Adaptive response to DNA and chromosomal damage induced by X-rays in human blood lymphocytes

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Nucleoid sedimentation, single-cell gel electrophoresis (comet assay) and premature chromosome condensation (PCC) technique were utilized to estimate the involvement of DNA strand breaks and chromosomal damage in radio-adaptive response of stimulated human lymphocytes. Conditioning of cells with 0.02 Gy X-rays rendered them more resistant to single- and double-strand DNA breaks produced by 1 Gy challenging treatment as revealed by the sedimentation behaviour of the nucleoids and the comet assay. Nucleoid sedimentation also demonstrated that adaptive reaction towards X-ray-induced DNA damage is favoured in the presence of oxygen. A concomitant decrease in the amount of interphase chromosomal breaks visualized by PCC under the same experimental conditions was observed. Data indicate that adaptation of human lymphocytes to X-rays is tightly linked to the reduced susceptibility towards generation of DNA and chromosomal breaks. It is proposed that the very persistence of DNA strand discontinuities might serve as a triggering signal for the adaptation of human lymphocytes against ionizing radiation exposure.

Introduction

Inducible repair processes as opposed to constitutive ones were initially detected and characterized in bacteria. One type of repair activity observed in Escherichia coli after treatment with alkylating agents was termed adaptive response (AR) (1). In general, this phenomenon occurs after treatment of the cells with a low dose of a clastogen. Such a conditioning provokes protective effect against the mutagen employed for the subsequent higher dose (challenging) treatment. Adaptive behaviour was found to be a characteristic feature of both mammalian and plant cells in their response to various mutagenic agents (2–4). Utilizing different biological end points adaptation to low level of alkylation, oxygen species and incorporated ³H-thymidine (³HdThd) or γ-rays has been reported (2,5–7).

The first report that cultured human lymphocytes exposed to low doses of radiation (either from the decay of the incorporated ³HdThd or from external X-rays) became refractory to the induction of chromosomal aberrations by a treatment with higher dose came from Olivieri et al. (8). Such a phenomenon has been attributed to the induction of chromosomal break repair. Radio-adaptive response in human lymphocytes was later analysed in a series of studies (9–15, for a review, see 16).

Several distinct features of the protective reaction of human lymphocytes against X-rays have been elucidated. As a rule, adaptation is triggered by a very low dose (the effective range is usually between 0.02 and 0.05 Gy) and manifested not earlier than 4 h after stimulation, i.e. it is not observed in dormant Go cells (17,18). AR is dependent not only on the rate of the initial damage but also on the time gap between adaptive and challenging treatments. AR was found to be effective for a relatively long time, approximately for three cell cycles (17,19). An important feature is also that cross-adaptation is reported for diverse types of initial and challenging treatments (20,21) and there is inter-individual variability among the donors (11,12).

Radio-adaptive response in rodent and human cells has been initially evaluated by the reduction in the frequencies of chromosomal aberrations (both of chromatid and chromosome type) as well as for sister chromatid exchanges (SCEs) and micronuclei induction (7,11,22–24). Up to now, however, the molecular basis of this reaction remains obscure. It was shown that AR is inhibited by 3-aminobenzamide and cycloheximide and there is de novo synthesis of several proteins in response to low-dose pretreatment (6,9,25). Although the character of the initial events, presumably DNA damage, is not yet elucidated, there are indications that after the initiating unidentified signal, a subset of components, including various protein kinases and early response genes regulating transcription machinery of the cell, are involved (26). A pivotal role of p53 protein in channeling of radiation-induced DNA double-strand breaks (DSBs) into adaptive repair pathways has been also proposed (27).

Premature chromosome condensation (PCC) is considered to be a reliable tool for studies of interphase chromosomes as they are visualized at different stages of the cell cycle (28). It is one of the most sensitive methods for monitoring initial chromosomal damage following exposure to ionizing radiation (29). As DNA strand scissions are among the major lesions induced from X-rays in DNA with the number of single-strand breaks (SSBs) being much higher than double-strand ones in a ratio of 30–40 : 1 within the low-dose range (30), we aimed our work on the role of DNA and chromosomal breaks in radio-adaptive response. Adaptation of phytohemagglutinin (PHA)-stimulated human lymphocytes was followed via monitoring of changes in DNA integrity by nucleoid sedimentation and comet assays. Chromosomal damage was visualized by PCC technique.

Materials and methods

Experimental material

Lymphocytes from two non-smoking healthy volunteers were obtained the day after the blood was collected from the Blood Bank, Leiden University Medical Centre. The layer with the mononuclear cells was isolated by centrifugation on histopaque-1077 (Sigma, Germany). Cells were washed with phosphate-buffered saline (PBS), suspended in Ham’s F10 plus 8% dimethylsulphoxide (DMSO)
and 40% foetal calf serum (FCS) and frozen at −100°C. Such storage over an extended period of time is known not to influence the results obtained using any of the assays employed in the present study. Lymphocytes from the same donors were used for each experimental procedure and replicate experiment.

** Culturing and irradiation conditions **

Lymphocyte samples were carefully thawed in 10 ml cold F10 and 40% FCS was added dropwise. Samples were centrifuged for 10 min, 250 × g, and suspended in 5 ml F10 and 5% FCS. After centrifugation (5 min, 250 × g) ～1.5 × 10^6 cells were suspended in 3 ml complete medium (Ham’s F10 containing 15% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 0.1 µg/ml streptomycin and 2% PHA) and incubated for 12 h at 37°C, 5% CO_2.

Prior to exposure of the cells to the priming dose, they were centrifuged (10 min at 250 × g) and suspended in 1 ml PBS^+ (1.8 mM CaCl_2 and 0.68 mM MgCl_2) with 0.1% FCS, or in 1 ml Ham’s F10. Irradiation was performed at 4°C with 0.02 or 0.05 Gy X-rays (Muller X-ray machine, 100 kV, 8 mA, 1.75-mm Al filter at dose rate 0.25 Gy/min). Cells were transferred in 3 ml complete medium with PHA and cultured for further 12 h. Challenging treatment with 1 Gy X-rays, given 24 h after culture initiation at a dose rate of 1 Gy/min, 1.5-mm Al filter, was performed under the conditions described above. In some experiments, O_2 and N_2 were flushed through 2.5 ml PBS plus 0.1% FCS for 10 min, cells were suspended in 200 µl of oxygen- or nitrogen-enriched PBS and challenged with 1 or 2 Gy X-rays. After centrifugation for 10 min (250 × g, 4°C), they were suspended in PBS or complete medium within 30 min and kept on ice for further analyses. In each experiment, three subcultures were used as controls—unirradiated cells, irradiated with priming dose alone and irradiated only with the challenge dose.

** Nucleoid sedimentation **

Nucleoid sedimentation assay was applied to measure the induction and repair of DNA SSBs and DSBs. After the challenging treatment, the cells were centrifuged at 4°C and suspended in 200 µl PBS^+ containing 0.1% FCS. Recovery was performed for 2 h in a complete medium at 37°C, 5% CO_2. Preparation and sedimentation of the nucleoids were done according to Cook and Brazell (31) as previously described (32) with some modifications (33). Briefly, ～10^6 cells in 100 µl medium were lysed in the dark at 4°C on top of a neutral 15–30% sucrose gradient containing 2 M NaCl and 30 µg/ml ethylenediaminetetraacetic acid (EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10) and treated with 1% Triton X-100 and 1% DMSO for 1.5 h in the dark. Slides were dried, placed on a horizontal electrophoresis unit, covered with cold electrophoresis buffer (300 mM NaOAc, 1 mM ethylenediaminetetraacetic acid) and left for 40 min to allow DNA unwinding. Electrophoresis was performed for 25 min at 25 V and 300 mA in the dark. The material was neutralized for 10 min in 0.4 M Tris, pH 7.5, and stained with 50 µl EtBr (2 µg/ml) in PBS.

Comet capture and analyses were performed by computerized image analyses system based on Aristoplan (Leitz Wetzler, Germany) fluorescence microscope coupled with a charge-coupled device camera. SSB induction was expressed as a migration coefficient (or tail moment) calculated by measuring the resulting comets. At least 40 randomly selected cells (viewed within 24 h after the electrophoresis) pooled from two independent experiments and four slides per experimental point were analysed.

**PCC assay**

The technique used was essentially that described by Pantelias and Maillie (36) with slight alterations. Briefly, interphase lymphocytes (3 × 10^6 to 4 × 10^6) were fused immediately after exposure to the challenging dose (1 Gy) with mitotic Chinese hamster ovary cells (1 × 10^6). Control samples were processed simultaneously. Polyethylene glycol (molecular weight 1450, 55% w/v in PBS) was used as a fusing agent. After fusion, the cells were incubated for 1 h in a complete growth medium containing colcemid (0.1 µg/ml). Air-dried preparations were made after hypotonic treatment (0.075 M KCl) and fixation with acetic acid and methanol (1:3). For scoring of PCC, slides were stained with 2% aqueous Giemsa. Data were obtained from two independent experiments and ~50 cells were analysed for each experimental point.

**Results**

Induction and recovery of DNA strand breaks in cultured human lymphocytes primed with low-dose X-rays and subsequently challenged with 1 Gy X-rays were analysed by nucleoid sedimentation technique. We failed to detect any difference of adaptive reaction in lymphocytes conditioned with 0.02 Gy but challenged with 0.5 Gy X-rays. As evident from the data on sedimentation behaviour of nucleoids when the challenging dose was doubled, conditioning treatment alone produced negligible amount of DNA scissions with comparable frequency for both doses applied (Figures 1 and 2). Sedimentation of the nucleoids from cells primed with 0.02 or 0.05 Gy and subsequently challenged with 1 Gy displayed some differences. In general, a decrease in the level of DNA strand breaks compared with the effect of 1 Gy alone was observed in both cases, but the effect was more pronounced after conditioning of the cells with 0.02 Gy where the difference between non-primed and primed lymphocytes was found to be statistically significant (P < 0.05). When the cells were allowed to recover for 2 h at 37°C after delivery of the challenging dose, the amount of detected DNA damage returned to the control level. In non-adapted lymphocytes irradiated with 1 Gy X-rays, some DNA breaks above the control were still persistent.

Data concerning the influence of oxygen and nitrogen conditions on the induction of DNA strand breaks in adapted and non-adapted cells obtained by nucleoid sedimentation are presented in Figure 3. When the lymphocytes were challenged in solution enriched in oxygen, the decrease of DNA damage was even more pronounced than the reduction described above.
On the contrary, when this treatment was performed under increased nitrogen concentration, such an effect was not only abolished but also the level of the induced DNA strand breaks in adapted cells was somewhat higher than that in the non-adapted ones. Challenging dose under the nitrogen conditions was doubled in order to have a similar extent of X-ray-induced DNA strand breaks as in the oxygen-enriched cell suspension.

The same protocol for culturing and irradiation of the cells was applied in order to measure the frequency of SSBs by single-cell gel electrophoresis (comet assay) under the alkaline conditions. This procedure combines the release of super-coiling and alkaline unwinding, which makes it particularly relevant to the single-strand breakage. Data obtained from the comet assay are presented in Figure 4. Based on the correlations found with nucleoid sedimentation, we estimated the frequencies of SSBs in lymphocyte cultures initially treated with 0.02 Gy and challenged with 1 Gy X-rays. As can be seen, conditioning treatment alone resulted in a slight increase of the induced breaks as compared to the control. When the average tail moment was compared in primed and unprimed cells, a pronounced statistically significant ($P < 0.05$) decrease of SSBs in lymphocytes pre-exposed to 0.02 Gy was detected.

Data on the chromosomal damage in adapted and non-adapted peripheral lymphocytes visualized by PCC technique are summarized in Table I. A substantial decrease ($\sim 25\%$) of the induced PCCs per cell was found after the application of adaptive and challenging treatment in comparison to cells treated with 1 Gy X-rays alone. This reduction is better exemplified by the percentage of distribution between the different classes of the PCCs observed. In all, 44% of the primed and subsequently challenged cells have restored the control value of 46 PCCs as opposed to $\sim 20\%$ in the population of cells treated with challenging dose only.

**Discussion**

It was previously shown that the adaptive potential of lymphocytes is manifested $\sim 30$ h after culture initiation, being strongly dependent on the time span between the priming and challenging dose (37). Some authors (17,23) failed to observe adaptation 9 h after the application of the priming dose when assayed for the induction of chromosome-type aberrations. Since only few lymphocytes are entering cell division earlier than 20 h after stimulation (38), our results indicate that adaptation on both DNA and chromosomal level is effectively
performed when the time gap between the conditioning and challenging treatment falls predominantly within the G1 stage of the cell cycle. The data support the notion for the lack of AR in dormant cells.

Results from nucleoid sedimentation studies show that adaptive reaction of lymphocytes strongly depends on the doses employed for adaptation and challenge. The effect was more pronounced when the cells were primed with 0.02 Gy than with 0.05 Gy. Obviously, the quantity of initial lesions eliciting adaptation effectively is a limited one, confirming earlier observations of AR dependence on a narrow range of doses (39). It has to be pointed also that we failed to observe any AR when lymphocytes primed with 0.02 Gy were challenged with 0.5 Gy X-rays. This is an indication that the AR manifested by DNA strand-break induction is also dependent on the ratio between the initial damage and that of the challenging treatment, being in accordance with other studies utilizing end points such as chromosomal aberrations, mutations towards 6-thioguanine resistance and cell killing (40). It can be also inferred that the observed adaptation of the cells measured by nucleoid sedimentation is presumably linked with alterations in DNA integrity and not with parameters like supercoil density or loop size, which were shown to remain unaffected (41).

Results concerning the recovery of the induced DNA strand breaks displayed practically the same levels in adapted and non-adapted cells, favouring the assumption that the repair potential of both populations is similar in magnitude, although the repair kinetics may differ (42). Some priming-induced increase in the repair capacity of the cells, however, cannot be excluded. It was shown that the application of low doses of ionizing radiation results in a significant decrease of the constitutive (steady state) levels of DNA strand breaks (43). Presumably, such a prior-activated protective mechanism might render the cells more resistant to a subsequent higher dose. As a whole, our data are more consistent with the notion that radio-adaptive response has to be rather attributed to the lowering of DNA damage produced by the challenging treatment than to the increased recovery potential of the cells (5,41,44).

The idea that radio-adaptive response in human lymphocytes might be triggered by the persistence of DNA discontinuities gains support also from our results obtained by single-cell gel electrophoresis under alkaline conditions. Cells initially treated with low dose of X-rays became more resistant for the induction of strand breaks within the DNA-superoiled looped domains by the challenging one. We have also applied sandwich enzyme-linked immunosorbent assay based on antibodies against single-stranded DNA in order to evaluate the amount of this type of damage in cells primed with 0.02 Gy and challenged with 1 Gy versus non-primed cells. A significant decrease in the single-stranded DNA in conditioned and subsequently challenged cells was again detected (L.M. Stoilov, G.P. van der Schans and A.T. Natarajan, unpublished results). Data obtained from the PCC experiments also show that adaptation results in a concomitant decrease of the interphase chromosomal breakage, thus eliciting the role of the primary chromosomal lesions in the defence mechanisms responsible for radio-adaptive response.

It was reported that AR to X-rays can be induced in human lymphocytes conditioned with H2O2 (25), an agent known to produce oxygen radicals similar to X-rays (45). A protective effect against radiation damage detected in human lymphocytes pre-exposed to H2O2 evaluated as frequencies of micronuclei in binucleated cells has also been reported (24). It was shown that in mammalian cells the oxygen enhancement ratios for DNA SSBs and DSBs are ~2–3 (46) and this led us to double the challenging dose under the nitrogen conditions in order to have similar levels of damage. Our results concerning the influence of oxygen and nitrogen on the AR indicate that the reduction of X-ray-induced DNA breaks is favoured in the presence of oxygen. This observation might have substantial implications for radiation therapy of solid tumours. The observed effect could be due to the elevated ability of cells to overcome the increased oxidative damage with the likely candidate involved being enzyme superoxide dismutase (SOD), known to reduce the level of radiation-generated oxygen species (47). This is supported by the data for higher activity of SOD in mammalian cells adapted with non-toxic doses of H2O2 (5). Experiments with embryonic cells from transgenic mice deficient in SOD have shown, however, that the AR does not always correlate with the amount of this enzyme (16,48). Such a discrepancy might be explained by the fact that expression of the gene CDC16 (known to be a part of the anaphase-promoting complex controlling progression through mitosis) is repressed after oxidative stress with H2O2 (49). Cell cycle delay might take place in adapted cells and make them more tolerant to a higher dose treatment, thus providing an additional time to activate their protective potential. Such a notion is supported by the finding that low-dose irradiation can alter significantly gene expression profiles of human lymphoblastoid cells including genes involved in the cell cycle control (50,51). Based on data obtained by comet assay, PCC and immuno-fluorescence labelling in non-dividing human cells, it was recently suggested that radio-adaptive response is most likely to be a reflection of the perturbation of cell cycle progression (52).

It was shown that human lymphocytes pre-exposed to low doses of X-rays exhibit cross-resistance not only to radiation but also to a variety of other challenging treatments such as mitomycin C, bleomycin, H2O2 and N-methyl-N-nitro-N-nitrosoguanidine (13,20,25,53). It was initially proposed that this might be due to repair activities coping efficiently with DNA lesions (54). On the other hand, the observed cross-adaptation could be a consequence from the wide spectrum of

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**Table I. AR of X-ray treated human peripheral blood lymphocytes, measured by PCC technique**

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Cells scored</th>
<th>PCCs per cell ± SD</th>
<th>Induced PCCs per cell</th>
<th>Distribution of PCCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45</td>
<td>46</td>
<td>0</td>
<td>100 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>0.02 Gy</td>
<td>46</td>
<td>46.06</td>
<td>0.06</td>
<td>97 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>1 Gy</td>
<td>50</td>
<td>48.6 ± 1.9</td>
<td>2.6</td>
<td>20 12 20 12 21 3 12</td>
</tr>
<tr>
<td>0.02 Gy + 1 Gy</td>
<td>42</td>
<td>47.8 ± 2.2</td>
<td>1.8</td>
<td>44 8 16 8 12 4 8</td>
</tr>
</tbody>
</table>

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and subsequently challenged cells was again detected (L.M. Stoilov, G.P. van der Schans and A.T. Natarajan, unpublished results). Data obtained from the PCC experiments also show that adaptation results in a concomitant decrease of the interphase chromosomal breakage, thus eliciting the role of the primary chromosomal lesions in the defence mechanisms responsible for radio-adaptive response.

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DNA lesions, induced by ionizing radiation—DNA SSBs and DSBs, base damage, DNA–protein cross-links, as well as radiation-induced oxidative damage. The involvement of DNA strand breaks in AR is emphasized from the observation that adaptation can be abolished by the inhibitors of the enzyme poly(ADP-ribose) polymerase (9), which is effectively stimulated in response to the induction of DNA DSBs (55). It was also reported for the increased DNA polymerase activity after low-level exposure to X-rays (56), an enzyme known to be responsible for the ligation of the DNA SSBs. All these observations together with our own results favor the assumption that initial DNA discontinuities might serve as a direct trigger of radio-adaptive response in human lymphocytes. Thus, the cells facing strand scissions, in order to be able to maintain properly such key nuclear functions like replication and transcription, are forced to activate their defense potential (natural, evoked or both) in order to keep the native status of DNA template.

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References


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