Persistence of Genetic Damage in Lymphocytes from Former Uranium Miners

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Key Words

Alpha radiation • Genomic instability •
Micronucleus-centromere test • Radon

Abstract

Lymphocytes from former uranium miners who finished work underground one or more decades ago were analysed with respect to possibly persisting genetic damage induced by their radiation exposure. A modified micronucleus-centromere test was used which determined the frequency of micronucleus-containing binucleate cells after cytochalasin B treatment and the percentage of centromere-free micronuclei, assessed with the help of immunofluorescence labeling of centromere protein B. Whereas the overall frequency of micronucleus-containing cells was not significantly elevated above the level found in a control group, former miners showed a greater percentage of centromere-free micronuclei, i.e. micronuclei containing only acentric fragments. Our results are in excellent agreement with those of an earlier uranium miner study and lend support to the assumption that genetic damage from alpha radiation can persist for many years after exposure, possibly due to genomic instability. The frequency of micronucleus-containing cells, but not the percentage of centromere-free micronuclei, significantly increased with time since last exposure in the mines. This can

be attributed, at least in part, to the fact that miners who have finished working underground longer ago tend to be older, and there is an increase of the frequency of micronucleus-containing cells with age.

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Of the different kinds of genetic damage used as markers for past exposure to chromosome breaking agents, so-called clastogens, some are stable and can therefore be detected even decades after exposure, whereas others are unstable and lose their detectability within a few years. To the first category belong chromosomal translocations which are either assessed with the help of chromosome banding [Awa, 1997] or after fluorescent in situ hybridization (FISH), also referred to as ‘chromosome painting’ [Lucas, 1997; Hande et al., 2005]. Translocations have, for instance, been found in the lymphocytes of atomic bomb survivors of Hiroshima and Nagasaki, and it has been shown that their frequency does not substantially change with time [Kodama et al., 2001]. The interpretation is that these types of damage are also induced in the bone marrow stem cells, and as they do not prevent cell division, lymphocytes containing translocations are produced again and again. In the second category – the unstable clastogen induced genetic damage – there are, for in-

stance, dicentric chromosomes and rings [Natarajan, 2002] or micronuclei [Müller and Streffer, 1994]. The frequency of lymphocytes containing this type of damage declines after exposure. There is some debate about the exact timing, but the half-life is most often found to be between 1 and 3 years [Chang et al., 1999; Natarajan and Kesavan, 2005; Thierens and Vral, 2009].

In some instances, genetic damage of the unstable type has been reported quite long times after exposure. For instance, Lee et al. [2002] found an overall decline of micronucleus frequency with 13 patients having undergone radiotherapy, but with 7 of them, the micronucleus frequency remained higher than before treatment for more than 6 years. This was presented as evidence of 'persistence' of this type of genetic damage but is still not incompatible with an average half-life of (damaged) lymphocytes of 1–3 years. Results with longer follow-up times have been reported for clean-up workers involved in the Chernobyl accident. Slozina et al. [2001] as well as others cited by them found the frequency of lymphocytes containing dicentrics and rings increased rather than decreased with time up to 12 years after exposure. Their data are in disagreement with those reports suggesting that damaged lymphocytes disappear from the peripheral blood with a half-life of 1 or 3 years, but it should not be dismissed easily especially as they come from several different laboratories.

Of special relevance for the current communication are 2 other studies. Mészáros et al. [2004] reported about persisting chromosomal damage in the form of dicentrics and acentrics in Hungarian uranium miners many years after they finished underground work. In the category of miners who finished 14–23 years ago, the frequency of dicentrics was still above 50% of the original value (no statistically significant difference), whereas the frequency of acentrics was around 25% of the original value (a highly significant reduction, but still more than would be expected assuming a half-life of 1–3 years). In another study on German uranium miners, Kryscio et al. [2001] did not find any enhanced frequency of micronucleus-containing lymphocytes 7–30 years after they finished underground work, but there was a higher percentage of micronuclei originating from acentric fragments. These micronuclei can be recognized after FISH with a centromere-specific DNA probe [Norppa et al., 1993; Vral et al., 1997].

Here, we report on a similar study conducted on Czech uranium miners who finished underground work 10–48 years ago. We were interested in repeating the analysis of Kryscio et al. [2001] but employed a modified micronucleus-centromere test which used immunofluorescence

labeling of centromere protein B instead of FISH with a centromere-specific DNA probe [Bejarano et al., 1993]. We compared our results of the miners with those of unexposed control persons, giving special consideration to the age-dependence of both the frequency of micronucleus-containing cells and the percentage of micronuclei containing only acentric fragments.

Material and Methods

Samples

Samples of heparinized blood were obtained by venipuncture from 60 miners from the area around Příbram in Central Bohemia where uranium mines were closed down in the early 1990s. Some workers, nevertheless, finished work underground as late as 10 years ago, as they were involved with the deinstallation activities. The workers' age was between 42 and 79 with a median of 50. Samples were also taken from 52 control persons (students and co-workers of the Faculty of Health and Social Studies in České Budějovice and the Faculty of Biomedical Engineering in Kladno, co-workers of the National Institute of Chemical, Nuclear and Biological Protection in Milín-Kamenná, and residents of a senior home in České Budějovice). The age in this group varied between 20 and 89 with a median of 55. None of the donors had any history of cancer. Transport to the laboratory at Milín-Kamenná took about 0.5 h from Příbram and 1.5 h from České Budějovice or Kladno. Information about age, kind of work, duration of work under exposure conditions, smoking habits etc. was gathered in interviews. Informed consent was obtained in written form from each participant on the study. Some personal dosimetry data of the miners were provided by the employer (DIAMO s.p., 100% owned by the Czech Ministry of Industry and Trade).

Cultivation of Blood Cells

Each sample was divided into 0.5-ml aliquots and topped up to 5 ml each with cultivation medium (RPMI 1640 with 20% fetal calf serum; both Gibco-Invitrogen, Paisley, Scotland). In order to stimulate lymphocyte proliferation, 15 μ l/ml phytohemagglutinin (Gibco-Invitrogen) was added. After 44 h of incubation at 37°C and 5% CO₂, 1.5 μ l/ml Cytochalasin B (Sigma, Deisenhofen, Germany) was added (final concentration 5.6 μ g/ml). Cultivation was continued for another 24 h under the same conditions. This procedure was first described by Fenech and Morley [1985].

Preparation of Slides

Cell suspensions were centrifuged at 1,000 rpm for 10 min. The supernatant was carefully sucked off and the sediment taken up in 5 ml lysis buffer (10 mM EDTA, 155 mM NH₄Cl, 10 mM KHCO₃) [following Zeni et al., 2003]. After whirling up the pellet, the cell suspension was immediately centrifuged at 1,000 rpm for 6 min. This sequence (sucking off the supernatant, adding lysis buffer, whirling up the pellet, centrifugation) was repeated once so that the overall time in lysis buffer was about 15 min. The cells were resuspended in (serum free) medium, centrifuged again at 1,000 rpm for 10 min, and taken up in hypotonic solution (medium/distilled water 1:3) for 3 min [following Thomson and Perry, 1988]. Cells were then cytospun onto uncoated slides at 1,200

rpm for 7 min (Rotofix32, Hettich, Tuttlingen, Germany). Fixation was carried out on the slides with 1% paraformaldehyde for 5 min at room temperature.

Immunostaining

Slides were dipped in 0.1% Triton X-100 for 5 min for permeabilization and incubated with an antibody against CENP-B (host: rabbit, H65, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) 1:50 diluted in 0.1% Triton X-100 in PBS for 1 h at room temperature. After rinsing 3 times with PBS, they were incubated with Alexa Fluor 488 goat anti-rabbit (Molecular Probes-Invitrogen, Paisley, Scotland) 1:500 in 0.1% Triton X-100 in PBS for 1 h at room temperature. Cell nuclei were counterstained with DAPI. Finally, the slides were rinsed 3 times with distilled water and left to dry for 10 min [following Abend et al., 1995]. Cover slips were mounted with the help of ProLong Gold anti-fading medium.

Scoring and Statistical Analysis

The slides were scored under a fluorescence microscope Olympus IX71 with a 40× objective and 400× total magnification. A total of 500 binucleate cells were scored per donor. The scoring was done 'blind', i.e. without the scorer knowing to which subgroup the donor belonged. Results are given below as means and standard deviations if not stated otherwise. Statistical differences were assessed using the Student's t-test as available online (<http://in-silico.net/statistics/ttest/two-sample>). For the linear regression analysis, the LAB Fit Curve Fitting Software (V 7.2.39, Wilton and Cleide P. Silva) was used. It provides 95% confidence intervals for the best-fit regression line.

Results

Of the 60 blood samples from miners, 40 were of sufficient quality to determine the frequency of micronucleus-containing binucleate cells. Of the 52 samples from control persons, 42 could be used. The results were between 0 and 2 cells with a micronucleus per 100 binucleate cells, with an average of 0.63 ± 0.59 for the miners and 0.50 ± 0.42 for the controls. The number of micronuclei per 100 cells was 0.69 ± 0.56 for the miners and 0.54 ± 0.46 for the controls. This level overall compares reasonably well with data in the literature, although it seems to be in the lower range of what has been reported by others (for a review, see Müller and Streffer [1994]: 0.5–2.0). The difference between miners and controls was not significant ($p > 0.05$).

If the micronuclei observed in the miners were induced by alpha radiation, we should expect their dispersion index to be higher than that in the controls. This would be due to a higher percentage of cells with more than 1 micronucleus. The average dispersion index, however, was the same for miners (1.10 ± 0.25) as for controls (1.12 ± 0.28).

With respect to micronucleus content, i.e. the presence or absence of a centromere signal, our results were not entirely satisfying, because, as indicated, we observed somewhat fewer micronuclei than expected from the literature, and moreover, the antibody labeling of centromeres gave unambiguous results in less than half the cases. Therefore, only relatively small numbers of micronuclei could be analysed with respect to the presence or absence of a centromere signal. Nevertheless, the average percentage of unlabeled micronuclei, i.e. micronuclei containing only acentric fragments, was clearly greater in the miners than in the controls: 34 ± 38 versus $12 \pm 21\%$, and this difference was significant ($p < 0.05$).

When we calculated the frequency of unlabeled micronuclei by multiplying the frequency of micronuclei with the percentage of unlabeled micronuclei, we again obtained significantly different results, namely 0.23 ± 0.24 for miners and 0.06 ± 0.12 for controls ($p < 0.05$). This led to very similar values, namely 0.46 ± 0.29 and 0.48 ± 0.18 , respectively, for the frequency of centromere-positive micronuclei.

It would be interesting, of course, to see whether the micronucleus frequency or content could in any way be related to radiation exposure in the mines. Unfortunately, the dosimetric records of this group of miners were so inconsistent and incomplete that it was impossible to derive reasonable estimates of exposure in about a third of the cases. We therefore looked at the duration of work underground as an indicator of exposure, but no significant correlation with the frequency of micronucleus-containing cells or the percentage of centromere-free micronuclei was found (fig. 1).

We further compared a number of subgroups of miners, such as hewers versus other workers and smokers versus non-smokers, but the differences were small and had no statistical significance ($p > 0.05$; data not shown in detail).

The only correlation that we found was one between the frequency of micronucleus-containing cells and the time since last exposure in the mines, i.e. since finishing work underground. This statistically significant ($p < 0.05$) correlation is shown in figure 2. At least part of this correlation could come from a certain age-dependence of the frequency of micronucleus-containing cells. The time since last exposure was very closely correlated with age ($p < 0.001$; data not shown in detail) as would be expected as miners started and finished work at similar ages, and age was a factor influencing the frequency of micronucleus-containing cells. The correlation between the frequency of micronucleus-containing cells and age was

Fig. 1. Relationships between the duration of work underground and the frequency of micronucleus-containing binucleate cells (left) or the percentage of centromere-free micronuclei (right).

Fig. 2. Relationship between the time since last exposure and the frequency of micronucleus-containing binucleate cells (left) or the percentage of centromere-free micronuclei (right).

statistically significant in the controls ($p < 0.05$; fig. 3a); in the miners, a similar age-related increase was observed but without significance ($p > 0.05$; fig. 3b). The increase was 0.0063% per year of age in the controls and 0.0083% per year of age in the miners.

The percentage of centromere-free micronuclei did not correlate significantly with either the time since last exposure of the miners or with the age of the controls or the miners (fig. 2, 3). The plots against age of course reflect the difference in the average percentage between the controls and the miners.

Discussion

The most important finding reported here is that of an enhanced percentage of centromere-free micronuclei in uranium miners who finished working underground

10–48 (median 22) years ago. No significant increase in the frequency of micronucleus-containing cells was observed. These results closely resembled those of Kryscio et al. [2001] obtained with former German uranium miners. Whereas our values were $34 \pm 38\%$ for the miners and $12 \pm 21\%$ for the controls, theirs were $38 \pm 2\%$ for the miners and $24 \pm 2\%$ for the controls. Of course, their statistics were much better as they could analyze more micronuclei; this may speak in favor of hybridization with centromere specific DNA probes instead of labeling with antibodies specific for centromere proteins. Nevertheless, the observation of a significantly enhanced level of centromere-free micronuclei has now been repeated in a different group of miners with a different technique.

It is not really surprising that we did not find any correlation between the duration of work underground and the micronucleus frequency or content. This parameter was introduced just as an indicator of exposure, whereas

Fig. 3. Relationships between the age at analysis and the frequency of micronucleus-containing binucleate cells (left) or the percentage of centromere-free micronuclei (right) in controls (a) and miners (b).

measured doses would have been more appropriate. As mentioned above, however, the dosimetric records of this group of miners were too sketchy to be of use. Moreover, the only measurement recorded was the exposure to short-lived radon decay products, whereas we know from the analysis of more recent data that this would account for only about 25% of the overall effective dose, other components being the external gamma exposure (20%) and the exposure to long-lived alpha emitters (55%) [Marušíaková et al., 2011; Zölzer et al., submitted for publication]. It should also be noted that no correlation between the frequency of micronucleus-containing cells and the radon exposure (given in working level month) was found in former German uranium miners either [Müller et al., 2004].

Our observation that the frequency of micronucleus-containing lymphocytes increases with time since last exposure, i.e. since finishing underground work, seems a bit irritating at first. It does not resemble the findings of Kryscio et al. [2001] who rather reported a small, although insignificant, decrease. Similarly, the dicentric and acentric data published by Mészáros et al. [2004]

showed a decrease, albeit less than expected under the assumption of a lymphocyte half-life of 3 years and not significant in the case of dicentrics. On the other hand, data from the Chernobyl clean-up workers, as summarized by Slozina et al. [2001], indicated an increase in unstable chromosome aberration frequency with time after exposure. As mentioned in the introduction, we think that these findings cannot easily be dismissed, even though they are not in agreement with the general thinking about the stability of the genetic damage in question.

In our case, it seems possible that at least part of the increase with time since exposure came from the age-dependence of the frequency of micronucleus-containing lymphocytes. The increase observed in the controls (0.0063% per year of age) was the same as that reported in the literature (for review see Müller and Streffer [1994]: 0.004–0.008% per year of age). Among the miners, a similar increase was observed (0.0083% per year of age). Although in this case the increase was not statistically significant, we consider it possible that miners who finished working longer ago had a higher frequency of micronu-

cleus-containing lymphocytes simply because they tended to be older. The fact that the percentage of centromere-free micronuclei is neither age-dependent nor dependent on the time since exposure would seem to be compatible with this assumption.

Finally, we would like to address the possible reasons for the persistence of genetic damage long after exposure. Kryscio et al. [2001] have interpreted their results as evidence for genomic instability induced by alpha radiation. That this phenomenon indeed occurs especially with densely ionizing radiation has been demonstrated in many cell culture [Khadim et al., 2010] and animal [Hei, 2011] studies. The finding of an enhanced level of damage typical for clastogen action many years after exposure could be evidence of the same effect in humans exposed to alpha radiation. A word of caution, however, seems to be in place. As pointed out, for instance, by Natarajan and Kesavan [2005], we do not know enough about different lymphocyte subpopulations to exclude the possibility of the existence of a relatively long-lived small subpopulation which would retain a 'memory' of past exposures. The relative importance of such a subpopulation would be expected to depend on the exact exposure conditions – whole body, bone marrow, and/or lung as in our case.

Kryscio et al. [2001] reported a higher percentage of unlabeled micronuclei in miners suffering from bronchial carcinoma than in those who had no tumor. This is in agreement with other reports in the literature about an association between the occurrence of cancer and elevated levels of either micronuclei [Iarmarcovai et al., 2008] or chromosomal aberrations [Bonassi et al., 2008] in the peripheral blood lymphocytes. A case-control study showed that chromosomal aberrations do not simply reflect the disease status of the patients but are early markers of later cancer development [Hagmar et al., 1998]. While a related study suggested that the association was unrelated to carcinogen exposure [Bonassi et al., 2000], a large investigation of uranium miners did show a correlation between elevated aberration frequency and cancer risk both due to radon exposure [Smerhovský et al., 2001]. In our case, as none of the donors had a tumor, this question could not be addressed. However, the hypothesis that alpha radiation induces genomic instability, which persists long after exposure and is related to a higher cancer risk, seems to fit all these findings, our own and those of Kryscio et al. [2001] as well as those of Smerhovský et al. [2001].

There is no evidence for a continued production of unstable chromosome damage in atomic bomb survivors [Kodama et al., 2005] or radiotherapy patients [Vinnikov

et al., 2010]. Stable chromosomal damage, e.g. translocations, remains of course detectable in these cases many years after exposure [Awa, 1997; Tawn and Whithouse, 2003]. It would therefore seem that only (or preferentially) densely ionizing radiation in particular is able to induce genomic instability. On the other hand, the persistence of dicentrics in Chernobyl clean-up workers, mentioned in the introduction [Slozina et al., 2001], speaks against such an assumption, as exposure in these cases stems mainly from sparsely ionizing radiation [Nuclear Energy Agency, 2002].

An alternative explanation is a possible effect of long-lived incorporated radionuclides, evidence of which has been found with people living in contaminated areas around Chernobyl [Livingston et al., 1997]. The estimated doses were small in these cases but might of course be much bigger in the case of clean-up workers. We would then have to assume that unstable chromosome damage was produced not by genomic instability (alone) but rather by continued radiation exposure. The same might be true for our uranium miners, but in neither case have data from whole-body counts been published which would support (or reject) the assumption of long-lived incorporated radionuclides.

Somewhat irritating in this context are the findings reported for former plutonium workers. They do not show any signs of persistently high levels of unstable chromosome damage [Whitehouse and Tawn, 2001] and thus seem to be a counterexample to the assumption of induced genomic instability as well as to the assumption of continued exposure. Differences in biokinetics may play a role in this context. One would have to know more about the long-term organ distribution patterns of plutonium on the one hand and of those radioisotopes important for uranium miners on the other, such as uranium, thorium, radium, lead, bismuth, and polonium [ICRP, 1992, 1995]. And one would have to calculate doses delivered to blood as these should be reflected in the chromosomal damage of peripheral lymphocytes. We admit that final conclusions cannot be drawn at the moment, and further studies are needed to address the discrepancies between the different studies mentioned.

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