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Complex aberrations in lymphocytes exposed to mixed beams of ^{241}Am alpha particles and X-rays

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ABSTRACT

Modern radiotherapy treatment modalities are associated with undesired out-of-field exposure to complex mixed beams of high and low energy transfer (LET) radiation that can give rise to secondary cancers. The biological effectiveness of mixed beams is not known. The aim of the investigation was the analysis of chromosomal damage in human peripheral blood lymphocytes (PBL) exposed to a mixed beam of X-rays and alpha particles. Using a dedicated exposure facility PBL were exposed to increasing doses of alpha particles (from ^{241}Am), X-rays and a mixture of both. Chromosomal aberrations were analysed in chromosomes 2, 8 and 14 using fluorescence in situ hybridisation. The found and expected frequencies of simple and complex aberrations were compared. Simple aberrations showed linear dose–response relationships with doses. A higher than expected frequency of simple aberrations was only observed after the highest mixed beam dose. A linear-quadratic dose response curve for complex aberrations was observed after mixed-beam exposure. Higher than expected frequencies of complex aberrations were observed for the two highest doses. Both the linear-quadratic dose–response relationship and the calculation of expected frequencies show that exposure of PBL to mixed beams of high and low LET radiation leads to a higher than expected frequency of complex-type aberrations. Because chromosomal changes are associated with cancer induction this result may imply that the cancer risk of exposure to mixed beams in radiation oncology may be higher than expected based on the additive action of the individual dose components.

1. Introduction

The improving curability of cancer raises questions about the risk of long-term side effects of therapeutic procedures. In radiotherapy, an emerging problem is the undesired radiation exposure outside the treated volume that can give rise to secondary cancers [1]. In intensity modulated radiotherapy (IMRT) the undesired radiation exposure comes from leaking accelerator heads and from neutrons generated by the interaction of high energy photons with the collimator. In light ion beam therapy, it comes from the production of nuclear fragments, such as neutrons, protons, heavier ions and pions. These secondary particles may possess energies up to several hundreds of MeV and can be transported long distances outside the treated volume. In a cascade of events, they in turn produce secondary particles through nuclear interactions during their transport. Consequently, in IMRT and in light ion therapy normal tissues, both close to and relatively far from the treated volume, are exposed to a mixed, complex secondary radiation field [2].

Assessment of risk of secondary cancers induced by mixed fields requires the knowledge of absorbed doses delivered to normal tissues and organs and that of radiation weighting factors W_R. The latter originate from studies where relative biological effectiveness (RBE) values of different radiation qualities are determined. The interesting question is if the RBE of a complex radiation field can be calculated based on simple additivity of mixed beam components. In other words, can it be assumed that the different radiation components do not interact with each other to produce effects stronger than based on additivity? The studies that have been undertaken to address this issue did not yield clear results as some of them suggest synergism [3,4] while others additivity [5–7] (see [4] for more references). The reason for this controversy is not clear, although the application of different exposure conditions and radiation qualities is a likely confounding factor.

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A synergistic (interacting) action of two radiation types can occur through the resulting increased ionisation density and increased damage complexity. Indeed, it has been shown that the level of clustered DNA double strand breaks (DSB), and the degree of their complexity, increases with the linear energy transfer (LET) [8] and this can explain the known increase of RBE with LET. Complex DSB are more difficult to repair than simple DSB [9,10] so it is possible that the damage induced by combined action of two radiations is not repaired as efficiently as after the action of the single mixed beam components. We have previously shown that exposure of PBL to mixed beams leads to higher than expected frequencies of micronuclei [4]. Misrepaired complex DNA damage can be visualised at the level of chromosomes when analysed by fluorescence in situ hybridisation (FISH, also called chromosome painting). High LET radiation is known to efficiently induce complex chromosome aberrations that are defined as chromosomal exchanges involving three or more breaks in two or more chromosomes [11]. Using three colour painting we have previously shown that the fraction of complex aberrations in human peripheral blood lymphocytes (PBL) increases with LET [12]. Chromosomal changes are associated with cancer induction and increased damage complexity following exposure to high LET radiation is probably responsible for the high efficiency, per unit dose, of high LET radiation to induce cancer [13]. Therefore, increased damage complexity following exposure to mixed beams could suggest a higher than expected risk of cancer induction.

In the present investigation we used the same technique of three colour painting to study the spectrum of chromosomal aberrations in PBL from one donor exposed in vitro to mixed beams of alpha particles and X-rays. PBL from this specific donor were previously analysed for micronuclei, as described in [4]. We specifically focused on the frequency of complex aberrations in order to see if their frequency would be additive or synergistic in cells exposed to mixed beams. The experiments were performed using a dedicated mixed beam exposure facility [7]. The results indicate a higher than expected frequency of complex aberrations in PBL exposed to mixed beams.

2. Materials and methods

2.1. Blood collection and irradiation

9 ml blood was drawn from one healthy donor (male, 28 years old, non-smoking) at three occasions during a period of one year. On the day of collection the blood was aliquoted and irradiated with increasing doses of radiation so that dose–response relationships could be established. A few dose points (0.8 and 0.4 Gy X-rays as well as 0.27 Gy alpha particles) were performed twice due to the poor quality of slides and/or insufficient number of metaphases after the first exposure. The dose response relationships are thus composed of dose points coming from two independent experiments. Each experiment included a control. The work was approved by the local ethical committee at the Karolinska University Hospital (diarium number 2010/27-31/1).

Before setting up cell cultures, whole blood was irradiated and sham exposed on round polyamide (PA) disks (155 mm in diameter, custom-constructed in the Institute for Energy-JRC, Petten, Netherlands) as described earlier [4]. Prior to exposure, the discs were cleaned with 70% ethanol and warmed to 37 °C in an incubator. 0.25 ml blood was positioned in the centre of the PA disc, covered with a Mylar foil lid and spread out to an even layer. For alpha particles and mixed beams 4×0.25 ml and for X-rays 2×0.5 ml whole blood per dose point was exposed. The doses were 0.20, 0.40 and 0.80 Gy X-rays (X); 0.13, 0.27 and 0.54 Gy alpha particles (α) and $0.20X + 0.07\alpha$, $0.40X + 0.13\alpha$ and $0.40X + 0.27\alpha$ Gy mixed beams. Assuming a nucleus diameter of 10 μm the alpha doses correspond to the following average number of hits per cell and percentage of hit cells (the latter is given in brackets): 0.07 Gy: 0.48 (38%); 0.13 Gy: 0.89 (59%); 0.27 Gy: 1.85 (84%); 0.54 Gy: 3.71 (97%). The values were calculated using the methodology described in [14,15].

2.2. Mixed beams exposure facility

Both the technical and dosimetric details of the mixed beams exposure facility are described elsewhere [7]. Aspects related to dose distribution in PBL are discussed in [4]. In short, the facility consists of an alpha irradiator and an X-ray tube, the former positioned inside and the latter underneath an incubator. The alpha exposure facility incorporates a ^{241}Am source as well as a movable shelf for positioning the cells on PA discs at defined distances from the source. The total activity of the source is 50 ± 7.5 MBq, with an active area of 180 mm × 180 mm and a 2 revolutions per second rotation during exposure. The average LET peak of the alpha particles is 90.92 ± 8.55 keV μm^{-1}. The dose rate is 0.265 Gy/min: 0.2404 Gy/min alpha particles with a 0.0246 Gy/min beta component. No collimator was used.

The X-ray tube was operated at 190 kV, 4.0 mA without the built-in filters. The X-rays were filtered by the steel bottom of the incubator as well as the 12.0 mm bottom plate and the 8.0 mm movable shelf of alpha facility (both aluminium) before reaching the blood layer. The dose rate for X-rays was 0.068 Gy/min in the bottom and 0.052 Gy/min in the top position of the movable shelf (representing X-rays alone and simultaneous exposure, respectively) [7].

2.3. Blood culturing

Immediately after exposure and sham-exposure 0.5 ml blood was added to 5.5 ml PBL culturing medium consisting of RPMI 1640 (R8785, Sigma–Aldrich, Stockholm, Sweden) supplemented with 20% foetal calf serum (10106-169, Gibco, Invitrogen, Stockholm, Sweden), 10 μg/ml phytohemagglutinin (15212-046, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (15240-096, Gibco). 20 μM bromodeoxyuridine (BrdU, Sigma–Aldrich) was added to the cultures 1 h after irradiation. Cells were incubated for 55 h at 37 °C in tightly closed culture tubes (BIO-ONE 10 ml, Greiner, Sollentuna, Sweden). 52 h after culture start colcemid (Sigma–Aldrich) was added at a concentration of 5.56 μg/ml. Calyculin A (Sigma–Aldrich) was added to a final concentration of 50 nM 53.5 h after culture start, inducing premature chromosome condensation (PCC) (the dual use of colcemid and calyculin being based on the study of [16]). 55 h after culture start cells were harvested according to standard procedures. Briefly, cells were swollen in 37 °C 0.075 M KCl solution for 15 min, followed by fixation and subsequent washes in 3:1 methanol:acetic acid (VWR International AB, Stockholm, Sweden). Lymphocytes were spread on clean, wet microscope slides. The slides were quality-checked and stored at −20 °C.

2.4. Fluorescence plus Giemsa (FPG) differential staining of chromatids

Before hybridisation, slides were treated for differential staining of chromatids. The treatment allows identification of second division metaphases and also results in a bright staining of centromeres, enabling discriminating between 2A (dicentrics) and 2B (translocations) exchanges in G2 and anaphase spreads. Slides were thawed and left to dry at room temperature for about 10 min. The slides were submerged in bisbenzamide (Hoechst, Sigma–Aldrich) in darkness for 20 min and thereafter covered with PBS (phosphate buffered saline), overlaid with another slide and UV-irradiated for 40 min using a 254 nm lamp. The slides were allowed to dry before the chromosome painting was started.

2.5. Fluorescence in situ hybridisation (FISH) chromosome painting

The frequency of chromosomal aberrations was analysed in chromosomes 2, 8 and 14. The decision to select the three chromosomes was based on the fact that they represent one large, one medium and one small chromosome. Moreover, we have successfully used this painting cocktail in our previous study [12]. The fraction of the male human whole genome DNA represented by the chromosomes 2, 8 and 14 is 0.0803, 0.0488 and 0.0338, respectively [12].

The probes for the direct labelling of the chromosomes were purchased from Metasystems (Althusheim, Germany). Hybridisation was carried out according to the protocol from the manufacturer, with the following modifications. Based on earlier experience and assay optimisation, the slides were treated with RNAse for 40 and with pepsin for 2–3 min instead of 30 and 10 min given in the protocol. Hot plate incubations were only initiated after the working solutions had been pipetted onto slides. The probe for chromosome 2 was labelled with FITC (fluorescein isothiocyanate), and the chromosome 8 probe with TRITC (rhodamine). Chromosome 14 was stained by a 1:1 mixture of FITC and TRITC, thus giving it an orange appearance. Slides were counterstained with DAPI (0.2 μg/ml, 4′,6-diamidino-2-phenylindole, Sigma–Aldrich), washed in PBS, dried, mounted with Vectashield (Immunkemi, Järfälla, Sweden) and sealed with rubber glue.

2.6. Image acquisition, analysis of aberrations and calculation of primary breaks

Images were captured by a Cool Cube 1 CCD camera (Metasystems) coupled to a Nikon Eclipse E800 (Nikon, Tokyo, Japan) fluorescence microscope equipped with filters for DAPI, FITC and TRITC. A 100× oil immersion objective was used and images were stored and processed with the ISIS image analysis system (version 5.3, Metasystems). Visualisation, enlargement and colour enhancement is available for each colour channel separately as well as for the full image. Image capture, classification and analysis of chromosomal aberrations were performed by two persons (E. S. and M. D-K.). First-division metaphases and anaphases along with G_2-PCC were scored. Only cells with clear centromeres were scored for G_2-PCC and anaphases. Each chromosome was evaluated separately. Aberrations were classified as dicentrics (2A, one- or two-way), translocations (2B, one- and two-way) [17], rings (centric and acentric) and acentric fragments. Dicentrics and translocations included