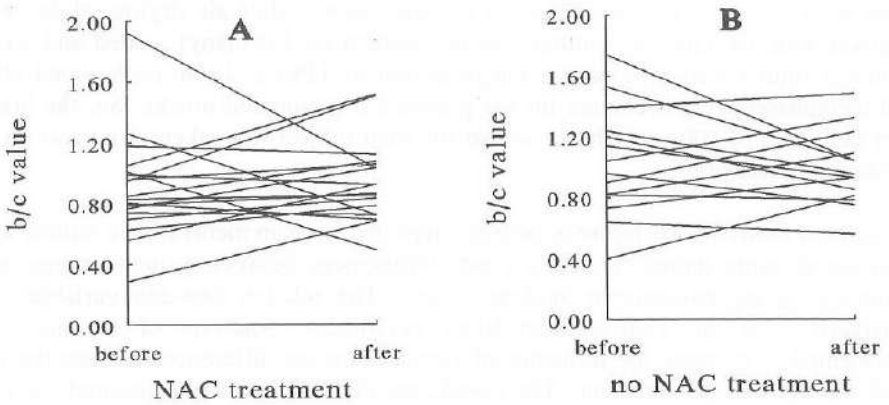

FIGURE 1 The individual b/c levels are plotted of the first and the second measurement. Panel A represents the patients who obtained NAC supplementation and panel B represents the control patients without NAC supplementation.

TABLE 1 *Patient characteristics*

NAC patient	age	smoke[a]	alcohol[b]	tumor site	treatment	interval[c]	difference[d]
1	73	3	3	oral cav	surgery	9	- 0.54
2	44	2	3	oral cav	surgery	6	- 0.52
3	51	3	2	oral cav	surgery + RT[e]	3	- 0.45
4	71	1	1	larynx	RT	4	- 0.07
5	68	3	2	larynx	surgery	3	0.39
6	69			larynx	RT	2	0.46
7	65	3		larynx	RT	5	0.07
8	72	3	2	larynx	RT	3	0.09
9	57	3	1	larynx	RT+sur+chemo	2.5	0.36
10	59	3	3	oral cav	surgery	4	- 0.06
11	71	2	1	larynx	RT	2	0.15
12	61	3	1	larynx	surgery	4	- 0.11
13	39	1	1	oral cav	surgery + RT	4	- 0.02
14	61	2		larynx	RT	2.5	0.26
15	49	3	3	oral cav	RT	7	0.19
16	65	3	2	larynx	RT	9	0.13
17	57	2	2	larynx	RT	3	0.02
18	69	2	3	pharynx	surgery	4	0.22
19	78	1	3	oral cav	surgery + RT	3	- 0.49
patient without NAC intervention							
1	38	2	2	pharynx	surgery + RT	≥12	0.20
2	74	3	3	oral cav	surgery	≥12	0.33
3	79	2	2	larynx	surgery	≥12	- 0.70
4	59	3	3	larynx	surgery + RT	≥12	- 0.29
5	66			pharynx	surgery + RT	≥12	- 0.49
6	51	2	3	pharynx	surgery + RT	≥12	0.28
7	64			pharynx	surgery + RT	≥12	0.27
8	69	1	1	oral cav	surgery	≥12	- 0.21
9	57	3	2	pharynx	surgery	≥12	- 0.08
10	62	1	1	oral cav	surgery + RT	≥12	0.41
11	49	2	2	oral cav	surgery	0.5	- 0.04
12	67	3	3	pharynx	surgery	0.5	- 0.21
13	77	1	2	oesophagus	sur+RT+chemo	1	- 0.35
14	45	2	2	larynx	surgery + RT	0.5	0.09

[a] cumulative tobacco smoking was assessed as pack years. 1=non smoker; 2=<30 pack years; 3=≥30 pack years

[b] cumulative drinking was assessed as unit years. 1=non drinker; 2=<100 unit years; 3=≥100 unit years

[c] interval = months between first and second measurements

[d] difference between b/c value of the first and second measurement

[e] RT: radio therapy

The relation of two measurements with their difference can for the main part be explained by mathemetical necessity (regression to the mean). This implies that, due to the clear interaction between the difference and the value of the first measurement, this latter variable has to be included as covariate in the further analysis of variance. This resulted in the finding that no other variable influences the difference between repeated measurements.

Discussion

In this study, the only significant interaction with the difference between repeated measurements was the b/c value of a person. This can partly be explained by "regression to the mean", however whether other influences also play a role in this phenomenon needs further investigation in larger patient groups.

The finding that the intake of 600 mg NAC daily does not influence the mutagen sensitivity of a person is in contrast from what was expected from *in vitro* studies in which protective effects were reported on the interaction between bleomycin and NAC [Trizna *et al.*, 1993]. However, the concentrations used in those experiments (10 mM NAC) may not be reached in the *in vivo* situation for which peak plasma levels of $\pm$ 12 μM NAC were reported [Pendyala and Creaven, 1995]. Another reason for the lack of influence of NAC intake may be that during the three days of culturing the lymphocytes in the mutagen sensitivity assay the influence of NAC is diluted. The plasma is diluted ten times in the culture medium, and thiols can be metabolized at the time the bleomycin is added. Fortunately, the potentiative effect which NAC can directly have on bleomycin-induced DNA damage [Cloos *et al.*, 1995] was also not found in this model system.

The results, in addition, show us to be careful regarding the identification of high risk individuals on the basis of one mutagen sensitivity measurement. In practice, the repeated measurements of patients for risk estimation is not easily applicable. However, for those patients who are in a b/c area between 0.7 and 1.0 a second measurement may be worthwhile to prevent false stratification on whether the patients is hypersensitive or not. More specific analysis of repeated sampling is necessary to substantiate this postulation, and has to be one of our main goals for further investigations on the concept of individualized high risk identification using this mutagen sensitivity assay.

The non-modifiable character of mutagen sensitivity is in line with our earlier findings that mutagen sensitivity is a constitutional factor. We hypothesize that mutagen sensitivity is a biomarker which can not be modulated by external factors such as smoking, alcohol, or nutrients. Therefore, we conclude that mutagen sensitivity can not be used to monitor the efficacy of NAC supplementation in protecting against carcinogenesis.

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Chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Knowledge regarding which patients who are curatively treated for head and neck squamous cell carcinoma (HNSCC) are at the highest risk for multiple primary tumors (MPT) is of utmost importance for the improvement of survival. This subpopulation can then be targeted for intensive behavioral interventions, surveillance through screening and enrolment in chemopreventive programs.

One objective of this study was the investigation whether the mutagen sensitivity can be used as a risk factor for the development of HNSCC. Moreover, it was hypothesized that the hyper-sensitive subpopulation is at the highest risk for the development of a multiple primary tumors (MPT). Our results indicate that mutagen sensitivity can in fact be used for the improvement of risk assessment of HNSCC (chapter 4). In addition a hyper-sensitive subgroup of patients who are at the highest risk to develop MPT could be identified (chapter 3).

Knowledge about the mechanisms underlying mutagen sensitivity may be helpful to get insight into the individual basis of a person's cancer susceptibility. Using this approach we may, in the future, be able to tailor prevention according to individual requirements. Possibly, the unraveling of the mutagen sensitivity phenotype will also render information about the process of human carcinogenesis. Our results (chapters 5, 6 and 7) indicate that the defect underlying mutagen sensitivity will more likely originate from impaired processing of DNA damage than from imperfect detoxification of carcinogens.

In this chapter some interesting aspects of mutagen sensitivity will be discussed and future perspectives will be addressed.

Risk Assessment

Mutagen sensitivity underscores the importance of genetic factors in the development of cancer. Our results clearly show an improvement of risk estimations for HNSCC when besides smoking and alcohol drinking behavior also the mutagen sensitivity phenotype is considered. It is acknowledged that mutagen sensitivity plays a role in the development of many environmentally related cancers [Hsu *et al.*, 1989, Spitz *et al.*, 1995]. The small but important differences in mutagen sensitivity are likely to account for increased cancer risk in environmental and occupational exposures. This emphasizes that traditional epidemiology which is mainly focussed on the identification of environmental causes of cancer can be improved when biological markers of susceptibility would be included. The need for a multi-disciplinary approach which involves different fields including epidemiology, tumor-biology and clinical medicine is highlighted by the current perception that carcinogenic exposure and genetic susceptibility act in concert to determine cancer risk [Caporaso and Goldstein, 1995].

Multiple primary tumors

It has been well established in the present study that mutagen sensitivity is increased in head and neck squamous cell carcinoma (HNSCC) patients, particularly those who have developed multiple primary tumors. However, to investigate the potential of the mutagen sensitivity assay to predict which curatively treated HNSCC patients are at the highest risk to develop MPT we have designed a prospective patient study.

During the last two years, mutagen sensitivity samples of approximately 300 HNSCC patients have been stored for future analysis. The characteristics of these patients concerning, age, gender, smoking and alcohol drinking habits, tumor site, tumor stage and occupation were collected and recorded in a database. Since about 3% of these patients develop a second primary tumor each year we should be able to determine within a few years whether mutagen sensitivity is an important factor in predicting the development of second primary tumors. The b/c levels of patients who will have had a second malignancy will be scored and compared to the b/c levels of a matched group of HNSCC patients who have not developed any secondary malignancies (nested case-control approach).

The potential of mutagen sensitivity to predict which patients would benefit most from chemoprevention is also currently under investigation in a large clinical chemoprevention study at the MD Anderson Cancer Center, Houston Texas [dr W.K. Hong, personal communication].

To use the mutagen sensitivity assay for a more individualized approach such as this prospective patient study it will be worthwhile to re-evaluate the hyper-sensitivity border. The border of a mean number of breaks per cell ≥ 1 is until now still based on the normal distribution of the control population (mean plus the standard deviation). The large number of mutagen sensitivity data that are currently available of HNSCC patients in combination with the data on repeated measurements should provide us the tools to analyze the validity of this hyper sensitivity border. This is important in order to make optimal use of the mutagen sensitivity assay as a screening test of individual HNSCC patients who can be selected for intensive intervention.

Constitutional basis of mutagen sensitivity

On the basis of our results so far we argue that mutagen sensitivity can be used as a biomarker for cancer susceptibility. Its constitutional character, however, precludes its use as an intermediate endpoint for intervention studies. The mutagen sensitivity assay which is performed on the peripheral blood lymphocytes of a person is probably not subject to variation in plasma levels of certain nutrients (chapter 7). The tissue from which the tumor originates, *e.g.* the mucosa of the upper aerodigestive tract, on the other hand, may be influenced by the nutrients. It is often noted that a blood test with bleomycin can never be conclusive about the sensitivity of the mucosa for cigarette smoke.

Since mutagen sensitivity is constitutional it should be present in all somatic cells of the body. We are currently evaluating a novel, more "true to life", model for the assessment of mutagen sensitivity. Cells of the area of interest, namely oral keratinocytes and fibroblasts of the upper aerodigestive tract, will be tested for their sensitivity to genotoxic compounds. Not only the different cell types can be compared but it is also interesting to see whether the inter-individual variation in carcinogen susceptibility can also be shown in these cells. The inter-individual variation will not only be determined for the standard bleomycin treatment but also for compounds to which subjects are commonly exposed such as cigarette smoke condensate.

Possibly, this alternative model of mutagen sensitivity will enhance the sensitivity of the detection of inter-individual differences in sensitivity for cigarette smoking induced DNA damage compared with the traditional mutagen sensitivity assay. Moreover, this approach will render the opportunity to investigate more physiologically the mucosa-carcinogen interactions.

So far, it has been validated that mutagen sensitivity is a constitutional factor, however, whether it has a genetic basis can not be concluded from this study. To prove a hereditary basis of the intrinsic sensitivity to clastogenic compounds family studies have to be performed. Indications of a distinct hereditary basis for mutagen sensitivity are described in studies in which cancer patients from cancer prone families were compared to their first degree relatives without cancer [Knight *et al.*, 1993; Hsu *et al.*, 1985; Liang *et al.*, 1989]. Since familial clustering of HNSCC has been found [Copper *et al.*, 1995] we plan to investigate HNSCC families with one or more positive proband in more detail using the mutagen sensitivity assay. The spouses of the patients will be measured as control subjects.

It has been postulated that the chromatid breaks that are induced by bleomycin are not random but occur at specific sites which may differ among patients with several tumor types [Dave *et al.*, 1994]. These putative fragile sites are of importance since they may indicate important chromosomal regions in which genes involved in malignant transformation may be located. We will further examine these site specific bleomycin induced breakpoints by including this cytogenetic analysis in our family study. Further research of this aspect of mutagen sensitivity may render new information regarding the human carcinogenesis process.

Implication of mutagen sensitivity for treatment

Recently, some authors have argued that the knowledge of a person's mutagen sensitivity may be valuable for cancer treatment. For breast cancer it has been described that a hypersensitive phenotype (as measured in irradiated fibroblasts) correlated with a better response to radiotherapy [West *et al.*, 1994]. In this respect we may in a future analysis also be able to evaluate whether hypersensitive HNSCC patients have responded better to their radiotherapy compared to

nonsensitive patients. Besides a better response of the tumor it can be postulated that the normal tissue will also suffer more damage in hyper-sensitive persons. Dr Pollak [1995] for instance, speculates that for hypersensitive patients the risk of chemotherapy-induced second malignancies is significantly higher than for the general population. Analysis of the data of the prospective trial of Spitz *et al.* [1994] showed no significant differences in the percentage of hypersensitive- compared to nonsensitive patients who both developed second malignant tumors and received chemotherapy [Spitz *et al.*, 1995a].

Although the above mentioned aspects are interesting, mutagen sensitivity is far from being used for decisions regarding tumor treatment strategies. According to the current state of affairs the usage of the mutagen sensitivity assay will mainly be valuable for the improvement of risk assessment [Olden, 1994].

Mechanisms underlying mutagen sensitivity

Mutagen sensitivity underscores the concept of cancer susceptibility. This latent chromosomal instability may provide us with a valuable tool to study more closely the process of carcinogenesis. Genetic instability has often been described as important factor for the carcinogenic process, especially with emphasis on the cancer prone syndromes such as Ataxia telangiectasia (AT). Some authors speculate that the mutagen sensitive individuals are in fact AT heterozygotes [Spitz *et al.*, 1993]. The interesting hypothesis can then be formulated that these AT heterozygotes correspond to the most sensitive subgroup in the mutagen sensitive population.

Cancer is a genetic disease and the rate of the human carcinogenesis process will be enhanced by genetic instability since DNA damages will be fixed. Normally, a cell will respond to DNA damage by inducing a cell cycle arrest. This will offer the cell time to repair or the cell might decide to go into apoptosis. The decision as to what a cell will choose is regulated by specific cell cycle checkpoints. The G2 checkpoint which delays the onset of mitosis seems to be important for clastogen-induced double strand breaks. In AT patients this checkpoint is defective since cells from AT patients do not show a G2 phase delay after double strand break induction. The cells that pass this checkpoint with the impaired delay exhibit an increased number of chromatid breaks. This phenomenon is visualized in Figure 1. Obviously, this fixed damage will render daughter-cells which either have lost or gained genetic information possibly resulting in growth advantage. Clearly the process of human carcinogenesis will be accelerated this way.

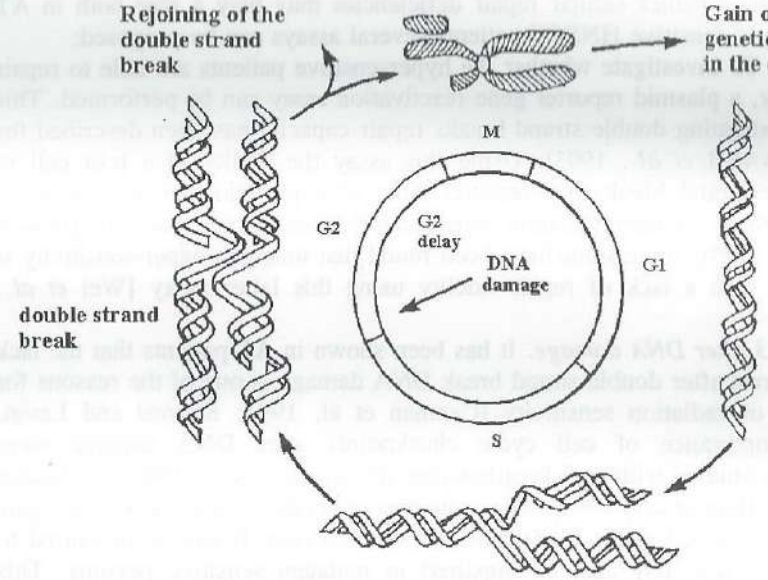

FIGURE 1 Double strand breaks which are introduced in the late S or G2 phase of the cell cycle will be fixed as chromatid breaks in mitosis. G2 delay may be controlled by checkpoints which involve activation of for instance Cyclin B and CDC2. However, impaired activation of p53 which is involved in G1 arrest also influences the protein cascade necessary for G2 delay.
(modified from Kaufmann, 1995)

Ultimate proof that our hypersensitive patients are in fact AT heterozygotes will become available now the AT gene has been cloned. The ATM gene has been located on 11q22-23 and has a transcript of 12 Kb which is mutated in all AT patients [Savitsky *et al.*, 1995]. This implies that a single gene is responsible for the disease although AT has a least four complementation groups. The ATM gene product has been related with a DNA-dependent protein kinase catalytic subunit which is involved in DNA double strand break repair and V(D)J recombination [Hartley *et al.*, 1995].

Since the occurrence of double strand breaks seems to be of crucial importance in mutagen sensitivity and the latent chromosomal instability in AT heterozygotes, the investigation on the mechanisms underlying mutagen sensitivity in future studies will be directed analogous to what has been reported for AT.

DNA repair

To elucidate whether similar repair deficiencies may play a role both in AT patients and hyper-sensitive HNSCC patients several assays can be proposed:

Repair fidelity. To investigate whether the hypersensitive patients are able to repair breaks correctly, a plasmid reporter gene reactivation assay can be performed. This method of investigating double strand breaks repair capacity has been described for AT patients [Powell *et al.*, 1993]. Using this assay the ability of a host cell to repair a double strand break in a reporter gene of a plasmid can be measured. Particularly, defects in recombination repair can be established this way [Powell and McMillan, 1994]. Indications have been found that mutagen hyper-sensitivity is also correlated with a lack of repair fidelity using this latter assay [Wei *et al.*, 1995].

Induction of p53 after DNA damage. It has been shown in AT patients that the lack of cell cycle arrest after double strand break DNA damage is one of the reasons for the high level of radiation sensitivity [Canman et al, 1994; Khanna and Lavin, 1993]. The importance of cell cycle checkpoints after DNA damage were demonstrated in studies with oral keratinocytes [Gulujuva *et al.*, 1994] and human lymphoma cells [Fan *et al.*, 1994]. The induction of proteins involved in cell cycle checkpoints such as p53, p21, GADD45, MDM2 or cyclin B can be measured to evaluate whether this may also be impaired in mutagen sensitive persons. This technique may then be performed in future studies using non-transformed lymphocytes in order to perform large scale screening.

Repair of single strand breaks particularly in the G2 phase of the cell cycle. The radiation sensitivity of the AT patients is thought to be due to impaired DNA repair particularly in the G2-phase of the cell cycle. We have shown a clear difference in single strand DNA repair in oral cavity and larynx cancer patients in fresh blood (peripheral blood lymphocytes in G0 phase of the cell cycle). This did not correlate with the double strand breaks measured by the b/c levels in the G2 phase of cultured lymphocytes (Chapter 5). It will be interesting to see whether this difference in radiation sensitivity in G0 phase cells can also be found in G2 phase cells. In order to investigate this, the repair capacity of radiation induced damage can be measured in cultured lymphocytes which are selected (Fluorescence Activated Cell Sorting) for being in the G2 phase of the cell cycle.

Detoxification

After analysis of the possibilities mentioned above, it might become clear that DNA repair is not the (only) important factor which determines the mutagen sensitivity. In that case, research can be directed towards mechanisms concerning detoxification or metabolic activation of carcinogens. This will for instance involve the analysis of the polymorphic genes involved in detoxification such as glutathione-s-transferase-μ and Cytochromes P450.

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SUMMARY

Tobacco smoking and alcohol abuse are major risk factors for the development of head and neck squamous cell carcinoma (HNSCC). However, only relatively few individuals who are being exposed actually develop HNSCC. Therefore, it is now generally acknowledged that individual susceptibility to cancer must play an important role as well. In this study an *in vitro* assay was used to determine a person's intrinsic susceptibility to cancer. This so-called mutagen sensitivity assay is based on the detection of chromosomal instability in mitogen stimulated cultured peripheral blood lymphocytes. This chromosomal instability is latent and only becomes visible after *in vitro* induction of DNA damage. The mean number of chromatid breaks per cell, induced by the DNA-damaging compound bleomycin in the S-G2 phase of the cell cycle, is a measure for this mutagen sensitivity.

We have first evaluated the assay predominantly in control persons. It was found that the mutagen sensitivity score is not influenced by age, gender, smoking, and alcohol use (chapter 2). Moreover, the importance of using duplicate cultures and scoring of two times fifty metaphases on coded slides was established in this part of the study. Knowing the constitutional and reproducible character of mutagen sensitivity we conducted a case-control study (chapter 3). We found a statistically significant increase in mutagen sensitivity in HNSCC patients compared to control persons, particularly in patients who had developed multiple primary tumors in the respiratory and upper digestive tracts. This latter finding substantiated the hypothesis that the mutagen sensitivity assay can be used to identify patients at the highest risk to develop multiple primary tumors. However, proof for this hypothesis has to come from prospective patient studies.

It was also postulated in this study that mutagen sensitivity only becomes important for HNSCC risk when a person is exposed to tobacco. However, due to the limited number of subjects we were not able to prove this hypothesis. In order to achieve this we combined our data with those of the M.D. Anderson Cancer Center, Houston, Texas and the Memorial Sloan Kettering Cancer Center, New York (chapter 4). We then found a dramatic increase in odds ratios for head and neck cancer from 10.6 (95% confidence interval; 4.5-25.8) in subjects who are heavy smokers to 45.1 (95% confidence interval; 17.0-120) in subjects who are besides heavy smokers also mutagen hypersensitive. The use of alcohol potentiated the effects of smoking, resulting in an odds ratio of 57.5 (95% confidence interval; 17.5-188) in hypersensitive persons. This unique set of patient data allowed us to perform multiple logistic regression analysis and we found that a hypersensitive phenotype did not appear to result in an increased cancer risk in non-smokers (relative risk 0.63, $p=0.25$), whereas there was a dose dependent association in individuals exposed to tobacco (relative risks ranging from 1.75 to 17.4; likelihood ratio test $p<0.0001$). We could prove the model that mutagen sensitivity measures how a person is able to deal with carcinogenic assaults. These results indicate a latent genetic susceptibility which only becomes apparent when a person is exposed

to carcinogens. The importance of utilizing both susceptibility markers and exposure data for the identification of persons at high risk for cancer is highlighted. The enhanced accuracy in risk estimation by the knowledge of both factors makes it possible to define susceptible subgroups of HNSCC patients who can be targeted for intensive behavioral interventions, surveillance through screening, and enrolment in chemopreventive programs.

Besides for risk estimation, mutagen sensitivity is also valuable for a better understanding of human carcinogenesis. This stimulated further research on the underlying mechanisms of mutagen sensitivity. One mechanism could be the ability of a person to repair DNA strand breaks. This could not easily be evaluated using the mutagen sensitivity assay and therefore an elegant immunochemical assay was used which measures induction of radiation induced strand breaks as well as subsequent repair (chapter 5). The amount of initial DNA damage was not different between patients and control persons. In contrast to larynx tumor patients, oral cavity tumor patients showed a significantly slower repair capacity compared to control persons. A slow repair is not necessarily unfavorable compared to a fast repair system since the latter may be more error prone. The difference between larynx and oral cavity cancer patients indicates a difference in the way DNA damage is processed between several sites within the head and neck region where HNSCC develops. The mutagen sensitivity was significantly higher in the HNSCC patients compared to the control persons. No correlation was found between mutagen sensitivity and both initial DNA damage and repair, neither in HNSCC patients nor in control persons. These results indicate that factors other than initial DNA damage or repair of single strand breaks induced by radiation are responsible for the observed differences in the bleomycin induced chromosomal damage among persons.

One such other factor underlying mutagen sensitivity might be the ability of a cell to detoxify compounds that induce the DNA damage. The role of detoxification in cancer is substantiated by epidemiological studies in which micronutrients with anti-oxidant properties have been found to be protective. Currently, clinical trials are being performed to investigate the possibility of inhibiting or preventing the process of carcinogenesis by the supplementation of anti-oxidants such as N-acetyl-cysteine.

To investigate (chapter 6) the influence of N-acetylcysteine and its metabolites glutathione and cysteine on bleomycin-induced DNA damage the supercoiled double stranded PM2 bacteriophage DNA was exposed to Cu^{2+}-bleomycin in the presence and absence of these thiols and the potent anti-oxidant ascorbic acid. In addition, by transfecting the PM2 bacteriophage to its host bacteria we gained further insight into the biological relevance of such damage. It was found that the presence of these compounds lead to a considerable enhancement of bleomycin-induced single- and double-strand breaks and a concomitant decrease of the biological activity of PM2 DNA in a dose dependent way. Cu^{2+}-bleomycin under our conditions was not active by itself. A hypothesis was formulated as to how bleomycin works: Cu^{2+}-

bleomycin which is quite stable to ligand-substitution reactions is transported to the nucleus. Most probably after binding to DNA Cu^{2+} exchanges for Fe^{3+} bound to DNA. Fe^{3+}-bleomycin is then reduced to Fe^{2+}-bleomycin, a process which is potentiated by the added antioxidants, and subsequentially activated by O_2. Ascorbic acid just enhanced the DNA-damaging properties of bleomycin, whereas the thiol compounds in addition influenced the type of DNA damage. The biological inactivation was larger than expected from the number of double strand breaks that were induced. This is probably caused by double-damages such as single strand breaks with closely opposed alkali-labile sites or base-damages. When we consider bleomycin as a model compound for various other carcinogens, the supplementation of anti-oxidants in the light of chemoprevention may not always provoke the desired protective effect and may depend on subtle redox-equilibria within the cells.

The DNA damage in this latter study was induced in a cell-free system, however, in the literature the effects of anti-oxidants on bleomycin-induced DNA damage have also been described in cell culture experiments. We were in the unique position to be able to determine the influence of a daily intake of 600 mg N-acetylcysteine on bleomycin induced damage using the mutagen sensitivity assay (chapter 7). Patients were analyzed (n=19) who had various periods of N-acetylcysteine supplementation. In addition a patient group (n=14) who did not receive N-acetylcysteine supplementation was analyzed as a control. Our results indicate that N-acetylcysteine did not influence the mutagen sensitivity score. The lack of effect of N-acetylcysteine supplementation again points towards the constitutional character of mutagen sensitivity.

In conclusion, mutagen sensitivity renders a valuable contribution to the estimation of cancer susceptibility. The mechanisms underlying mutagen sensitivity may be very diverse. Based on the literature and our results we hypothesize (chapter 8) that in most cases the mechanism will be similar to the latent chromosomal instability found in heterozygotes of the Ataxia telangiectasia syndrome.

SAMENVATTING

Het roken van tabak en het overmatig gebruik van alcohol zijn belangrijke risico factoren voor de ontwikkeling van kanker, in het bijzonder plaveiselcel-carcinoom in het slijmvlies van het hoofd-halsgebied (PCHH). Toch doen deze tumoren zich slechts bij een kleine minderheid van de zware rokers/drinkers voor. Tegenwoordig wordt dan ook algemeen aangenomen dat voor het krijgen van dit type kanker naast blootstelling ook een individuele aanleg een belangrijke rol speelt.

In het onderzoek, beschreven in dit proefschrift, werd gebruik gemaakt van een *in vitro* test om een intrinsieke ontvankelijkheid van een persoon voor kanker te bepalen. Deze zogeheten mutageen gevoeligheids test is gebaseerd op het detecteren van chromosomale instabiliteit in gekweekte (delende) perifere bloedlymfocyten. Deze chromosomale instabiliteit is latent aanwezig en wordt pas zichtbaar na het induceren van DNA schade in de celkweek. Het gemiddelde aantal chromatide breuken per cel, die door de DNA beschadigende stof bleomycine in de S-G2 fase van de celcyclus worden veroorzaakt, is een maat voor de mutageen gevoeligheid.

De studie werd begonnen met het evalueren van de test bij gezonde controle personen. De mutageen gevoeligheids score van een persoon blijkt niet beïnvloed te worden door leeftijd, geslacht, rookgedrag en alcohol gebruik (hoofdstuk 2). Bovendien kon vastgesteld worden dat het voor de nauwkeurigheid van de bepaling van belang is dat per bloedmonster twee onafhankelijke kweken ingezet worden. Van de gecodeerde microscoop glaasjes worden dan uiteindelijk per persoon twee keer vijftig metafasen gescoord. Uitgaande van het constitutionele en reproduceerbare karakter van de mutageen gevoeligheid werd een patiënt-controle studie uitgevoerd (hoofdstuk 3). Patiënten met PCHH hebben een statistisch significant verhoogde mutageen gevoeligheid vergeleken met gezonde controle personen. Dit geldt in het bijzonder voor PCHH patiënten bij wie zich meerdere primaire tumoren hebben ontwikkeld in het hoofd-halsgebied, de longen of de slokdarm. Deze bevinding ondersteunt de hypothese dat de mutageen gevoeligheids test gebruikt kan worden om patiënten te identificeren die een zeer hoog risico lopen op het ontwikkelen van meerdere primaire tumoren in de bovenste voedsel- en of luchtweg. Deze hypothese zal echter getoetst moeten worden in prospectieve studies. Daartoe hebben wij nu al een groot aantal patiënten monsters opgeslagen. Over enige jaren kan dan geanalyseerd worden of de test goed voorspelt welke patiënten een tweede tumor zullen ontwikkelen.

In hoofdstuk 3 is de hypothese geponeerd dat voor het bepalen van het risico op het ontwikkelen van PCHH mutageen gevoeligheid alleen van belang is bij patiënten die roken. Deze hypothese kon in het eigen onderzoek niet getoetst worden vanwege het beperkte aantal personen dat geanalyseerd kon worden. Dankzij goede contacten deed zich de mogelijkheid voor onze gegevens te combineren met die van het M.D. Anderson kanker centrum in Houston, Texas en die van het Memorial Sloan Kettering kanker centrum in New York, waardoor over een veel

grotere groep patiënten met PCHH en controle personen bij wie de mutageen gevoeligheid was bepaald, beschikt kon worden (hoofdstuk 4). Nu kon een aanzienlijke toename van het relatieve risico (odds ratio) op kanker van 10.6 voor stevige rokers (95% betrouwbaarheids interval; 4.5-25.8) naar 45.1 voor stevige rokers waarvan tevens bekend was dat ze mutageen overgevoelig waren (95% betrouwbaarheids interval; 17-120), aangetoond worden. Het gebruik van alcohol versterkt het effect van roken, hetgeen resulteert in een odds ratio van 57.5 in overgevoelige personen (95% betrouwbaarheids interval; 17.5-188). Dankzij deze unieke bundeling van patiënten gegevens kon tevens multipele logistische regressie analyse worden uitgevoerd. Aangetoond kon worden dat een overgevoeligheid bij niet-rokers niet resulteert in een verhoogd risico op kanker (relatief risico; 0.63, $p=0.25$), terwijl er een dosis afhankelijke associatie gevonden werd met het roken van sigaretten (relatief risico liep van 1.75 op tot 17.4; waarschijnlijkheidstest $p<0.0001$). Met deze analyse is aangetoond dat mutageen gevoeligheid bepaalt hoe goed een persoon in staat is om zich te weren tegen kankerverwekkende stoffen. Bovendien werd aangetoond dat deze gevoeligheid latent is en alleen zichtbaar van invloed is wanneer een persoon bloot staat aan carcinogene stoffen.

Met deze resultaten is duidelijk aangegeven dat het bij het inschatten van het risico op kanker van belang is om zowel individuele aanleg voor kanker als gegevens over blootstelling aan kanker verwekkende stoffen in ogenschouw te nemen. Op deze wijze zal het in de toekomst mogelijk zijn een subgroep van PCHH patiënten te identificeren die een hoog risico heeft meer tumoren in de bovenste voedsel- en/of de luchtweg te ontwikkelen. Die groep zou dan in aanmerking kunnen komen voor ingrijpende veranderingen in levensstijl, geïntensiveerde screening tijdens de follow-up en deelname aan chemopreventieve programma's.

Tevens werd onderzoek verricht naar de mechanismen die ten grondslag zouden kunnen liggen aan de mutageen gevoeligheid. Er zijn in principe twee mechanismen denkbaar; detoxificatie van de potentieel kanker verwekkende stof of herstel van toegebrachte DNA schade.

In hoofdstuk 5 zijn experimenten beschreven om het herstel van toegebrachte DNA schade kwantitatief te meten. Vers bloed (lymfocyten in de G0 fase van de celcyclus) werd bestraald en de hoeveelheid schade (enkel-en dubbel strengs breuken) werd gemeten met een immunochemische test. Het bleek dat tussen controle personen, patiënten met mondholte kanker en patiënten met larynx kanker de hoeveelheid toegebrachte schade niet verschilde. Wel bleek dat er een verschil was in de snelheid van DNA herstel. De patiënten met mondholte kanker konden de schade minder snel herstellen dan larynx kanker patiënten en controle personen. Nu is het niet noodzakelijkerwijs zo dat een relatief snel schade herstel gunstig is. Een snel schade herstel zou gepaard kunnen gaan met een groter aantal fouten. Deze resultaten duiden op een mogelijk verschil in de manier waarop DNA schade verwerkt wordt tussen verschillende lokaties van het slijmvlies van het hoofd-hals gebied. Vanzelfsprekend werd bij deze populatie ook de mutageen gevoeligheid

bepaald. Ook in deze studie hadden de PCHH patiënten een significant hogere mutageen gevoeligheid vergeleken met de controle personen. De hoeveelheid toegebrachte schade door straling en het herstel hiervan waren echter niet gecorreleerd met mutageen gevoeligheid. Dit wijst erop dat bij chromosomale instabiliteit waarschijnlijk andere factoren een rol spelen dan de hoeveelheid en het herstel van de door straling toegebrachte schade die gemeten kon worden met de immunochemische test.

Een factor die bij mutageen gevoeligheid eventueel ook een rol kan spelen betreft het vermogen van een persoon om kanker verwekkende stoffen onschadelijk te maken. Dat dit van belang kan zijn bij het ontstaan van kanker is gebleken uit epidemiologische studies: van micro nutriënten met anti-oxidatieve eigenschappen is een beschermende werking beschreven. Tegenwoordig worden klinische studies uitgevoerd om na te gaan of het carcinogenese proces voorkomen of vertraagd kan worden door aanvulling van de voeding met anti-oxidatieve stoffen zoals N-acetylcysteïne. Om te onderzoeken of N-acetylcysteïne en zijn metabolieten glutathion en cysteïne van invloed zijn op bleomycine geïnduceerde DNA schade werd de bacteriofaag PM2 blootgesteld aan Cu^{2+}-bleomycine in de aan- en afwezigheid van deze thiolen en de krachtige anti-oxidant ascorbine-zuur (hoofdstuk 6). Bovendien kon inzicht verkregen worden in de biologische relevantie van deze schade door de beschadigde bacteriofaag te transfecteren naar zijn gastheer bacterie. Deze anti-oxidatieve stoffen blijken de werking van bleomycine te versterken door een toename aan enkel- en dubbel strengs breuken en een daarmee gepaard gaande afname van de biologische activiteit van de bacteriofaag. De Cu^{2+}-bleomycine bleek onder de gebruikte omstandigheden niet zelf actief te zijn. Aan de hand van enkele aanvullende proeven kon een model opgesteld worden voor de mogelijke werking van bleomycine: Cu^{2+}-bleomycine is vrij goed bestand tegen ligand-substitutie reacties en zorgt voor het transport naar de kern. Waarschijnlijk bindt Cu^{2+}-bleomycine eerst aan DNA voordat de koper-ionen uitgewisseld worden met DNA gebonden ijzer-ionen. Fe^{2+}-bleomycine wordt dan gereduceerd tot Fe^{3+}-bleomycine, een proces dat versterkt wordt door de toegevoegde anti-oxidanten. Hierna wordt het complex geactiveerd door O_2.

Voorts werd gevonden dat ascorbine-zuur alleen de DNA beschadigende eigenschappen van bleomycine kan versterken, terwijl de thiolen ook nog invloed hebben op het soort DNA schade. De biologische inactivatie van de bacteriofaag is groter dan op basis van de dubbel strengs breuken verwacht kan worden. Dit wordt waarschijnlijk veroorzaakt door enkel strengs breuken met in de tegenoverliggende streng alkali-labiele plaatsen of base-beschadigingen. Als er vanuit gegaan wordt dat bleomycine een goede modelstof is voor andere kanker verwekkende stoffen dan kan geconcludeerd worden dat het toevoegen van anti-oxidanten aan de voeding niet altijd de gewenste beschermende effecten heeft. Mogelijk zal de invloed afhangen van het subtiele redox-evenwicht dat in de cellen aanwezig is.

De DNA schade beschreven in hoofdstuk 6 werd geïnduceerd in een cel-vrij systeem. De versterking van DNA schade door anti-oxidanten is echter ook

beschreven in celkweek experimenten. Wij waren in de unieke positie om in patiënten het effect van dagelijkse inname van 600 mg N-acetylcysteïne op de bleomycine geïnduceerde DNA schade te bepalen met behulp van de mutageen gevoeligheids test (hoofdstuk 7). Bij 19 patiënten die gedurende verschillende perioden N-acetylcysteïne toegediend kregen, werd de mutageen gevoeligheid voor en na behandeling gemeten. Bovendien werd een groep van 14 patiënten getest die deze stof niet werd toegediend. De resultaten wijzen uit dat N-acetylcysteïne geen invloed heeft op de mutageen gevoeligheid. Dit geeft nogmaals aan dat mutageen gevoeligheid een constitutionele factor betreft.

Concluderend kan gesteld worden dat de mutageen gevoeligheid een waardevolle bijdrage levert aan het bepalen van de individuele aanleg voor kanker van de slijmvliezen in het hoofd-halsgebied. De mechanismen die hieraan ten grondslag liggen zijn mogelijk divers. Gebaseerd op de literatuur en op onze eigen resultaten gaan wij ervan uit dat het mechanisme in de meeste gevallen vergelijkbaar zal zijn met de chromosomale instabiliteit die gevonden wordt bij heterozygoten van het Ataxia telangiectasia syndroom. In hoofdstuk 8 wordt aangegeven hoe dit verder onderzocht kan worden.

Het Chinese idiogram voor *Crisis*
bestaat uit twee aaneensluitende karakters.
Het ene is *Gevaar* en het andere is *Mogelijkheid*.
Aldus is de schoonheid van gevaar dat het
een evenredige mogelijkheid tot
verandering in zich draagt.

- Yatri -

危机

CURRICULUM VITAE

Jacqueline Cloos werd geboren op 11 september 1962 te Amsterdam. Ze behaalde in 1979 haar eindexamen HAVO op het Stedelijk Lyceum te Zutphen. Met het plan ooit tuinarchitecte te worden heeft ze in 1982 de Middelbare Tuinbouw School in Hoorn afgerond. Na 2 jaar VWO op de Scholengemeenschap Rooswijk te Zaandijk, is ze in 1985 begonnen met de studie biologie aan de Vrije Universiteit in Amsterdam met als studierichting Medische Biologie.

Haar interesse in het kankeronderzoek werd tijdens de studie gewekt vanwege de veelzijdigheid van het onderzoek. Haar hoofdvak stage werd gevolgd bij de sectie Biochemische Farmacologie van de afdeling Inwendige Geneeskunde /Oncologie onder leiding van dr G.J. Peters en dr C.L. van der Wilt. Hier verrichtte zij onderzoek naar de modulatie van het cytostaticum 5-fluorouracil. Een moleculair biologische stage van 9 maanden werd verricht bij de afdeling Bloedstolling van het Centraal Laboratorium van de Bloedtransfusie dienst te Amsterdam onder leiding van dr A. Leyte. Onderzoek naar functionele regio's van het factor VIII gen stond hierin centraal.

In de periode na het afstuderen heeft ze eerst een half jaar als adjunct onderzoeker gewerkt bij de afdeling Inwendige Geneeskunde/Oncologie. Deels was dit een voortzetting van het onderzoek naar 5-fluorouracil (dr G.J. Peters). Voor het andere deel was dit onderzoek naar de beschermende werking van de thiol WR2721 op de beenmerg toxiciteit na behandeling van muizen met het cytostaticum carboplatin (prof.dr W. van der Vijgh).

In Augustus 1991 is ze begonnen met haar AIO-baan bij de afdeling Keel-Neus- en Oorheelkunde, van het ziekenhuis van de Vrije Universiteit te Amsterdam waarvan dit proefschrift het resultaat toont. Het pionierswerk naar mutageen gevoeligheid is een waardevolle opzet gebleken voor verder onderzoek naar een mogelijk genetische aanleg voor het ontstaan van hoofd- halskanker. Met deze onderzoeksvraagstelling zal zij zich de komende twee jaar als post-doc bezighouden op dezelfde afdeling.

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DANKWOORD

Nu het boekje zo goed als af is, besef ik waarom de meeste mensen beginnen met het lezen van het dankwoord: zij zien één naam op de kaft staan en zij realiseren zich meteen dat dit proefschrift nooit door één persoon tot stand kan zijn gekomen. Dit neemt niet weg dat ik het een moeilijke opgave vind om een zo volledig mogelijke opsomming te geven van de mensen die hieraan hebben bijgedragen. Nochtans ga ik een poging wagen.

Allereerst wil ik mijn promotor prof. dr G.B. Snow noemen. Prof Snow, ik dank u voor de gelegenheid die u mij heeft geboden om het promotie onderzoek op uw afdeling te verrichten. De interesse die u altijd getoond heeft in mijn werk heb ik als zeer motiverend ervaren. Uw praktische kijk op dit onderzoek heeft er zeker toe bijgedragen dat de essentie van dit proefschrift bij meer mensen duidelijker zal zijn overgekomen.

Mijn copromotor, dr B.J.M. Braakhuis, wil ik bedanken voor de plezierige samenwerking. Beste Boudewijn, als er iemand is, die nieuwe ideeën heeft of nieuwe proeven weet te verzinnen, dan ben jij het wel. Jouw enthousiasme leidde tot menig uurtje "brain stormen" waaraan mede het succes van dit project te danken is. Ik vind het dan ook een prettig vooruitzicht om de komende twee jaar met jou aan het onderzoek verder te werken.

Prof.dr A. Westerveld, ik waardeer het dat u referent wilde zijn van dit proefschrift. Langs deze weg wil ik u nogmaals bedanken voor het meedenken over de voortgang van dit onderzoek.

Alle andere leden van de beoordelings commissie, dr. H. Joenje, prof.dr J.A. Koeman, prof.dr A.T. Natarajan, prof.dr H.M. Pinedo, prof.dr J. Retèl en prof.dr I. van der Waal, vriendelijk bedankt voor uw tijd om het manuscript door te nemen en de bereidheid zitting te nemen in de promotiecommissie.

Dr T.C. Hsu, I would like to thank you for your hospitality and the charing of your knowledge of human genetics with me. These words are, of course, also directed to dr S. Pathak who insisted on keeping me in Houston untill I could read the slides with my eyes closed. I would like to take this opportunity to include all the people of the laboratory in my words of gratitude for not only did they teach me all the technical details of the mutagen sensitivity test, they also showed me many wonderful things in and around Houston.

Dr M.R. Spitz, besides for your hospitality in Houston I would like to thank you for the enthousiastic discussions about mutagen sensitivity. I am most proud that you had confidence in my "fragile" ideas and trusted me with your data. This latter holds of course also true for dr S.P. Schantz and dr Z. Zheng from the Memorial Sloan Kettering Cancer Center in New York.

The collaboration and friendship I had with Tomacs Krecicki, Maria Sasiadek and Jacek Cichosz from Wroclaw, Poland has been very nice. Thank you, and I hope we will have the chance to meet again and discuss our joint scientific interests.

De afdeling Antropogenetica bedank ik voor de getoonde interesse en de

enthousiaste hulp die ik van iedereen kreeg als ik weer eens wat vroeg. Dit geldt in het bijzonder voor Anneke Oostra die ik overladen heb met vragen. Hans Gille, ik heb veel van je geleerd, zowel wat betreft het labwerk als van jouw commentaren op mijn manuscripten. Dit laatste geldt natuurlijk ook voor Vincent Lafleur en Prof Retèl van de afdeling Oncologie. Met z'n vieren hebben we menig uur aangenaam gediscussieerd over proeven, model systemen en bovendien over de filosofische kanten van de wetenschap. Bedankt!

De mensen van de afdeling Genetische Toxicologie van TNO in Rijswijk ben ik zeer erkentelijk voor de prettige samenwerking, in het bijzonder dr G.P. van der Schans en Jaap Timmerman.

Met mijn collega's van de afdeling KNO heb ik ook altijd zeer prettig samengewerkt. Veel dank ben ik verschuldigd aan alle medewerkers van het "tumorbiologielab", de afdelingen 1 Noord en 1 West, de polikliniek, en het secretariaat, alle artsen en assistenten en Fred Snel (de spil van de afdeling). Ik ga natuurlijk vreselijk de mist in als ik alle namen moet vermelden, dus maak ik mij er op deze manier vrij simpel vanaf. Toch wil ik nog enkele personen noemen. Guus van Dongen en Ruud Brakenhof, bedankt voor jullie steun op wetenschappelijk en sociaal gebied. Ivar Steen, je bent een kleurrijk persoon met veel vakkennis. We hebben als perfect team samengewerkt. Bedankt hiervoor! De stagiaires wil ik bedanken voor het enthousiasme waarmee ze aan dit onderzoek hebben meegewerkt. Marianne van der Sterre stond aan de wieg van de mutageen gevoeligheidstest. Marianne, ik hoop dat je zo hier en daar gezellig langs blijft komen vlinderen. Simone Meier is begonnen met het kweken van keratinocyten voor het opzetten van een nieuw model systeem. Simone, met plezier denk ik terug aan onze belevenissen in Polen en ik heb er alle vertrouwen in dat je met de vele kwaliteiten die je bezit een positieve toekomst tegemoet gaat. Marieke van der Bijl heeft de proeven opgezet waardoor het nu mogelijk is de bleomycine gevoeligheid van verschillende celtypen te bepalen. Marieke, bedankt en heel veel succes in je verdere loopbaan!

Colin Reid now performs the screening of patient material for sensitivity to bleomycin. Colin, thank you for your enthousiasm for the research work which you have to perform next to your clinical obligations. Your sociability and your knowledge of the English language have already been very pleasant for me. I hope we will be able to write some nice articles about your work.

Marlies Ooms is momenteel bezig met een assay om DNA herstel te bestuderen. Marlies, bedankt voor je inzet. Als we het motto hanteren dat je met een lach veel kunt bereiken is voor jouw geen berg te hoog!

Dan zijn er natuurlijk ook nog vrienden die mij al veel langer steunen dan de afgelopen viereneenhalf jaar. Mijn ouders zijn mijn beste vrienden en ik wil hen bedanken voor de kansen die zij mij geboden hebben. Alle overige familie, vrienden en kennissen wil ik bedanken voor hun getoonde interesse in mijn werk. Ik hoop dat wij in de toekomst nog veel plezier kunnen maken met elkaar.

Ron, bedankt voor het koken, banden plakken, hulp bij computer problemen, enzovoort. Bovenal bedankt voor je vriendschap. Grow old with me!