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A comparison of bleomycin-induced damage in lymphocytes and primary oral fibroblasts and keratinocytes in 30 subjects

Jacqueline Cloos[1], Colin B.A.Reid[2], Marianne L.T.van der Sterre[1], Hilde Tobi[3], Charles R.Leemans[1], Gordon B.Snow[1] and Boudewijn J.M.Braakhuis[1,4]

[1]Department of Otolaryngology/Head and Neck Surgery, [3]Department of Epidemiology and Biostatistics, University Hospital Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands and [2]Rodney Williams and Garnett Passe Memorial Foundation, Melbourne, Australia

The number of chromatid breaks in peripheral blood lymphocytes (PBL) after exposure to bleomycin in the S/G_2 phase of the cell cycle (in the literature referred to as 'mutagen sensitivity') is associated with an increased risk of environmentally related cancers, including oral cancer. The aim of this study was to elucidate whether mutagen sensitivity measured in lymphocytes actually reflects chromosomal instability of normal cells in the areas in which tumors develop. Therefore, bleomycin-induced chromosomal damage in and growth inhibition of cultured oral fibroblasts and oral keratinocytes from 30 persons were compared with the standard mutagen sensitivity score in PBL. A correlation was found for the percentage of aberrant metaphases between PBL and oral fibroblasts but not for the number of breaks per cell. These data do not allow a conclusion to be drawn on the use of fibroblasts to study cancer risk. Within the fibroblasts it was found that a high number of breaks per cell was associated with less growth inhibition, indicative of damage-resistant growth. Oral keratinocytes were extremely sensitive to bleomycin, as indicated by a strong cell cycle block which resulted in a mitotic index too low to determine chromosomal breaks. Moreover, in the cell proliferation assay keratinocytes were found to be 100 times more sensitive as compared with fibroblasts. There was no correlation between bleomycin sensitivity of keratinocytes compared with fibroblasts from a single patient as measured by growth inhibition. This may be due to the strong influence of alcohol consumption by the subjects, which was found to increase the sensitivity of keratinocytes but not of PBL and fibroblasts. In conclusion, oral fibroblasts but not keratinocytes can be used to measure sensitivity for chromatid breaks. The apparent influence of environmental factors on keratinocytes makes them a useful source to study exposure characteristics but limits their application for the determination of genetic factors.

Introduction

From traditional epidemiology it has become clear that exposure to alcohol and tobacco are important risk factors for the development of many types of cancers, including head and neck squamous cell carcinoma (HNSCC) (Maier *et al.*, 1992). Moreover, there is increasing evidence that dietary factors also play a role (Zheng *et al.*, 1993). Understanding the carcinogenic process has often included studies on the accumulation of DNA damage caused by carcinogenic agents. Recently, it has been acknowledged that environmental exposure acts in concert with genetic characteristics to determine the risk for common cancers (Caporaso and Goldstein, 1995), such as lung cancer, colon cancer and HNSCC. It seems that, through inheritance of altered genes, susceptible individuals more easily acquire somatic mutations due to carcinogenic assault.

Differences in an individual's capability to deal with mutagenic assault can be measured using chromosomal aberration tests. One such reliable and simple assay (Hsu, 1983) measures sensitivity to a mutagen which probably has an hereditary basis (Bondy *et al.*, 1993). In the S/G_2 phase of the cell cycle DNA damage is induced by bleomycin, chosen for its clastogenic properties (induction of double-strand breaks, which are thought to be the lesions leading to chromatid breaks) and for its mechanism of action, which resembles that of environmental carcinogens (Pryor, 1982). Recently, Wei *et al.* (1996) presented similar results using a well-known environmental mutagen which is a constituent of burned tobacco (benzo[*a*]pyrene diol epoxide) to induce DNA damage in a comparable assay.

Using the bleomycin-based mutagen sensitivity assay, a large number of healthy persons and cancer patients have been screened. The results indicate that mutagen sensitivity is a constitutional and reproducible factor (Cloos *et al.*, 1993), which will be valuable for the identification of individuals at high risk for HNSCC (Cloos *et al.*, 1994, 1996).

Since a genetic factor should be present in all normal somatic cells, peripheral blood lymphocytes (PBL) are most convenient for the assay. These cells can be easily obtained from subjects and cultured under mitogen stimulation. However, it is often questioned whether the chromosomal instability as measured in the mutagen sensitivity assay actually reveals the chromosomal instability of the cells in the area in which tumors develop.

The aim of this study was to determine whether mutagen sensitivity can actually be extrapolated to the target cells of carcinogenesis in the head and neck region. Although keratinocytes are the target cells for the vast majority of HNSCC much emphasis has been given lately to the role of fibroblasts in the underlying stroma (Schor *et al.*, 1994). We exposed primary cultured oral fibroblasts and keratinocytes to bleomycin and compared this with the standard mutagen sensitivity assay using lymphocytes from the same subjects.

Materials and methods

Subjects

Normal oral tissue was collected from 27 healthy control persons and three HNSCC patients (Table I). Ages of the persons varied from 25 to 75 years with a mean of 49. Five of the subjects (17%) were female. Besides current smoking status ($n = 21$) and current alcohol use ($n = 23$), for some persons

[4]To whom correspondence should be addressed. Tel: +31 20 444 0905; Fax: +31 20 444 0983; Email: bjm.braakhuis@azvu.nl

Table I. Subject characteristics

Patient ID	Cancer	Age	Gender	Smoking		Alcohol consumption	
				Current	Cumulative[a]	Current	Cumulative[b]
P643	No	56	M	No	0	Yes	50
P644	No	48	M				
P649	No	50	M				
P650	Larynx	65	F				
P651	No	59	M	No	0	Yes	50
P654	No	53	M	No	0	Yes	75
P656	Oral cavity	49	F				
P657	No	64	F	No	45	Yes	45
P659	No	44	M	No	0	Yes	54
P671	No	36	M	Yes	13	Yes	19
P678	No	25	M	No	0	Yes	6
P679	No	57	M				
P680	No	49	M	Yes		Yes	6
P681	No	46	M	No	0	Yes	90
P682	No	50	M	No	8	Yes	10
P683	No	36	M	No	24	Yes	21
P685	No	58	F	No	30	Yes	18
P689	No	56	M	No	0	No	0
P690	No	55	M	Yes	33	No	0
P691	No	54	M	Yes	56	Yes	48
P692	No	39	M	Yes	13	Yes	10
P693	No	75	M			Yes	120
P695	No	33	M			Yes	41
P699	No	62	M				
P700	No	56	M	No	92	Yes	100
P701	No	31	M	Yes	12	Yes	45
P702	No	69	M	Yes	15	No	0
P703	No	32	F	Yes		No	0
P704	No	35	M	Yes		Yes	
P707	Larynx	44	M				

PBL, oral fibroblasts and oral keratinocytes were obtained from these subjects of whom three were cancer patients. M, male; F, female.
[a]Cumulative smoking was determined as the number of pack years, which is calculated as the number of years of smoking multiplied by the number of cigarette packs smoked daily. The number of packs is calculated based on 25 cigarettes per pack, as is common in The Netherlands.
[b]Cumulative drinking (unit years) was similarly calculated, in which one unit is defined as one alcoholic beverage (equivalent to 1.5 ml ethanol) daily. The blanks in the table represent missing data.

cumulative smoking (pack years; $n = 18$) and alcohol use (unit years; $n = 22$) could be assessed. The number of pack years was calculated as the number of years of smoking multiplied by the number of cigarette packs smoked daily. The number of packs is calculated based on 25 cigarettes/pack. Unit years was similarly calculated; one unit is defined as one alcoholic beverage [equivalent to 1.5 ml (100%) ethanol] daily.

Cell culture

Human peripheral blood lymphocytes (PBL). Lymphocytes were cultured at 37°C, 5% CO_2 for 72 h. Heparinized whole blood (0.5 ml) was diluted in 4.5 ml RPMI 1640 medium supplemented with 15% fetal calf serum (HyClone, Logan, UT), 1.5% phytohemagglutinin (Life Technologies, Paisley, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Bio Witthaker, Walkersville, MD). RPMI 1640 medium was prepared by dissolving powdered RPMI (Bio Whittaker) in aquadest and supplementing with 2 mM L-glutamine (Life Technologies), 2 g/l $NaHCO_3$ and 2 g/l glucose. After adjusting to pH 7.4 using HCl, the medium was sterilized using bottle-top filters (0.22 µm). Whole blood was either cultured on the day it was obtained or stored at 4°C for a maximum of 2 days before culturing.

Oral tissue. Tissue was obtained from uvulas removed at uvulopalatopharyngoplasty for snoring and/or obstructive sleep apnea of control persons without a cancer history. In addition, macroscopically normal tissue was obtained in one patient from the edges of cancer specimens in the oral cavity. From two HNSCC patients we obtained normal cells from the larynx after surgery. Isolation of cells from the tissue has previously been described by Reid *et al.* (1997).

Fibroblasts. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (HyClone), penicillin/streptomycin (final concentrations 50 U/ml and 50 µg/ml), gentamicin (final concentration 5 µg/ml; Sigma, St Louis, MO) and amphotericin B (final concentration 0.25 µg/ml; Life Technologies). The medium was prepared by diluting powdered DMEM with L-glutamine (Bio Whittaker) in water, supplementing with 5.0 g/l HEPES and 0.85 g/l $NaHCO_3$ and adjusting with NaOH to pH 7.4, after which the solution was sterilized using bottle-top filters (0.22 µm).

Keratinocytes. Keratinocytes were cultured in 6-well culture plates in keratinocyte growth medium (KGM) which was prepared following the guidelines of the supplier (Life Technologies). KGM comes as a kit consisting of 500 ml standard medium (low Ca^{2+}) and the additives epidermal growth factor (human recombinant, final concentration 5 µg/l) and bovine pituitary extract (final concentration 50 mg/l). To complete our KGM stock we also added gentamicin (final concentration 5 µg/ml) and amphotericin B (final concentration 0.5 µg/ml).

Chromosome break assay

Lymphocytes. This procedure has been described previously (Cloos *et al.*, 1993). In brief, the cells were cultured for 3 days and for the last 5 h incubated with 30 mU/ml bleomycin (a gift from Lundbeck, Amsterdam, The Netherlands). Mitotic arrest was induced by a 1 h treatment with colcemid (50 µg/ml) after 4 h bleomycin incubation. The cells were harvested by centrifugation and treated with a 0.06 M KCl hypotonic solution for 20 min at room temperature. The cells were fixed and washed with Carnoy's fixative (acetic acid:methanol 1:3). Metaphase spreads were prepared by dropping the cells on wet slides. After air drying, the slides were stained with a 5% Giemsa solution. On each slide 50 metaphases were scored for chromatid breaks (b/c) (based on a slightly modified CBIC system; Hsu *et al.*, 1996). In contrast to radiation, this bleomycin treatment produces only a small proportion of gaps and they were omitted from the analysis. When ⩾12 breaks were found per metaphase this was counted as 12. The mean number of breaks per cell and the percentage of aberrant metaphases (%am) (% of cells with at least one break) were calculated as measures of DNA damage.

Fibroblasts. One million cells were seeded in an 80 cm^2 culture flask and cultured for 30 h. For the last 2 h the cells were challenged with 0, 0.5, 1 or 2 mU/ml bleomycin. The cells were treated with colcemid (50 µg/ml) for the

Table II. Chromatid breaks after G_2 phase bleomycin treatment

Patient ID	Lymphocytes		Oral fibroblasts							
	30 mU/ml		0 mU/ml		0.5 mU/ml		1 mU/ml		2 mU/ml	
	b/c	%am	b/c	%am	b/c	%am	b/c	%am	b/c	%am
P643	1.3	48	0.16	12	0.5	36	0.82	44	1.16	56
P644	0.5	36	0.12	12	0.54	34	0.42	34	0.48	36
P649			0.12	12	0.26	24	0.62	44	0.5	42
P650	0.72	37								
P651	1.04	41	0.06	6	0.3	16	0.42	26	0.56	34
P654			0.06	6	0.34	24	0.66	32	1.08	34
P656	1.05	43	0.1	10	0.58	40	0.52	40	0.8	48
P657	1.82	68								
P659	1.21	53								
P671	0.77	43								
P678	0.68	43	0.08	8	0.58	40	1.08	60	1.92	70
P679	0.64	45								
P680	0.83	41	0.1	10	0.38	28	0.46	28	0.94	56
P681	0.49	35	0.08	8	0.24	16	0.44	30	0.88	50
P682	0.56	31	0.06	6	0.36	24	0.94	56	1.3	50
P683	0.6	35	0.06	6	0.98	50	1.04	46	0.84	44
P685	0.5	34								
P689	0.65	41	0.02	2	0.46	28	1.04	36	1.86	50
P690	0.6	36								
P691	0.72	42	0.06	6	0.38	30	0.92	42	1.11	50
P692	0.67	45	0.04	4	0.5	32	0.92	42	2.7	76
P693	0.95	59	0	0	0.4	24	1.34	50	nd	nd
P695	0.74	42	0.04	4	0.74	36	0.98	46	2.0	56
P699			0.08	8	0.5	34	0.4	30	0.46	32
P700	1.13	47								
P701	0.42	36	0.02	2	0.5	18	1.12	44	0.92	40
P702	0.71	42	0.14	14	0.2	18	0.36	26	0.72	48
P703	0.59	36	0.1	10	0.6	28	0.78	32	0.6	40
P704	0.88	46	0.08	8	0.44	34	0.74	40	1.44	52
P707	0.95	38	0	0	0.44	14	nd	nd	nd	nd

The mean number of chromatid breaks (b/c) and the percentage of aberrant metaphases (%am) were scored after damage induction with bleomycin. For blood the incubations were performed with 30 mU/ml bleomycin for 5 h, while fibroblasts were treated for 2 h with several concentrations of bleomycin. The missing data represent cultures that failed to grow. nd, not determined due to too low a mitotic index. The blanks in the table represent missing data.

last hour. After trypsinization and washing with phosphate-buffered saline, the same procedure was followed as described above for PBL.

Cell growth inhibition by bleomycin (sulphorhodamine B assay)

The sulphorhodamine B (SRB) assay was used to monitor growth of the cells. This assay has been described in detail by Skehan *et al.* (1990) and measures the amount of protein as an indicator of the number of cells present in the culture. In short, cells are plated in 96-well microtiter plates (Greiner, Alphen a/d Rijn, the Netherlands) at different cell concentrations (150 µl/well) and incubated at 37°C, 5% CO_2. For fibroblasts, 5000 and 10 000 cells/well were plated, with 2000, 3000 or 4000 cells/well for keratinocytes. This assay is not suited to PBL since it requires cells to adhere to the bottom of the wells. After 3 days growth (lag phase) the cells of one plate were fixed by addition of 50 µl/well 25% trichloroacetic acid solution and the plate was stored at 4°C. After 3 days incubation with several concentrations of bleomycin (day 6) the plates were fixed for at least 1 h at 4°C. Both day 3 and day 6 plates were then washed five times with tapwater and air dried. SRB solution (0.4% in 1% acetic acid) was added (50 µl/ well) and incubated at room temperature for at least 15 min to stain the fixed cells. The plates were then washed four times with 1% acetic acid and air dried. The remaining stain was dissolved in 200 µl 10 mM unbufferred Tris. The optical density (OD) was measured at 540 nm using a plate reader (Multiskan Biochromatic; Labsystems, Helsinki, Finland). Normal growth of cells (OD day 6 – OD day 3) without bleomycin was taken as 100%. The 25 and 50% inhibiting concentrations (IC_{25} and IC_{50}) were calculated by relating the bleomycin-treated cells to normal growth.

Statistics

For the 30 subjects used in this study we collected both blood and oral tissue. Some cultures failed to grow and therefore for some subjects some data are missing. All different analyses were performed on the maximal available number of subjects for each assay. Possible bias due to this strategy cannot be completely excluded. Associations between variables were calculated using Spearman rank (r_s) and Pearson correlation tests. Two-sided significance levels were calculated for all parameters and P values <0.05 were considered statistically significant. Multiple linear regression was performed to simultaneously test several variables adjusted for one another.

Results

The results of the chromosome break assay are summarized in Table II. This assay could not be performed on keratinocytes because of the low number of metaphases which were harvested. The number of cells participating in cell division is smaller in these primary keratinocytes as compared with PBL and fibroblasts. Moreover, the sensitivity to bleomycin was so high that the keratinocytes did not enter mitosis after incubation with concentrations as low as 0.05 mU/ml. At concentrations <0.05 mU/ml too few breaks could be observed.

For PBL the assay was performed on whole blood (see Materials and methods) as routinely performed in our laboratory. A high bleomycin concentration of 30 mU/ml for 5 h is required to induce chromatid breaks in PBL, since red blood cells absorb a considerable amount of the bleomycin. This standard assay gives similar results to when a 2 h, 4 mU/ml bleomycin incubation is used for isolated cultured PBL (Hsu *et al.*, 1990). The mean b/c level of PBL from the control subjects in this study population was 0.79 ± 0.32, which is similar to previously reported data (Cloos *et al.*, 1994). The b/c level of the cancer patients was higher (0.91 ± 0.17) although not significantly so, possibly due to the low number

of patients (n = 3). As we were principally interested in the correlation between normal cell types within one person, cancer and non-cancer patients were analysed as one group.

The chromosome break assay is not routinely performed on fibroblasts. For this study we used three different bleomycin concentrations (0.5, 1.0 and 2.0 mU/ml) and incubation for 2 h. When comparing the chromosome break assay data for PBL with fibroblasts (Table II and Figure 1) it was found that

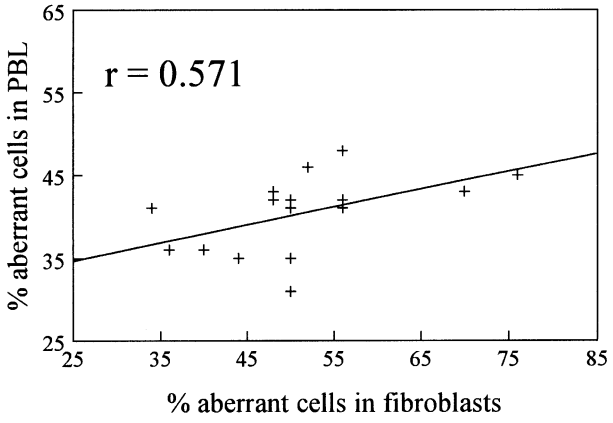

Fig. 1. Individual data of %am in fibroblasts are plotted against the corresponding data of %am in PBL for each subject. The linear curve fit depicts the correlation between the two parameters.

%am in PBL is correlated with %am in fibroblasts at a bleomycin concentration of 2 mU/ml (r_s = 0.571, P = 0.017). This bleomycin concentration is two times lower compared with what is commonly used in lymphocyte cultures without red blood cells (4 mU/ml bleomycin). This indicates that fibroblasts are a little more sensitive to bleomycin in this assay as compared with PBL. %am is a crude measure of chromosomal damage whereas the number of b/c gives a more specific value about how much the DNA is damaged. Using the refined DNA damage parameter b/c, no significant correlation was found between PBL and fibroblasts.

Growth inhibition by bleomycin (Table III) could be compared with chromosome breakage in fibroblasts, since for these cells both assays could be performed. The SRB assay by which growth inhibition was measured was comparable with results of viable cell counts. In order to measure cell growth inhibition several cell cycles have to pass. Therefore, in the SRB assay a chronic 72 h bleomycin treatment was used, in contrast to the G_2 phase treatment in the chromosome break assay. The concentration of bleomycin at which 25% of cell growth was inhibited (IC_{25}) was found to be a good parameter for growth inhibition in fibroblasts. It strongly correlates with cell growth at all the bleomycin concentrations used and with the often used IC_{50} value. Most data were available (n = 27) for the IC_{25} value (mean 1.5×10^{-7} M). There was a very good correlation (r = 0.602, P = 0.005) between the IC_{25} value and the b/c value at a bleomycin concentration of 0.5 mU/ml

Table III. Growth inhibition after 72 h exposure to bleomycin

Patient ID	Oral fibroblasts			Oral keratinocytes		
	IC_{50} ($\times10^{-10}$ M)	IC_{25} ($\times10^{-10}$ M)	Growth (%) at 0.1 μM	IC_{50} ($\times10^{-10}$ M)	IC_{25} ($\times10^{-10}$ M)	Growth (%) at 0.1 μM
P643				2.5	<1	0
P644				1.5	<1	0
P649	>10 000	344	61	39.2	4.4	0.4
P650				20.3	4.4	0
P651	8628	1096	75	21.7	6	0
P654	1942	346	56	27.8	4.7	4.9
P656	>10 000	4047	78			
P657	1833	383	54	41.8	16.7	0
P659	2198	508	54	8	<1	0
P671	798	161	47			
P678	>10 000	2753	80			
P679	>10 000	552	65			
P680	>10 000	479	67			
P681	>10 000	537	67			
P682	384	62	40	33.4	13.2	0
P683	4047	3764	61			
P685	>10 000	1000	74	35.4	27.1	0
P689	9716	469	65	177.2	83.8	19.6
P690	>10 000	9413	84	43.1	5.6	0
P691	>10 000	745	70	87.3	53.9	17.8
P692	>10 000	875	73	182.2	51.4	11.2
P693	>10 000	1195	75			
P695	>10 000	2356	77			
P699	1682	300	56			
P700	6813	485	65			
P701	10 000	687	66	18.8	<1	4.1
P702	8762	760	67	197.2	92.1	16.3
P703	>10 000	7371	80			
P704	10 000	793	70	9	2.2	0
P707	>10 000	848	73			

Growth inhibition was measured using a proliferation assay in which the number of cells is related to the amount of protein (SRB). Cells treated with several concentrations of bleomycin (10^{-5}–10^{-10} M) were compared with control growth of untreated cells. IC_{50} and IC_{25}, the concentrations of bleomycin at which 50 and 25%, respectively, of cell growth was inhibited compared with the untreated control growth. The missing values (blanks in the table) are due to failures in cell growth.

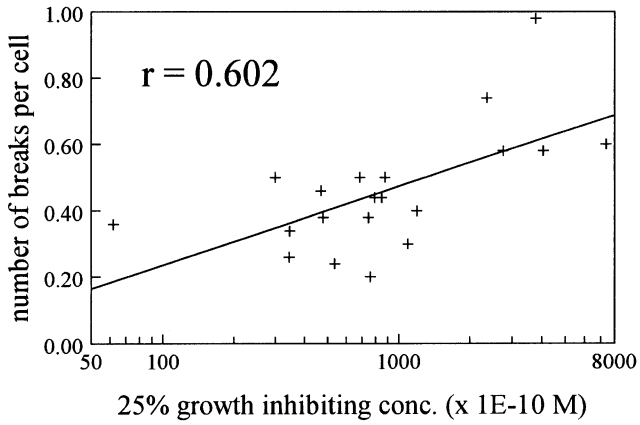

Fig. 2. Correlation between growth inhibition (IC_{25}) and mean number of b/c within each subject.

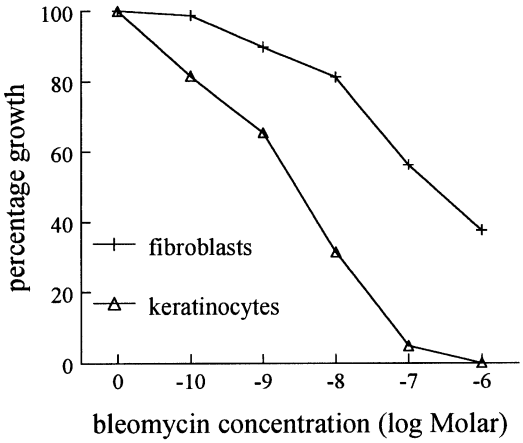

Fig. 3. A representative dose–response curve for keratinocytes and fibroblasts of one person (P654) as measured using the SRB assay (see Materials and methods). The growth of cells without bleomycin was set at 100%.

(~3.3×10^{-7} M) (Figure 2). Of the bleomycin concentrations that were tested in the chromosomal aberration assay this concentration is the most comparable with the IC_{25}. This positive correlation indicates that a higher b/c score is associated with a higher concentration of bleomycin necessary to inhibit 25% of growth. Thus, less growth inhibition is associated with a higher b/c level.

The SRB assay could not be performed using PBL since this assay requires cells that adhere to the bottom of the wells. There was no correlation between any growth inhibition variables for keratinocytes and fibroblasts. For both cell types it was found that the level of inhibition was not related to the growth rate of untreated cells. In all persons tested, keratinocytes are far more sensitive to bleomycin compared with fibroblasts, as indicated by the higher IC_{25} and IC_{50} values in fibroblasts. Moreover, at 1 μM bleomycin cells were killed in all keratinocyte cultures, which was not found for fibroblasts, even at a concentration of 10 μM. A representative example of a dose–response curve for fibroblasts and keratinocytes of one person as measured using the SRB assay is shown in Figure 3.

The lack of correlation between fibroblasts and keratinocytes might be explained by the fact that keratinocytes are in direct contact with the environment and exposure to toxic compounds influences their behavior. There was no correlation between smoking or alcohol use by the subjects and any of the variables for PBL and fibroblasts. In contrast, for keratinocytes the influence of these factors on growth inhibition was pronounced. The correlation between cumulative smoking (pack years) and IC_{50} was almost significant ($r_s = 0.548$, $P = 0.052$). Moreover, the correlation between unit years and IC_{50} was strongly negative ($r_s = -0.743$, $P = 0.04$). In multiple linear regression models other variables such as age and pack years and their interactions were added to find out whether they may account for this strong relation between IC_{50} and unit years. The influence of pack years on the IC_{50} is not significant in any model, with or without log transformation. The only variable which significantly influences the IC_{50} is unit years in all analyses, although it has to be emphasized that cumulative alcohol use was not known for all persons for whom the IC_{50} was measured ($n = 13$).

An unusual correlation was found between age and b/c in fibroblasts at 0.5 mU/ml bleomycin. This correlation was strong and negative ($r_s = -0.562$, $P = 0.006$), which cannot easily be explained. It was only observed at this specific bleomycin concentration and not with any other variable. If any correlation was to be expected it would be that older subjects would have more DNA damage. Using multiple regression analysis it was found that this correlation does not influence the association between IC_{25} and b/c at 0.5 mU/ml in fibroblasts.

Discussion

It has to be emphasized that due to culture failures data for some experiments are missing. A frequently encountered problem was that keratinocyte cultures did not yield enough dividing cells to carry out a SRB assay. All analyses were performed on the maximum available number of subjects.

For both chromosome breakage and growth inhibition large differences were found between persons. Chromosome breaks were measurable in PBL and fibroblasts, both of which are often used for these types of studies (Price *et al.*, 1991; Kaufman and Wilson, 1994). A good correlation was found between the two cell types for %am, which is a less refined parameter of DNA damage compared with the b/c value. There was no significant correlation between the number of breaks in PBL and the number of breaks in fibroblasts within the subjects. This b/c parameter is very precise as to how much the cells are damaged. A few cells with many breaks have a large impact on the b/c score, which may explain the lack of a significant correlation using this parameter. The fact that a good correlation was found for cells with at least one break (%am) suggests that whether a cell has any persistent chromatid damage is more important than the number of breaks present in the damaged cell. One cell with several breaks may be less significant in the carcinogenic process than several cells with one break, since the former cell will certainly die and will not give rise to fixed mutations.

When a cell recognizes DNA damage it has several options to deal with the damage. It is often postulated that the cell can enter apoptosis and die or growth can be arrested at several cell cycle checkpoints to allow DNA repair (Weinert and Lydall, 1993; Goodger and Morgan, 1997). In line with the latter phenomenon, it can be postulated that in persons with a high b/c score cell growth is not arrested to allow time to repair the DNA damage properly because of impaired inducement of cell cycle blocks. Our results support this

hypothesis, because there was a strong correlation between diminished growth inhibition (IC_{25}) in fibroblasts and the number of b/c. This indicates that a high level of chromatid breaks is associated with a short time to repair the damage. A mutagen hypersensitive phenotype may therefore be related to defects in cell cycle checkpoints.

Unfortunately, the chromosome break assay could not be performed in keratinocytes, but our results show that fibroblasts are far less sensitive with respect to growth inhibition by bleomycin compared with keratinocytes. It seems that keratinocytes have a functionally different mechanism in response to damage, since they stop proliferating as soon as DNA damage occurs. Whether they undergo apoptosis and die cannot be concluded from these results, however, the SRB assay data indicate that cells are lost even at low doses of bleomycin (<0.1 μM). Growth inhibition in fibroblasts was not correlated with that of keratinocytes within the subjects. This lack of correlation may be due to functional differences between the two cell types, including the fact that keratinocytes are in direct contact with the environment. This is especially important for direct interaction with compounds from tobacco smoke and with alcohol. There was no influence found on any of the measured parameters of external factors such as smoking and alcohol intake of the subjects for PBL and fibroblasts, while there was for keratinocytes. The negative correlation which was found between unit years and IC_{50} indicates that when a person has a history of extensive alcohol use keratinocytes become more sensitive to the growth inhibitory effects of bleomycin. Although the influence of smoking on keratinocytes was less pronounced, it is interesting to note that increased pack years was associated with less growth inhibition. The pronounced influence of these factors on growth inhibition implies that the cells have acquired changes due to exposure which are fixed and passed on to daughter cells. Otherwise, it may be expected that culture of the cells for so many weeks in the absence of alcohol and smoke particles would have diminished their effects on cell growth.

It seems that exposure to tobacco smoke induces resistance to bleomycin. Possibly, the cells acquired a mechanism to detoxify various toxic compounds. In the case of alcohol exposure the explanation for the increased sensitivity to bleomycin is not so obvious. It has been postulated that alcohol acts as a co-carcinogen (Hsu *et al.*, 1991) and its metabolite acetaldehyde has clastogenic potential (Singh and Khan, 1995). Not only alcohol itself in the drinks but trace amounts of congeners may be responsible for the effect (Blot, 1992). This holds particularly true for apple brandy (calvados) (Yamada *et al.*, 1992).

Both our study and others (Wang *et al.*, 1992; Maier *et al.*, 1994; Colucci *et al.*, 1997) show that alcohol and tobacco use have a significant effect on oral mucosa cells. This is in line with the fact that both are major risk factors for the development of HNSCC. These phenomena are very interesting and more research is warranted as to the underlying mechanisms of the prolonged effects of both alcohol and tobacco.

In conclusion, oral fibroblasts as well as PBL of a person may be used to determine their susceptibility to chromosome damage in the mutagen sensitivity assay. However, the correlation between PBL and fibroblasts was not straightfroward. Clearly other factors specific for cell type and not for genetic/constitutional characteristics play an additional role in sensitivity to carcinogenic assault. From this study it cannot be concluded which cell type might be best to predict cancer risk. To elucidate that, a large study has to be performed in which chromosome damage in fibroblasts of cancer patients and control persons is compared. It must be emphasized that the use of PBL is faster and less tedious, therefore fibroblasts are not nearly as convenient as PBL. Primary oral keratinocytes are not suitable for chromosomal assays due to the low number of metaphases and the high sensitivity of the cells for DNA damage. This high sensitivity of keratinocytes for DNA damage was detected in both assays mainly as significant growth arrest (e.g. a low mitotic index and a low IC_{50} value). Moreover, the apparent influence of exogenous environmental factors on keratinocytes makes them a useful source to study exposure characteristics but limits their applicability for the determination of endogenous genetic factors.

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