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Relationship between chromatin structure, DNA damage and repair following X-irradiation of human lymphocytes

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ABSTRACT

Earlier studies using the technique of premature chromosome condensation (PCC) have shown that in human lymphocytes, exchange type of aberrations are formed immediately following low doses (<2 Gy) of X-rays, whereas at higher doses these aberrations increase with the duration of recovery. This reflects the relative roles of slow and fast repair in the formation of exchange aberrations. The underlying basis for slow and fast repairing components of the DNA repair may be related to differential localization of the initial damage in the genome, i.e., between relaxed and condensed chromatin. We have tried to gain some insight into this problem by (a) X-irradiating lymphocytes in the presence of dimethyl sulfoxide (DMSO) a potent scavenger of radiation-induced •OH radicals followed by PCC and (b) probing the damage and repair in two specific chromosomes, 18 and 19, which are relatively poor and rich in transcribing genes by COMET–FISH, a combination of Comet assay and fluorescence in situ hybridization (FISH) techniques.

Results obtained show (a) that both fast appearing and slowly formed exchange aberrations seem to take place in relaxed chromatin, since they are affected to a similar extent by DMSO, (b) significant differential DNA breakage of chromosome 18 compared to chromosome 19 in both G0 and G1 phases of the cell cycle as detected by Comet assay, indicating that relaxed chromatin containing high densities of transcriptionally active genes shows less fragmentation due to fast repair (chromosome 19) compared to chromosome 18, and (c) that relaxed chromatin is repaired or mis-repaired faster than more compact chromatin.

1. Introduction

Ionizing radiation (IR) is an efficient inducer of chromosome aberrations (CA), which represent the outcome of very complex events, involving the conversion of DNA double strand breaks (DSBs) and other lesions through various DNA repair pathways into microscopically detectable events. Though IR is expected to induce DNA lesions in a random manner in the genome, some studies using FISH technique with chromosome specific DNA probes, have reported that the frequency of radiation-induced chromosome exchanges was proportional to the length or to the DNA content of target chromosomes [1–3], whereas other studies have reported that the induction of chromosome exchanges is non-random among the chromosomes studied [4–10]. The possible causes for this reported non-random distribution of exchanges have been attributed to various factors such as, differential primary damage of DNA and its repair which could be influenced by transcriptional activity [11–14], chromatin structure [4,5,15,16] and gene density [17,18] of the studied chromosomes. This implies that CA we observe, which are the consequences of mis-repair of IR induced DSBs, may not reflect the initial distribution of DNA damage in the genome. Earlier studies using the technique of premature chromosome condensation (PCC) by fusion with mitotic cells, have shown that in human lymphocytes, exchange type of aberrations are formed within a few minutes, following low doses (<2 Gy) of X-rays where the two lesions required to produce the exchange are significantly generated by one ionization track, whereas at higher doses where the probability of the two lesions being produced by two independent tracks is higher, these aberrations increase with the duration of recovery [19,20]. This reflects the relative roles of slow and fast repairing components of induced DNA double strand breaks (DSBs) leading to CA. The underlying basis for the slow and fast repairing components of the DNA breaks may be related to the differential localization of the damage in the genome, i.e., between relaxed and condensed regions. The genome is highly het-

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erogeneous and it has been shown that genes are clustered among chromosomes in a sub-set of R bands, most of which are known as T bands containing the highest concentration of CpG islands typical of relaxed regions. While G bands are late replicating, relatively AT-rich, highly condensed and transcriptionally silent, R and T bands are early replicating, less condensed with unmethylated DNA and accessible HpaII sensitive sites on chromosomes [21].

We have tried to elucidate the relative roles of slow and fast repairing components of induced DSBs in relaxed and condensed regions by means of cytogenetic analysis in prematurely condensed chromosomes (PCC) and alkaline Comet assay in unstimulated (G0) human lymphocytes irradiated with X-rays in the absence and presence of dimethyl sulfoxide (DMSO). DMSO, an efficient and selective scavenger of radiation-induced •OH radicals, can be used as a probe for events taking place in regions of different chromatin compactness, since DNA damage induced by •OH radicals is known to be high in more opened chromatin [22–25]. Furthermore, we probed the damage and repair in transcriptionally silent condensed regions of chromosomes or from relaxed regions with very high gene density by COMET–FISH, a combination of Comet assay and fluorescence in situ hybridization (FISH) techniques [26] in human chromosomes, 18 and 19, which possess ideal properties in respect to their structural organization to be considered as models of condensed and relaxed chromatin respectively. Both chromosomes are similar in size, but exhibit very different chromatin organisation and banding patterns. While chromosome 19 has a very high gene density with strong hybridization signals for T bands, chromosome 18 exhibits very few active genes and very little hybridization signals for T bands [27,28].

2. Materials and methods

2.1. Cell preparation and treatment conditions

Lymphocytes were isolated from bags of "buffy coat" generated from approximately 500 ml of heparinised fresh venous whole blood drawn from three healthy male donors, supplied by a local hospital. "Buffy coats" were diluted 1:1 in phosphate-buffered saline (PBS) and lymphocytes separated using Ficoll–Histopaque 1077 (Sigma). Briefly, 15–20 ml diluted "buffy coat" was layered over 12.5 ml Ficoll–Histopaque in 50 ml polystyrene conical centrifuge tubes (Falcon) and centrifuged for 35 min at 450 × *g* at room temperature. During centrifugation, mononuclear cells and platelets were concentrated in a fluffy white layer below the plasma. The cell layers were collected with Pasteur pipettes and washed twice with fresh culture medium. Then the cell pellets were resuspended by gentle vortexing, and cell suspensions with a final concentration of 1×10^7 cells/ml in PBS were prepared. Greiner® 14 ml conical cell culture tubes contained 0.5 ml of cell suspension (5×10^6 lymphocytes) and 4.5 ml medium (Ham's F-10, supplemented with 20% foetal calf serum heat-inactivated at 56 °C, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.4 mM L-glutamine and 2% Hepes buffer). All the chemicals used for lymphocyte cultures were supplied by Gibco®. Cultures were irradiated with 3 Gy X-rays at 300-kV and 6 mA at a rate of 1.0 Gy/min directly prior to stimulation with 2% phytohaemagglutinin (PHA 15 Murex, Italy) when lymphocytes are in a G0 phase (unstimulated cultures), or 12 h after stimulation with PHA when lymphocytes are in the G1 phase of the cell cycle.

For treatments in the presence of dimethyl sulfoxide (Sigma–Aldrich), CAS registry number: 67-68-5, cell suspensions were inoculated in 4.5 ml PBS containing DMSO (2 M final concentration).

Human lymphocytes from treated cultures were processed to prepare Comet slides, immediately after irradiation (0 h recovery time) and always on ice or placed in an incubator at 37 °C for the appropriate recovery time (0.5, 1, 2, 4 and 24 h), or fused with mitotic CHO cells to obtain PCC immediately after irradiation (0 h recovery time) and at 2 and 4 h recovery times. Mitotic CHO cells collected by mitotic shake off and frozen at −80 °C in complete growth medium containing DMSO 10% and 0.2 μg/ml colcemid, were thawed immediately before use. Control cultures both in the absence and presence of DMSO were not irradiated.

2.1.1. Cytogenetic analysis in prematurely condensed (PCC) human chromosomes

Cell fusion between mitotic CHO cells and G0 lymphocytes was mediated by polyethylene glycol (PEG) according to Pantelias and Maillie [29].

Briefly, mitotic cells and appropriately treated human lymphocytes (see Section 2.1) were mixed in a ratio of 1:3 in round-bottom culture tubes. After centrifugation, the cells were suspended in 2 ml of Ham's F-10 medium without serum and centrifuged again. The supernatant was discarded without disturbing the pellet and 0.25 ml of PEG (MW 1450 from Sigma–Aldrich) was added and left for 1 min. For the next 3 min, 2 ml of phosphate-buffered saline (PBS) were added drop wise; the tube was gently shaken after each drop. The cell suspensions were centrifuged and resuspended in 0.5 ml culture medium containing colcemid (0.2 μg/ml) and incubated at 37 °C for 1 h [30,31]. Finally cells were treated with hypotonic KCl solution (75 mM) for 10 min and fixed in methanol–acetic acid (3:1). Cell suspension was dropped onto pre-cleaned wet slides, air-dried and aged for at least 1 week. In order to improve detection of dicentric chromosomes in PCC, we employed fluorescence in situ hybridization (FISH) using centromeric DNA probes directly labelled with rhodamine (TRITC) which identify the centromeric region of each human chromosome (Appligene-Oncor). To apply pan-centromeric probes, slides were denatured for 4 min at +80 °C with 70% formamide/2× SSC and dehydrated by serial ethanol washing (70, 90 and 100%). The pan-centromeric probe was pre-warmed for 20 min at 37 °C, 6 μl mixed with 14 μl hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC), denatured at +80 °C for 10 min and chilled on ice. Twenty microliters of the probe were put onto each slide, which were then sealed and hybridized at 37 °C overnight in a moist-chamber. Then, slides were preincubated twice with 50% formamide in 2× SSC at 42 °C for 5 min, the coverslips were removed and washed once with 0.005% Tween 20 in PBS. Finally slides were dehydrated by serial ethanol washing (70, 90 and 100%), and embedded in 25 μl Vectashield (Vector Laboratories) with DAPI at a final concentration of 0.15 μg/ml.

2.1.2. "Conventional" alkaline Comet and FISH/alkaline Comet assays

Comet slides were prepared following published protocols for the alkaline "Comet assay" [32] with modifications for FISH with whole chromosome painting probes, as previously described [33,34]. Briefly, 10 μl of cell suspension was mixed with 65 μl of 0.7% (w/v) low-melting point agarose (Bio-Rad Lab.) and sandwiched between a lower layer of 1% (w/v) normal-melting point agarose (Bio-Rad Lab.) and an upper layer of 0.7% (w/v) low-melting point agarose on microscope slides (Carlo Erba, Milan, Italy). Triplicate slides were prepared from each treatment. Slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) containing 10% DMSO and 1% Triton x-100 (ICN Biomedicals Inc.) at 4 °C overnight. Following lysis, slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH ≥ 13) and incubated for 25 min at 4 °C to allow the DNA to unwind and to express the alkali-labile sites. Electrophoresis was carried out at 4 °C for 15 min at 30 V (1 V/cm) and 300 mA. After electrophoresis, slides were immersed in 0.3 M sodium acetate in ethanol for 30 min. One set of slides from each treatment series was dehydrated by passing them through an alcohol series (2 min at 70, 85 and 100%), air-dried and stained with 20 μg/ml ethidium bromide immediately before analysis of DNA breakage by conventional Comet assay (tail moment), as described in Section 2.2.2.

The remaining two sets of slides from each treatment series were dehydrated by passing them through an alcohol series (2 min at each 70, 85 and 100%), dried thoroughly at 37 °C before detection of probes and counterstained by COMET–FISH analysis. This procedure was adopted to improve stabilization of the gel, which otherwise disintegrates during hybridization. Dual-colour FISH was performed as follows: flow-sorted human chromosomes 18 and 19 (kindly supplied by Prof. M. Rocchi, University of Bari, Italy) were amplified and directly labelled with digoxigenin-11-dUTP or with biotin-16-dUTP (Boehringer, Mannheim, Germany), respectively, by degenerative oligonucleotide primer-polymerase chain reaction (DOP-PCR). 400 ng of each labelled probe, mixed with 100 μl of 70% deionised formamide in 2× standard sodium citrate solution (SSC), was applied to cells on the air-dried slides. Cells and probes were denatured at +74 °C for 5 min before cover slips (24 mm × 50 mm) were sealed with rubber cement over the gels. Hybridization was performed overnight at +37 °C in a humid chamber. Slides were then washed in 50% formamide, 2× SSC (pH 7.0) for 5 min at +42 °C followed by three washes in 0.01× SSC for 5 min at +60 °C and a final wash with 4× SSC, 0.05% Tween 20 (pH 7.0) for 5 min. Biotinylated probes were detected with avidin–FITC, biotin–avidin and avidin–FITC (Vector Laboratories, Burlingame, CA, USA). The probes labelled with digoxigenin were detected with sequential mouse anti-digoxigenin, sheep anti-mouse–digoxigenin and sheep anti-digoxigenin–TRITC (Vector Laboratories, Burlingame, CA, USA). Finally slides were dehydrated, dried and mounted in Vectashield (Vector Laboratories) containing 0.15 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and antifade.

2.2. Analysis

2.2.1. PCC analysis

PCC slides were analyzed with a Leitz Ergolux microscope equipped with single band pass filters for DAPI, FITC, TRITC and a fully automated metaphase finder (MetaSystems GmbH, Altlussheim, Germany). An Isis digital imaging system (MetaSystems GmbH, Altlussheim, Germany) was used to capture, digitalise, annotate and print fluorescence images. A minimum of 100 PCC plates were scored for each test point per experiment. Chromosomes with two or more centromeric signals were scored as dicentric or polycentric chromosomes (which were converted into dicentrics for the purpose of calculation) while chromosomes without centromeric signal were classified as fragments. A typical example of PCC is shown in Fig. 1.

2.2.2. Conventional alkaline Comet assay

Slides stained with DAPI were examined at 40× magnification using an automated image analysis system specific for Comet assays (Comet Assay III; Perceptive