Evaluation of genomic instability and cancer prevention

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OBJECTIVES: This study aimed at verifying the damage index acquired from the environment and from an inherited condition in the leukocytes of workers occupationally exposed to X-radiation and antineoplastic drugs, patients with Down syndrome, Fanconi anemia, and controls. MATERIAL AND METHODS: Cytokinesis-block micronucleus assay (CB-MN) and single-cell-gel electrophoresis (SCGE) were employed in 22 workers potentially exposed to X-radiation and 22 controls matched for age, sex, and smoking habits from a hospital in southern Brazil. The same evaluation was employed in 12 individuals who had been occupationally exposed to antineoplastic drugs and in 14 patients with Fanconi anemia (FA), 30 with Down syndrome (DS), and 30 controls, in order to examine the sensitivity of the techniques to detect specific genome instability. RESULTS: Both CB-MN and SCGE showed increased genetic damage in the cells of exposed individuals. In individuals handling antineoplastic drugs, no statistically difference was found when using CB-MN; however, the mean value of SCGE was significantly higher in exposed individuals when compared to controls. Down syndrome presented an increase just in the SCGE technique; the frequency of micronuclei and dicentric bridges was similar to that found in controls. Both CB-MN and SCGE showed increased genetic damage in the cells of individuals with Fanconi anemia. The high frequency of micronuclei seems to be due to clastogenic events, since the frequency of dicentric bridges was also elevated. DISCUSSION: Both methods are efficient for monitoring mutagenic events in exposed populations or individuals presenting genetic instability. CB-MN represents a longer time of exposure, while SCGE detects momentary DNA damage and/or repair activity. The combination of both techniques is recommended to monitor chronically exposed populations. Changes in lifestyle may constitute an important way of preventing carcinogenesis, either in individuals presenting increased risk and in the general population.

Key-words: Antineoplastic agents; Down syndrome; Fanconi anemia; cytokinesis-block micronucleus assay; single-cell-gel electrophoresis.

Avaliação da instabilidade genômica e prevenção de câncer
OBJETIVOS: Este estudo teve como objetivo avaliar o nível de mutagênese em indivíduos normais não expostos, em trabalhadores expostos à radiação ionizante e drogas antineoplásicas e em indivíduos portadores de doenças genéticas, comparando os níveis de mutagênese herdada com aqueles adquiridos por exposição a mutágenos.

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MÉTODOS: A técnica de micronúcleo em linfócitos do sangue periférico e a técnica do cometa ou eletroforese em célula única foram empregados em 22 trabalhadores potencialmente expostos à radiação X, 12 potencialmente expostos a drogas antineoplásicas, 34 controles adultos, 14 pacientes com anemia de Fanconi (AF), 30 com síndrome de Down (SD) e 30 controles infantis, do Hospital de Clínicas de Porto Alegre.

RESULTADOS: As duas técnicas mostraram um aumento no dano genético em células de indivíduos expostos a radiação-X. Nos indivíduos que manuseiam drogas antineoplásicas, não foi encontrada diferença significativa com a técnica de micronúcleo; no entanto, o índice de dano avaliado pela técnica do cometa foi significativamente maior em indivíduos expostos em relação a controles. Pacientes com síndrome de Down apresentaram um aumento no índice medido pela técnica do cometa; a freqüência de micronúcleos e pontes dicêntricas foi semelhante ao valor encontrado em controles. Tanto a técnica de micronúcleo como a técnica do cometa mostraram aumento de dano genético nas células de indivíduos com anemia Fanconi. A alta freqüência de micronúcleos parece ser devida a eventos clastogênicos, uma vez que a freqüência de pontes dicêntricas também se encontrava elevada.

DISCUSSÃO: As duas técnicas são eficientes na monitoração de eventos mutagênicos em populações expostas ou em indivíduos que apresentam instabilidade genética. A técnica de micronúcleo representa um tempo maior de exposição, enquanto que a técnica do cometa detecta dano momentâneo ao DNA e/ou atividade de reparo. A combinação das duas técnicas é recomendada para monitorar populações cronicamente expostas. Mudanças no estilo de vida podem constituir uma forma importante de prevenir carcinogênese, tanto em indivíduos que apresentam risco aumentado como na população em geral.

Unitermos: Agentes antineoplásicos; síndrome de Down; anemia Fanconi; técnica de micronúcleo; técnica do cometa.

Introduction

The mutagenic effect of ionizing radiation has been extensively studied (1-4), and an increase in chromosomal aberrations has been observed in occupationally exposed workers when the permissible levels of radiation are exceeded (5).

Antineoplastic drugs comprise chemicals that block the cellular growth, resulting in the death of dividing cells; as neoplastic cells are in active growing state, they preferentially die. Antineoplastic drugs include alkilants (e.g. cyclophosphamide), antimetabolic agents (e.g. fluorouracyl), spindle poisons (e.g. vincristine), antibiotics (e.g. doxorubicin), and hormones (e.g. diethylstilbestrol). These drugs are in general teratogenic and are also frequently mutagenic. The mutagenic effects are mainly confirmed by chromosome mutation studies in patients submitted to acute or chronic treatment with antineoplastic drugs.

Among the individuals occupationally exposed to mutagenic and carcinogenic agents, the group handling antineoplastic drugs deserves special attention. Waksvik et al. (6) and Nikula et al. (7) reported a significant increase in structural chromosome aberrations in the lymphocytes of nurses handling antineoplastic drugs. An increased frequency of sister-chromatid exchange was also observed (8,9). Machado-Santelli et al. (10) observed an increased number of micronucleated exfoliated cells in the buccal cavity of nurses handling antineoplastic drugs. In other studies, no differences were observed between controls and workers exposed to antineoplastic drugs regarding the frequency of sister-chromatid exchanges and chromosome aberrations (11-14).

Individuals with Down syndrome (DS) or trisomy 21 are known to be at high risk for malignant diseases (15,16), and their cells are reported to exhibit increased sensitivity to various

mutagens, e.g. radiation (17-20), chemicals (21,22), and viruses (23).

Fanconi anemia (FA) is inherited as an autosomal recessive trait, and its outstanding clinical manifestations are generalized failure of the bone marrow leading to pancytopenia, major anatomical defects, especially of the radius, thumb, and kidney, mild mental and growth retardation, and patchy brown pigmentation of the skin (24,25). Elevated spontaneous chromosome breaks and rearrangements (26-28), in addition to mitotic disturbances, such as anaphase bridges, aberrant fragments, and micronuclei (29) are seen in almost all FA patients. The diagnosis can be made unequivocally by combining clinical data with cytogenetic evaluation of the frequency of aberrations induced by DNA cross-linking agents such as diepoxybutane or mitomycin C (27,30).

Micronuclei (MN) are expressed mutations and need cell division to appear. The concomitant analysis of dicentric bridges when determining micronucleus frequency does not involve much extra work, and may serve as a reference to the type of mechanism of mutagenesis (clastogenic or aneugenic). The damage detected by single-cell-gel electrophoresis (SCGE) assay (or comet assay) is repairable and does not need cell division; under alkaline conditions, it assesses double and single-strand breaks and alkali-labile sites. Although, in general, the comet assay may be considered as a more sensitive method to assess DNA damage, different mechanisms are involved when positive results appear in the MN test or the comet assay. Combination of the alkaline SCGE and the cytokinesis-block micronucleus assay (CB-MN) seems to be recommendable to assess genetic instability.

Materials and methods

The study was carried out at Hospital de Clínicas de Porto Alegre, a university hospital in the city of Porto Alegre, Brazil. The total sample consisted of 22 individuals who had been occupationally exposed to low levels of X-ray, 12 individuals who had been occupationally exposed to antineoplastic drugs, 34 nonexposed control individuals matched for age, sex, and smoking habits, 14 FA patients, 30 DS patients, and 30 infant control individuals. In X-ray-exposed individuals, exposure was measured by personal $CaSO_4$ thermoluminescent dosimeters. All

individuals answered the personal health questionnaire published by the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) (31).

Peripheral blood samples were collected and directly used in the CB-MN and comet assays. For CB-MN, cytochalasin B (Cyt B, Sigma) was added to the lymphocyte culture at 44 h (4.5 mg/ml) (32,33). Cells were fixed in 3:1 methanol:acetic acid without hypotonic treatment, and the suspension was dropped onto clean slides and stained with Giemsa. Two thousand binucleated cells per individual were assessed for the presence of micronuclei and dicentric bridges (nucleoplasmic bridges between daughter nuclei) (34).

A standard protocol for the comet assay was applied according to Speit and Hartmann (35). The slides were prepared by mixing 5 ml of whole blood with 90 ml low melting point agarose (0.5%). Immediately after the addition of low melting point agarose to a microscope slide precoated with agarose, a coverslip was placed onto the slide, and the tray was kept in the refrigerator for 5 min until the agarose layer hardened. After that, the coverslip was gently removed, and the slide was lowered into cold, freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Na Sarcosinate, to which 1% Triton X-100 and 10% DMSO had been added). After at least 1 hour at 4 ℃, the slides were gently removed from the lysing solution, and the excess liquid was blotted away on the back and edges. The slides were then placed at 4 ℃ on an horizontal gel box filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) until the liquid completely covered the slides, which were then left in the tray for 30 min before the power was turned on. Electrophoresis was carried out for 20 min at 25 V (0.9 V/cm), 300 mA. All the steps described above were carried out under red light to avoid the induction of DNA damage.

After electrophoresis, the slides were gently removed from the tank, and neutralizing buffer (0.4 M Tris, pH 7.5) was added drop-wise to the slides three times at 5-min intervals. The slides were drained, and 70 ml ethidium bromide (40 mg/ml) was added. A coverslip was then placed onto the slide. The analysis was performed immediately after the staining. For evaluation of DNA damage, 50 cells per subject were analyzed at a magnification of 200 X under a fluorescent microscope (Zeiss), equipped with a 560 nm

excitation filter and a 590 nm barrier filter. The cells were visually classified into five categories, according to tail intensity, from undamaged (=0) to maximally damaged (=4) (36). Therefore, the total score for each subject (50 cells) could range from 0 (all undamaged) to 200 (all maximally damaged).

Statistical differences between controls and treated samples were determined with the non-parametric Mann-Whitney *U*-test. The Spearman test was used to analyze correlations.

Results

For the group exposed to X-radiation, both CB-MN (P=0.008) and alkaline SCGE ($P = 0.001$) showed increased genetic damages in cells of exposed individuals. The frequency of dicentric bridges was analyzed and also showed a significant increase ($P = 0.016$). A statistically significant correlation was detected between age and genetic damage in both micronucleus frequency and comet values.

For the group of workers handling antineoplastic drugs, no statistical difference was found for micronucleus and dicentric bridge frequency between exposed individuals and controls ($P = 0.129$ and $P = 0.373$, respectively). However, the mean value of SCGE was significantly higher in the exposed group than in controls ($P = 0.0006$).

Finally, when CB-MN and SCGE were employed in leukocytes from 14 FA, 30 DS, and 30 control individuals, DS presented an increase just in the SCGE technique ($P < 0.001$); micronucleus and dicentric bridge frequencies were similar to those found in controls. Both CB-MN and SCGE techniques showed increased genetic damage in the cells of individuals with FA: all results were statistically significant, with $P < 0.001$.

The high frequency of micronuclei seems to be due to clastogenic events, since the frequency of dicentric bridges was also elevated. The micronucleus frequency was significantly correlated with dicentric bridges ($P < 0.001$) and with the damage index from comet assay ($P = 0.011$) (Spearman Correlation Test). The results of the comet and CB-MN assays in subjects occupationally exposed to X-radiation and antineoplastic drugs and in DS and FA patients are summarized in table 1.

Discussion

Increased frequencies of chromosome aberrations and micronuclei are well known among individuals occupationally exposed to ionizing radiation (5,37-39). Qualities of the radiation to which populations were exposed differed in most of the studies, and there was frequently more than one kind of radiation involved. Chromosome abnormalities have been described in populations exposed to low doses of radiation, and doses ranged from zero to 500 millisieverts (mSv) (37,40-42). In the present study, we investigated workers exposed only to X-radiation, and the accumulated dose range was from 0.2 to 121.8 mSv. We did not find any correlation between genetic effects and the doses recorded by the dosimeters. This result is in agreement with a previous study (42), in which 26 workers were studied and the accumulated dose ranged between 2.3 and 131.7 mSv.

The CB-MN assay has the advantage of detecting both acentric chromosome fragments due to DNA breakage during interphase and chromosome loss resulting from chromosome lagging during anaphase. Micronucleus formation can indicate damage caused by an exposure occurred long before blood was withdrawn for analysis. The concomitant analysis of dicentric bridges in the micronucleus test does not cause much extra work, and may serve as a reference to the type of mutagen (clastogenic or aneugenic). As expected, the significantly increased frequency of dicentric bridges in the present study suggests a clastogenic action in individuals exposed to X-radiation.

Under the alkaline conditions used here, the comet assay detected double and single-strand breaks, as well as alkali-labile sites. The increased comet values in the peripheral blood of radiological workers exposed to X-ray found in the present study indicate a higher level of radiation-induced primary DNA damage. Although the comet assay may be considered more sensitive for the assessment of induced DNA damage, the micronucleus test has the advantage of detecting the manifestation of damages, such as chromosomal aberrations. Some previous studies already comparatively investigated these two endpoints (43-45), but none of them was carried out *in vivo*. The high values of standard deviation found in the comet assay in the present

study elucidate the high interindividual variation of this technique (46,47). While the evaluation of micronuclei is limited to lymphocytes, the comet assay detects damage in all leukocytes that constitute a heterogeneous mixture of cells. Lifespan of subpopulations may vary from weeks to decades (lymphocytes), while other cells have a short half-life ranging from 7 to 24 hours (granulocytes) (47).

In the case of chronic exposure, comparing the levels of damage revealed in a cytogenetic technique with those found in the comet assay may provide information about concurrent *versus* past exposure levels. The micronucleus frequency represents a longer time of exposure with a cumulative effect, while the comet assay detects momentary DNA damage and/or repair activity. In the present study, blood samples were collected during the work interval, and the comet assay effects on exposed individuals might reflect concurrent exposure.

Antineoplastic drugs include unrelated chemical agents that can inhibit tumor growth; the most commonly used include clastogenic and aneugenic drugs. The group exposed to antineoplasic drugs presented a frequency of micronuclei and dicentric bridges similar to the control group. However, when SCGE was applied to the same blood samples, increased values were detected in exposed individuals. SCGE does not require cell division. This damage may have originated minutes or hours before the blood was withdrawn for analysis. Repair of this damage is cell-type dependent.

Workers handling antineoplastic drugs can be exposed through inhalation of aerosolized drugs, transdermal absorption, and accidental ingestion. Increased genetic damage in workers occupationally exposed to antineoplastic drugs has been related with careless handling of these substances (10,48-51). In the present study, pharmacists and nurses have used safety covers and have followed strict guidelines to work with antineoplastic drugs.

Smoking was not a frequent habit in our subjects. This fact could explain that there was no correlation between smoking habit and detection of genetic effects. The increase in micronucleus frequency in relation to age in the present study is in agreement with other studies (33,52,53). A significant increase in the comet values in relation to age was also found. This result

is in disagreement with some studies using comet assays to monitor human populations (54,55). However, Singh et al. (56) reported an age-related increase in the levels of DNA damage in lymphocytes of the blood of non-smoking subjects, using a procedure with increased sensitivity of the pH>13 assay by increasing the duration of electrophoresis from 20 to 40 minutes. When the exposed and control groups were tested separately, the unexposed individuals did not show a correlation with age. When the cytogenetic endpoints were compared with years of employment, no correlation was found. These results may indicate a greater sensitivity to X-radiation in older individuals.

DS is considered a premature aging syndrome, and it has been compared with other disorders like Werner syndrome, Cockaine syndrome, and ataxia-telangiectasia. Children with DS are generally known to present a higher risk for developing leukemia. It could be shown that by triplication of a special fragment of chromosome 21, the dosage of Cu/Zn superoxide dismutase is elevated, resulting in a disturbance of the very sensitive balance of enzymes responsible for oxygen radical metabolism (20,57). Radicals damage the DNA and gradually exhaust the repair capacity of cells, with consequent chromosome breaks and micronucleus accumulation; this causes mitosis impairment and delay in the cell cycle. Thus, similarly to other diseases with completely different genetic backgrounds, via the impairment of DNA repair, DS leads to the common symptoms of premature senescence. At the chromosomal level, sensitivity of cells of DS patients to X-ray exposure is elevated in relation to cells of normal controls, although the ratio of sensitivity found in each study varies (17-20).

The results of the analysis of micronuclei and dicentric bridges in individuals with DS was similar to controls. Weirich-Schwaiger et al. (58), in a study of senescence and DNA repair, demonstrated that in young individuals with DS, the spontaneous micronucleus frequency was similar to young controls, but they observed a striking increase of micronucleus frequency at the final stages of cells growing in vitro. In contrast, the number of chromosome breaks did not follow this increase, which shows a possible age-related susceptibility to aneugenesis in these cells. The sensitivity ratio, measured by chromosome aberrations, has been

Table 1. Mean frequencies of micronuclei (MN), dicentric bridges (DB), and comet assay damage scores (SCGE) in groups exposed to X-ray and antineoplastic drugs, Down sindrome and Fanconi anemia patients, and controls

Group	n	Mean age	MN[2]	DB[2]	SCGE[1]
Exposed to X-radiation	22	37.18	17.68±4.69	5.95±3.14	17.73±10.51
Controls	22	32.5	14.36±5.18	3.91±2.07	8.54±7.11
Exposed to antineoplastic drugs	12	34.75	13.50±6.05	2.83±1.85	20.83±10.19
Controls	12	34.42	11.08±5.96	2.75±2.67	8.08±5.16
Down syndrome	30	0.72±1.80	10.17±3.64	2.77±1.65	13.4±8.21
Fanconi anemia	14	9.74±6.10	21.71±3.38	7.57±1.70	26.50±22.38
Controls	30	3.54±4.86	9.30±4.47	2.73±1.31	8.67±11.65

[1] DS was calculated by adding the score (0 to 4) of each of the 50 cells analyzed (maximum per individual: 0; minimum: 200).
[2] In 2000 binucleated cells.

suggested to decrease with age (59). Such behavior is important to consider, because in the present study, the sample was formed by very young individuals (mean age = 0.72±1.80), and the DNA damage index found in the comet assay was higher when compared to controls.

FA is characterized as a DNA repair disorder, since cells derived from patients are hypersensitive to DNA cross-linking agents. Although the primary defects in FA patients are not known, biochemical evidence supports either a direct defect in the removal of DNA cross-links or a defect in the ability of cells to respond to oxidative stress, as a result from the interaction with cross-linking agents. The localization of chromosome aberrations in FA shows a non-random pattern in the breakpoints correlated with the chromosomal location of fragile sites, oncogenes, and points involved in cancer-rearrangements, suggesting a possible relationship with the high predisposition to cancer observed in this disease (60,61).

Fenech et al. (1) have evaluated the performance of the CB-MN assay as an in vivo dosimeter in a study with cancer patients undergoing fractionated partial body radiotherapy obtaining a dose-related increase in MN in all the patients studied. The high frequency of micronuclei seems to be due to clastogenic events, since the frequency of dicentric bridges was also elevated in the same order of the micronuclei (>2x). FA patients also presented a proportionally higher DNA damage index in SCGE than in the CB-MN assay.

In short, our study demonstrates the presence of genotoxic effects in workers exposed to X-irradiation with two independent genetic endpoints and indicates that the combination of the comet assay with the micronucleus test may be useful for the monitoring of populations chronically exposed to genotoxic agents. Avoidance of environmental stress on genetically predisposed patients may finally result in an increase in individual life expectancy.

Genetic damage, inheritance and environment

The different characteristics between the micronucleus test and the comet assay show the importance of this concomitant analysis both for quantitative studies and for understanding the mechanisms that form the alterations and action of DNA repair. Workers exposed to mutagenic agents have an inherited capacity to repair DNA damage; this capacity is polymorphic and makes individuals more or less sensitive to exposure to mutagenic agents. Individuals carrying unfavorable genetic heritage concerning the maintenance of genomic stability may have a high frequency of genetic alterations even if they are apparently not exposed to mutagenic agents or if they are exposed only to

agents commonly present in the environment. The two assays used in this study provided information regarding the number and type of alterations found in these two groups of individuals.

Daily use of antimutagens and anticarcinogens will probably be the most effective procedure to prevent cancer. There are several ways of preventing or reducing mutagenesis. Chemical agents that act in the repair of DNA damage or in the metabolism of mutagenic agents may be effective. Several examples illustrate that antimutagenic effects are often specific for certain types of mutagens and/or tests. This way, if antimutagens have an impact on human diseases, it is essential that they be specifically directed against the most common mutagens found in the environment, according to the habits and activities of each one of them (62).

Carcinogenesis is related to genetic changes in somatic cells produced by mutagens and carcinogens. The molecular mechanisms of human carcinogenesis are very complex, involving multiple genetic changes in established cell lineages; these alterations are usually more numerous in cells of more malignant tumors. The carcinogenic process requires a long time to be completed, and precancerous changes may persist for many years. Recent findings on the complex alterations observed in human cancer, the presence of new genotoxic substances in our daily life, new promoters of cancer, and new precancerous changes are important advances that serve as basic information to consider ways of preventing cancer and developing recommendations for the improvement of life quality (63).

A lot of attention has been given to studies that suggest the involvement of active oxygen and free radicals in an array of pathological events, cancer, and some aging processes. Oxygen is indispensable for aerobic organisms; however, it is also believed to be responsible for undesirable effects. Species of oxygen such as hydrogen peroxide, superoxide radical, simple oxygen, and other radicals are considered agents that attack polyunsaturated fatty acids in the cellular membrane, leading to a lipid peroxidation. Several studies have suggested that lipid peroxidation may result in destabilization and disintegration of the cellular membrane, leading to problems in the liver, some diseases, aging, and susceptibility to cancer (64).

Lipid peroxidation is a problem related not only to the food-processing industry, but also to the human body itself. However, in living systems, certain enzymes and endogenous antioxidants are used to inhibit peroxidation processes by removing active oxygen, reducing peroxides, and capturing radicals. There are also several compounds in nature that act as antioxidants, and are believed to inhibit carcinogenesis and delay aging (65).

A great number of metabolites extracted from vegetables and used in our diet were tested in terms of their antimutagenic capacity. In microbial tests, several of them showed to inhibit the mutagenic effect of toxic elements (66).

Vitamin C, at a given concentration, diminishes the frequency of sister-chromatid exchange in lymphocytes of the human peripheral blood. The effect of vitamin C as an inhibitor of mutagenicity for various carcinogens is attributed to its antioxidant action. Some studies have also emphasized the anticarcinogenic effect of vitamin C, which is an effective protector against the carcinogenic activity of ionizing radiation and chemical agents. Vitamin C is the most effective agent in the inhibition of mutations induced by ethylmethanesulfonate (EMS). In the presence of vitamin C at a concentration of 100 mg/ml, EMS-induced mutations were reduced to almost one-fourth of the frequency found in control cultures treated only with EMS (67).

Fiber-rich diets may protect the organism against some types of carcinogenic agents through the their absorption; this way, these agents are expelled from the digestive tract. A group of mutagens identified in roast beef, fish, chicken, and grain products (68,69) may have their effects neutralized in the presence of a fiber-rich diet (62).

The levels of most environmental carcinogens, including heterocyclic amines formed in the preparation of some food items, are much below the minimum level necessary to induce cancer in laboratory animals. However, cancer development is the final result of multiple sequential genetic transformations. There is still much to be understood about cancer prevention, but interesting results have been observed as a result of changes in lifestyle. It should be noted that some types of cancer have disappeared. Cases of cancer in X-ray workers caused by exposure to ionizing radiation are also extremely rare (63). Some changes in the lifestyle of people daily exposed to mutagens should be adopted. The following measures should be observed in order to prevent cancer:

1. Follow a balanced diet, avoiding excess of calories and fat.
2. Avoid excessive consumption of alcohol.
3. If you are a smoker, smoke as little as possible, or try to stop smoking.
4. Avoid excessive exposure to sunlight.
5. Do moderate physical exercises regularly.

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