MONITORING OF DNA AND CYTOGENETIC DAMAGE IN LYMPHOCYTES FROM PERSONS WITH SKIN CANCER DISEASES

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Abstract

There is a lot of interest in the studies that would help to understand whether there is a casual association between cancer and various types of molecular or cytogenetic damage detected in human cells. One major oncogenesis process is activation of proto-oncogenes by point mutations or chromosomal translocation. There are substantial evidence that indicates that the loss of heterozygosity of certain chromosomes is involved in human carcinogenesis. Our study aimed to elicit the possible association between cancer and DNA and cytogenetic abnormalities induced in lymphocytes of people bearing various categories of skin cancer cells. Fresh blood was collected by venipuncture from 25 individuals (including nine prior to cancer treatment). All patients were nonsmoking males, however 42.3 % of them were former smokers. Blood samples were divided into two parts and in the first part of samples cytogenetic studies were performed immediately, while from the second part lymphocytes were isolated and stored at -70°C for further studies in vitro. In the later one a single cell gel electrophoresis assay (SCGE) known as a Comet assay was performed to study individual susceptibility to the induction of DNA damage by UV or radiation and to estimate variability in cellular repair capabilities. An average of 220 per sample of good metaphase spreads in the first mitotic division, and 100 per sample in the second division, were accepted for analysis of cytogenetic damage. Chromosome and chromatid type aberrations were scored in the cells in the first mitosis and expressed as total aberration frequency including gaps and excluding gaps. Sister chromatid exchanges, high frequency cells and proliferative rate index were screened and evaluated in the second mitosis. Each of the patient revealed exceeding in at least one of the cytogenetic biomarkers level from the biomarker's level detected in a reference group. In order to estimate susceptibility of people to environmentally induced damage, the isolated lymphocytes were irradiated with 2 Gy dose of X-rays or 6 J/m^2 of UV radiation and the single cell gel electrophoresis (SCGE assay) was performed. To compare various individual capabilities to repair damages induced, an incubation of cells in the presence or absence of cellular processes starting agent was done prior to DNA damage analysis. A statistically significant higher response to UV and a lower capability to repair UV induced damage in skin cancer patients were observed. On average no difference between control and skin cancer patients in the induction of DNA damage by X-rays was observed, though correlations between highest cytogenetic damages detected in lymphocytes of skin cancer patients and the lowest capability to repair DNA damage were noticed.

INTRODUCTION

In general, the objectives of biological monitoring are to identify one or several measurements of critical change in the host resulting in or associated with the pathological change of great concern. It is unlikely that a single biological marker for any particular genotoxic effect will be found because of the dynamics and adaptability of biological systems and multiplicity of the reactions and mechanisms involved in genotoxic end-points affecting either structure or function. This is of particular interest if any of the damage detected could be used as an early biomarker of

diseases. Among the selected from several critical markers appropriate for the biological monitoring is inherent susceptibility associated with genetic traits. There is also a lot if interest in the studies that would help to understand whether there is a casual association between cancer and various types of molecular or cytogenetic damage detected in human cells. One major oncogenesis process is activation of proto-oncogenes by point mutations or chromosomal translocation. There is substantial evidence that indicates that the loss of heterozygosity of certain chromosomes is involved in human carcinogenesis.

Our study aimed to elicit the possible association between cancer and DNA and cytogenetic abnormalities induced in lymphocytes of people bearing various categories of skin cancer cells. Some of the measurements are still going on, however our preliminary results are presented in this paper.

MATERIAL AND METHODS

Sampling

Fresh blood was collected from donors by venipuncture. All skin cancer patients prior to cancer treatment were nonsmoking males, however 42.3 % of them were former smokers. Reference groups contained 40 persons of healthy donors [2]. The donors for the reference groups were chosen from two industrial and countryside areas of Southern Poland (the same region from which patients under the study came). The industrial area controls were administrative staff of the petroleum plant close to a highly polluted region, and the countryside regions were two villages in Krakow vicinity and two neighboring villages with a low level of pollution [19] and a low level of total cancer cases [25]. The averages of age in the reference groups were 40 and 60 years. Among the reference group 36% were smokers and 64% were non-smokers, and 44% were females. Individuals in the healthy control group were of similar socio-economic status and were represented by farmers and foresters, skilled state workers, teachers or clerks with liberal or technical education and a low medium income. Interviews were performed with a questionnaire considering health, life styles and habits, job conditions, and possibilities of hazardous exposure leading to increased genotoxic risk. The questionnaires were in agreement with the recommendation for biological monitoring type of study [4]. The whole blood samples were transported in heparinized tubes to the laboratory of DREB at the Institute of Nuclear Physics. Then, blood samples were divided into two parts. From the first part appropriate lymphocytes isolation was done and lymphocytes were in stored at -70°C for further DNA analysis. From the second part culturing of samples was processed as soon as possible. Results of all the analysis performed were compared to the average levels observed in the reference group.

Blood culturing and cytogenetic screening:

The samples of heparinized whole blood were incubated at 37°C using Eagle`s medium supplemented with 20% of fetal calf serum and antibiotics. Lymphocytes were stimulated with LF-7, a Polish substitute of PHA [2,5,6,7] and cultured, with an addition of a proper amount of BrdU, for 48h, in case of chromosomal aberrations and for 72 h, in case of SCE cultures. Two hours before the end of culturing colcemid was added (0.1 μl/ml) to each sample. In the cultured samples fixation and staining were performed by standard cytological procedures either for CA or SCE analysis [5,6,7,12,24]. Chromosome and chromatid type aberrations were scored in the cells in the first mitosis and expressed as total aberration frequency including gaps (TAbF), and excluding

gaps (AbF). Sister chromatid exchanges (SCE) were screened in the second mitosis and breaks per each cell containing at least 44 chromosomes were counted. High frequency cells (HFC- percent of cells displaying number of exchanges per cell higher than the 95% of the population distribution), and PRI - proliferative rate index (MI+ 2xMII+ 3xMIII) /(MI+ MII+ MIII), were evaluated as reported elsewhere [1,23].

COMET ASSAY

Single cell gel electrophoresis- SCGE is a method that allows visualization and measuring of DNA damage in individual cells, it is also known as a "Comet assay". The comets are formed during electrophoresis by different migrations of the broken DNA pieces released from the cores of nucleus. The image of the cells remainds "comets" because of the migration of DNA fragments from the cell nucleus towards the anode. The length of the "comet" tail gives information about the degree of DNA damage. The migrating fraction of DNA is visualized by staining with fluorophor and quantified by using an epifluorescence microscope. The simplest method of assessing DNA damage is the evaluation of the comet size from its tail extension [3,8,16]. All assay procedures and requirements for the experimental condition were as described elsewhere [8].

Chemicals

For SCGE procedure following chemicals were applied: normal melting temperature agarose (NMA), low melting temperature agarose (LMA) were purchased from GIBCO BRL, Paisley, Scotland; Triton X-100 from Aldrich Chemie, Steinheim, Germany; sodium sarcosinate, EDTA, ethidium bromide, DMSO, RPMI 1640, Tris and Histopaque from Sigma Chemicals Company, St. Louis, MO, USA; sodium chloride, sodium hydroxide from POCH, Lublin, Poland.

Cryopreservation

Heparinized whole blood collected from each donor (9ml) was diluted 1:1 with RPMI 1640 and kept at 4^0C for 10 min. Lymphocytes were separated by centrifugation over 4ml of Histopaque at 200 g for 30 min. Than lymphocytes were removed and washed twice with RPMI 1640. Lymphocytes suspended in RPMI were counted in a hemocytometer. For the cryopreservation the cells were resuspended in a freezing medium consisting of 10% DMSO, 40% RPMI and 50% fetal calf serum. The cell suspension was transferred into plastic freezing vials in aliquots of 1.8-2 x106 cells. Vials were placed in a -70^0C.

Thawing

The vials were submerged in a 37^0C water bath until the last trace of ice was melted. The thawed cells were quickly transferred to centrifuge tubes containing 15 ml of a thawing medium (50% fetal calf serum, 40% RPMI 1640, 10% Dextrose). Cells were centrifuged at 200 x g for 10 min and the cells were resuspended in RPMI 1640. Cell viability, usually over 94% was determined using the trypan blue exclusion technique.

DNA challenge assay: X-rays and UV irradiation:

Isolated lymphocytes were washed in cold PBS, and resuspended in the same medium at 4^0C. To assess predictability of the cells from people investigated to induce damage by genotoxic agent, isolated cells were irradiated with 2 Gy dose from X-rays machine (Philips MCN 323

operated at 250 kV and 10 mA). Target to X-ray tube distance was 17,3 cm giving an average dose rate of 2 Gy/mm. For UV irradiation, 60 W quartz lamp (ë predominantly 254 nm) was used. In the condition of UVC exposure dose rate was 0,2 J m^{-2} s^{-1}. Cells were irradiated at 20^{0}C, and to avoid a cellular repair process they were immediately transferred to 4^{0}C, then incubated at 37^{0}C for 2h in the presence or absence of proper amount of LF-7.

Slide preparation

Slides (two repetitions of each slide) were prepared according to the standard procedure described elsewhere [3,8,16]. Slides were immersed for 1 h at 4^{0}C in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate), with 1% Triton X-100 and 10% DMSO added just before the use. Then the slides were washed with H_2O distilled (at 4^{0}C) and placed side by side in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH) to a level of about 0.25 cm above the slides. After 20 min of unwinding of the DNA, the electrophoresis was conducted at 4^{0}C for 20 min at 25 V, 300 mA. All of the steps after lysis were conducted under yellow light to prevent any induction of additional DNA damage. After electrophoresis the slides were gently washed 3 times for 5 min with Tris buffer (0.4 M Tris pH 7.5). The slides were stained with 60 ml of 17 mg/ml ethidium bromide in distilled water and covered with a coverslip. Before analysis the slides were stored in a light proof box containing moist PBS at 4^{0}C.

Slide analysis

The slides were examined at 200x magnification using an epifluorescence microscope (Olympus BX-50) equipped with a 100 W mercury lamp and an excitation filter of 515-560 nm, and a barrier filter of 590 nm. The CCD camera was used to get the image of the comet and automatic evaluation of the comet size was performed with an application of Komet 3.0 software from Kinetic Img. 100 cells were measured per the agent dose (50 cells from replicated slides). Tail length (the length of the comet tail measured from the edge of the comet head) and tail moment (the fraction of DNA in the tail multiplied by the tail length) parameters were used as a measure of the DNA damage.

Statistics

Statistics in cytogenetic data, T-test, Anova and multivariate stepvise regression analysis were applied from SPSS program for Windows. The mean values of Tail Length, Tail Moment and statistics were calculated by Komet 3.0 Kinetic Img.

RESULTS:

Table 1 presents results of cytogenetics measures, and average values for the two reference groups characterized by different average age values. The results of the analysis that exceeded significantly (more than 2SE) the levels of biomarkers observed in older reference group are presented in bold characters. Table 2 shows the influence of confounding factors on the biomarkers levels detected in the group.

Table 1.
Comparison of biomarkers detected in the group investigated with averages from reference groups

Code	CD	TAbF /cell	AbF /cell	AbC %	Endo %	Tetra %	SCE /cell	PRI	HFC %
1	1	0.010	.005	0.98	.00	.00	7.45	2.09	7.0
2	2	0.019	.000	1.95	.50	.00	6.65	2.49	1.0
3	1	0.012	.006	1.15	.00	1.07	6.92	1.67	4.5
4	2	0.069	.042	6.89	1.89	1.17	7.81	2.12	8.1
5	3	0.050	.037	4.98	.64	.62	7.71	2.03	13.0
6	1	0.024	.015	2.44	.49	.49	8.01	2.25	11.0
7	3	0.020	.005	1.95	.00	.00	7.44	2.25	5.0
8	3	0.242	.101	11.9	.0	.00	5.98	1.87	1.0
9	1	0.186	.063	9.10	.0	.00	4.36	2.06	0.5
ALL		.053±.016	.019	3.28	0.60	0.53	7.40	2.12	7.5
Ref[40]	no	.032	.017	6.30	nd	nd	7.00	2.40	6.7
Ref[60] ±SE	no	.040 ±.011	.021 ±.006	3.70 ±0.95	0.0	0.0	7.10 ±0.90	2.09 ±0.15	8.0 ±3.0

CD-histopathology of cancer cells (WHO coding system). First division: TAbF,AbF-aberration frequency (including and excluding gaps respectively), AbC-percent of aberrant cells, Second division: Endo - percent of endoreduplicated and tetraploidcells respectively, SCE-sister chromatid exchanges, HFC-high frequency cells (percent of cells displaying exchanges higher than the 95% of the reference group), PRI-proliferative rate index PRI = $(M_1+2M_2+3M_3)/(M_1+M_2+M_3)$,

Owing to a very small group, the variation between biomarkers within different histopathology subgroups is statistically insignificant, though it presents an consequent tendency. Highest values of chromosomal damage was observed among patients with a cell type diagnosed as (CD=3). Patients with a cell type diagnosed as CD=2, showed the highest probability to express endo-reduplicating and tetraploid cells. Their lymphocytes were the fastest in entering the second and third divisions, although they showed the lowest level of detected SCE and HFC. The lowest level of biomarkers detected in the first mitosis (TabF,AbF, AbC) and associated with the lowest percent of endoreduplicating cells was observed among the patients with diagnosis No 1, types of cells. Probably due to the fact that none of the patients was a recent smoker, this factor did not influence the measures, however, the average age of nonsmokers is significantly higher than the one of the former smokers (68.6 vs 48.4 $p< .0005$). Age of the expression of the dissease was significantly bigger and cytogenetic damages were lower in the patients who had not reported any

cancer in the immediate families, although at the time of skin cancer diagnose, the levels of SCE, and HFC, were significantly higher than in the patients reporting other cancer developments in immediate families ($p < .005$).

Table 2
Influence of confounding factors on the biomarkers levels detected in the group

Factor			No	TabF/cell± SE	AbF /cell	AbC %	Endo %	Tetra %	SCE /cell	HFC %	PRI
	61	All	7	.053 ± .034	.026	4.1	.62	.47	7.1	6.7	2.10
CD	64	1	4	.040 ± .020	.016	2.6	.27	.45	7.02	6.5	2.01
	62	2	2	.049 ± .021	.025	4.9	1.33	.70	7.35	5.3	2.27
	56	3	3	.072 ± .030	.037	5.1	.39	.31	7.33	8.3	2.08
CiF	65*	0	7	.052 ± .037	.029	4.3	.62	.58	7.4**	7.5**	2.03
	44.7	1	2	.075 ± .020	.021	4.3	.63	.00	5.9	0.8	2.35
GF	61	0	5	.060 ± .027	.028	4.0	.30*	.36	7.0	6.7	2.01
	76	1	2	.069 ± .057	.042	6.9	1.89	1.17	7.8	8.1	2.12
MT	59	0	3	.016 ± .013	.005	1.6	.00	.54	7.2	4.7	1.96
	65	1	5	.071 ± .040	.037	5.3	.83	.53	7.3	8.4	2.09
S	68.6***	0	5	.053 ± .042	.029	4.4	.77	.68	7.2	7.9	2.00**
	48.4	1	4	.053 ± .021	.020	3.5	.34	.14	7.2	5.0	2.26

Biomarkers abbreviations as in the Table 1. CiF; Cancer in a family (0 no, 1 cancer reported in immediate family), Genetic Factor 1 = reported problems with progeny, MT - other than cancer medical treatment, S- smoking; 0 never smoke, 1 - gave up smoking more than half a year ago, 2 - recent smoker,

Variability in the DNA damage detected in skin cancer patients' lymphocytes is presented in Table 3. Fig.1a and b show distribution of DNA damages (Comet Length and Moment Tail

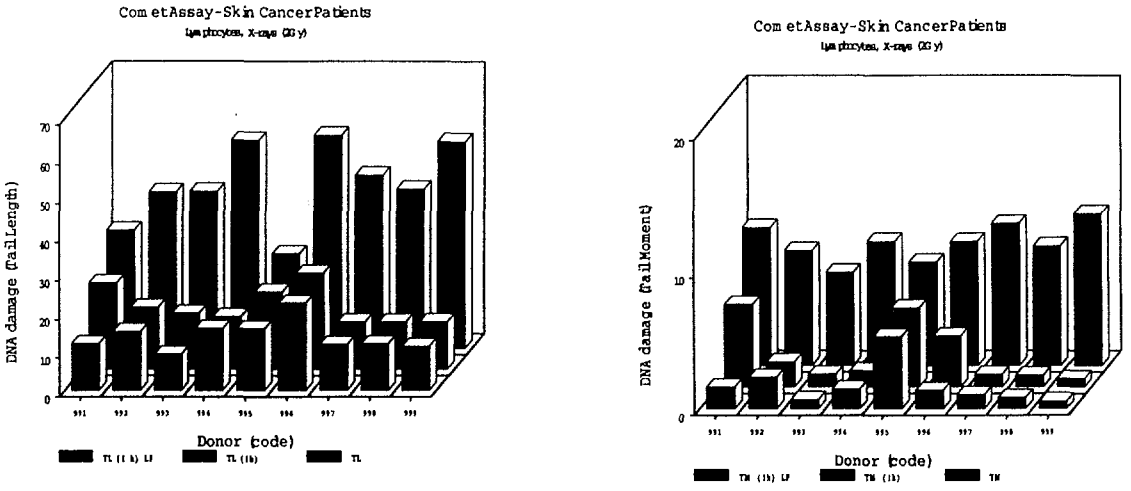

Fig. 1 a and b.
Distribution of the DNA damages (Comet Length and Moment Tail respectively) detected by the comet assay in the lymphocytes of skin cancer patients, and distribution of damages after challenging X-ray radiation dose (2Gy) in the cells stimulated (LF) or not to cellular processes of division.

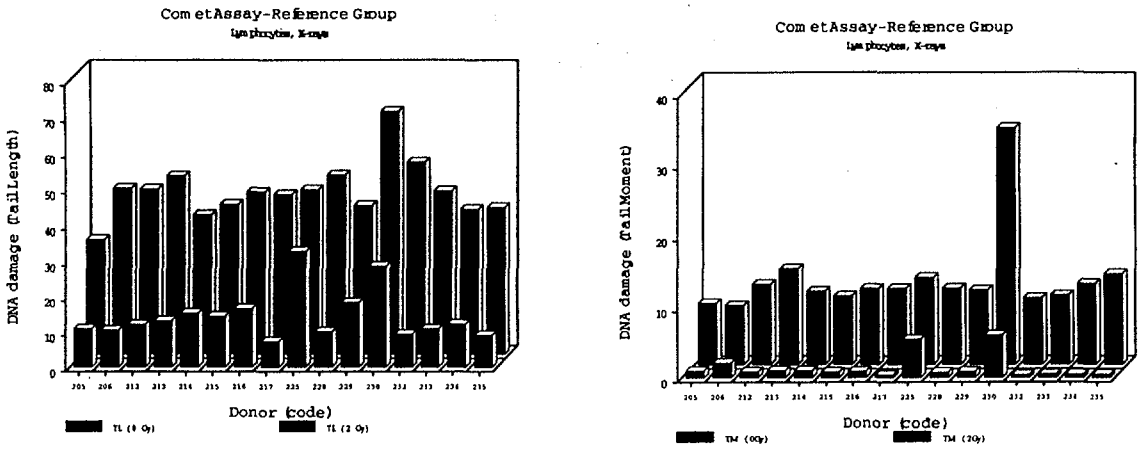

Fig. 2 a and b.
Distribution of the DNA damages (Comet Length and Moment Tail respectively) detected by the comet assay in the lymphocytes of donors from reference group, and distribution of damages after challenging X-ray radiation dose (2Gy) in the cells stimulated (LF) or not to cellular processes of division.

Table 3
Variability in the DNA damage detected by SCGE assay in lymphocytes of skin cancer patients

Code of the sample	Tail Moment ± SD	Tail DNA ± SD	Tail Length ± SD
1	2.21 ± 1.12	6.96 ± 1.40	13.78 ± 1.96
2	1.37 ± 0.80	7.29 ± 2.01	13.56 ± 3.97
3	0.59 ± 0.38	4.27 ± 1.05	8.21 ± 1.95
4	1.88 ± 0.62	7.37 ± 1.58	19.37 ± 1.91
5	2.80 ± 2.56	8.45 ± 2.63	13.89 ± 4.96
6	1.50 ± 0.47	6.72 ± 2.31	18.25 ± 3.89
7	1.29 ± 0.41	6.72 ± 1.22	14.71 ± 1.93
8	1.36 ± 0.43	8.90 ± 2.49	13.65 ± 1.71
9	0.46 ± 0.16	4.27 ± 1.01	9.92 ± 1.55

respectively) detected by the comet assay in the lymphocytes of skin cancer patients. There is also shown the distribution of damages observed as cell responses to the challenging X-ray radiation dose (2Gy) in the cells stimulated or not to cellular processes of division. There is seen the significant influence of cellular processes in the repair of the DNA damages induced. There is also shown the distribution of damages observed as cell responses to the challenging X-ray radiation dose (2Gy) in the cells stimulated or not to cellular processes of division. Figure 2 a and b show the distribution of the DNA damages (Comet Length and Moment Tail respectively) detected by the comet assay in the lymphocytes of people from this reference group. Comparing the average for the skin cancer patients and healthy group values of the DNA damage there is no visible difference in capacity of repair processes of DNA damages induced by radiation (18.9% and 18.2% respectively, after incubation of cells in the presence of stimulating cellular processes agent). However, the highest response to X-rays is observed in the skin cancer patients with codes 4, 8 and 9, which correlates with the highest values of cytogenetic damages detected in lymphocytes of those people (Table 1).

Figures 3 a and b and 4 a and b show variability in the cellular response in the same groups under the study, but detected after challenging cells with the exposure to UV radiation. All types of DNA damage measures (expressed as; TDNA- DNA in a comet tail, CM - comet's moment and CL - comet length) both in the absence and presence of LF-7 agent, are significantly higher in lymphocytes of skin cancer patients than those of the reference group (LF-7=0; p(tDNA) =.004, p(TM) = .004, p(TL)=.001 LF-7=1: p(tDNA) =.0008, p(TM) = .0000, p(TL)=.0000).

DISCUSSION:

The genotoxic hazard depends on the very complex mixture of agents and confounding factors. Among the possible factors influencing the hazardous level to genetic material are: diet, smoking habits, alcohol consumption, agrochemicals, UV, noise and vibrations, organic and

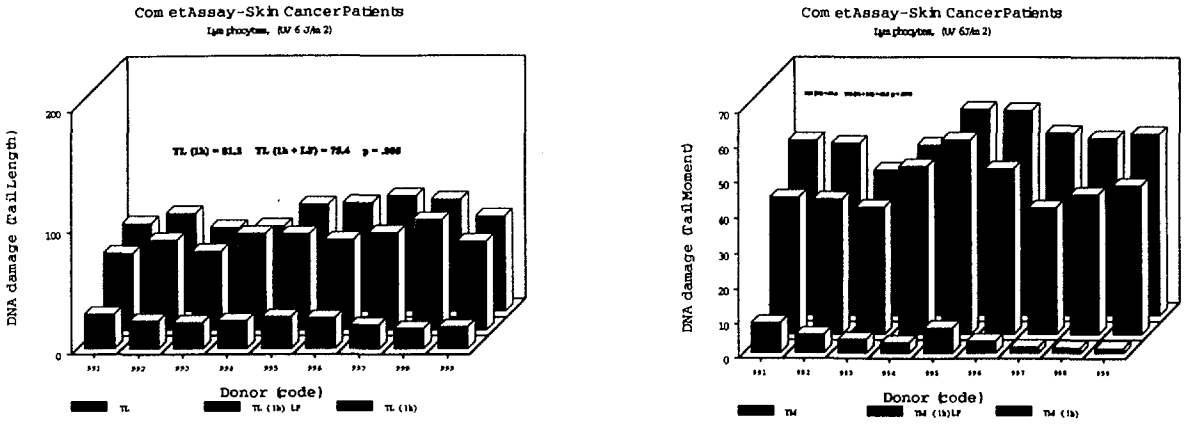

Fig. 3 a and b
Variability in the cellular response and capability to repair DNA damage induced by challenging dose of UV radiation by lymphocytes from skin cancer patients.

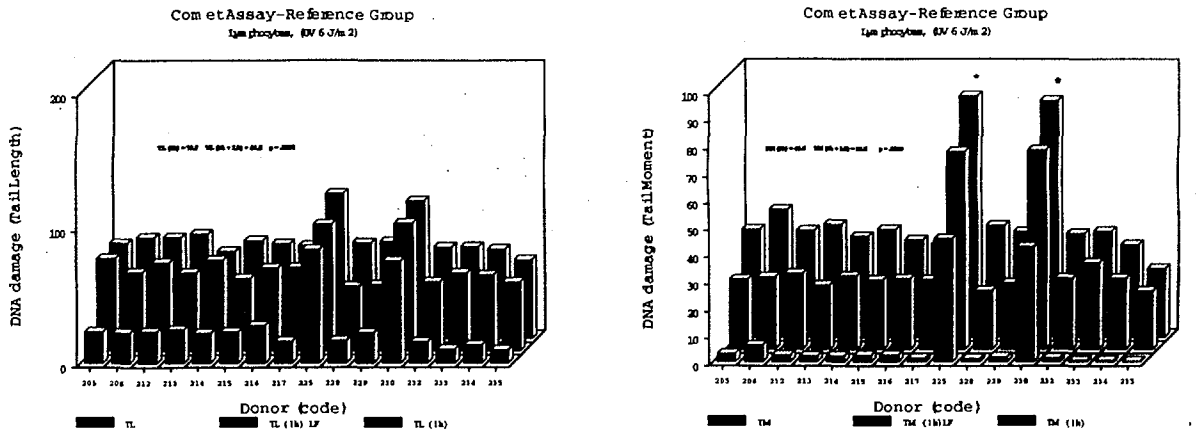

Fig. 4 a and b
Variability in the cellular response and capability to repair DNA damage induced by challenging dose of UV radiation by lymphocytes from reference group.

nonorganic dusts, even extreme temperatures [9,18,20]. In reacting synergistically, it may restrain human genomic function [11,17,21]. In the studies by Cebulska-Wasilewska et al. [7] a multivariate showed the existence of casual association between environmental exposures, cytogenetic damages and cancer. Performed by White et al. [22] the studies have shown possible pathways associating cytogenetic damages leading to melanoma development. Cytogenetic investigation of untreated uveal melanoma has shown that the most frequent abnormality was monosomy 3, which occurred in approximately 60% of cases. The karyotype of the pigmented tumor revealed monosomy 3, whereas the nonpigmented tumor showed two apparently normal chromosomes 3. Molecular

studies on DNA that was extracted from normal lymphocytes and separately from both areas of the tumor supported authors hypothesis that the abnormality of chromosome 3 played a central role in the molecular pathogenesis of uveal melanoma and that some melanomas developed acquired homozygosity (isodisomy) by loss and duplication of the remaining, presumably abnormal, chromosome 3. Presented results of our studies also suggest that some of the cytogenetic damage present in the cells may be associated with consequent abnormalities in ploidy level.

It is well known that UVC-radiation (λ=254nm) cause pyrymidine dimmers and 4,6 pyrimidine photoproducts [13]. UV irradiation itself doesn't induce DNA breaks, which could be directly detected by the Comet assay though, DNA damages are produced in cellular repair process during incubation. An early step in repair is the incision of DNA close to a lesion (approximately 14 nucleotides) by a cellular enzyme (i.e. UV endonuclease). It is possible to follow the kinetics of repair process from incision through repair synthesis to ligation [13]. In the presented paper, the aim of whis is to study individual repair capacity variation, we have used a SCGE assay to estimate the influence of exposure and incubation on DNA damages level and their subsequent repair capacity in UV irradiated lymphocytes. The data obtained by Genter et al. [14] showed that PHA-induced differentiation of human lymphocytes leads to an increase in the intensity of repair after UV-irradiation of these cells and the repair is completed in a shorter time. Our former results showed statistically significant influence of the LF-7 on the repair of the DNA damages induced with different doses of UV. The data presented above showed statistically significant higher response to UV and lower capability to repair UV induced damage in skin cancer patients than in healthy donors. On average, no difference between control and skin cancer patients in the induction of DNA damage by X-rays was observed, though correlations between the highest cytogenetic damage detected in lymphocytes of skin cancer patients and the lowest capability to repair DNA damage was observed. Elavathil et al. [11] investigated the prognostic value of tumor cell DNA content determined with flow cytometry. Multivariate analysis with the stepwise Cox proportional hazards regression model did not confirm a cellular DNA content as an independent prognostic factor. However, the age at diagnosis, the largest tumor dimension and the presence of spindle vs. the mixed or epithelioid cell pattern were significant predictors of death from uveal melanoma. Our results show that UV induced damage repair capacity measured by the Comet assay could be a good predictive assay, and much faster and probably more accurate than classic cytogenetics.

Acknowledgments

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