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Analysis of inversions and sister chromatid exchanges in chromosome 3 of human lymphocytes exposed to X-rays

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It has been shown repeatedly that exposure of G_1 cells unifilarily labelled with 5-bromodeoxyuridine (BrdU) to X-rays leads to sister chromatid exchanges (SCE) when the cells are allowed to grow for one further cycle in the absence of BrdU. It has been suggested that damage induced by ionizing radiation does not lead to 'true' SCE and that the observed SCE are 'false', resulting from structural chromosomal aberrations, especially interstitial inversions. We used a painting probe for the p14 region of human chromosome 3 and anti-BrdU antibodies to analyse the frequency of radiation-induced SCE in that chromosome. This method allowed us to discriminate between para- and pericentric 'true' and 'false' SCE. Our results indicate that most radiation-induced SCE do not result from inversions.

Introduction

It is generally assumed that sister chromatid exchanges (SCE) are a consequence of DNA replication on a damaged template (Latt, 1981) and can only arise when DNA damage is not repaired in an error-free way before the cell enters S phase. Various models seek to explain SCE as either a mechanism of DNA repair (Ishii and Bender, 1980) or damage bypass (Evans, 1977; Shafer, 1977; Painter, 1980; Cleaver, 1981; Schubert, 1990). Recent publications indicate that SCE induction is not influenced by a defect in mismatch repair (Afzal *et al*., 1995) and that SCE occur predominantly within damaged, transcriptionally active regions of the genome (Cleaver *et al*., 1996).

Chemical mutagens, especially those capable of alkylating DNA, induce SCE very effectively (Latt, 1981; Perry and Thomson, 1984). In contrast, ionizing radiation is a poor inducer of SCE and is only effective when applied to cells in G_1 with chromosomes unifilarily substituted with BrdU (Littlefield *et al*., 1979; Renault *et al*., 1982; Mühlmann-Diaz and Bedford, 1995). The nature of radiation-induced SCE is a matter of debate because ionizing radiation induces mainly single- and double-strand breaks, which are expected to be repaired before the cell enters S phase (Szumiel, 1998). Wolff *et al*. (1974) were the first to suggest that SCE induced by low LET radiation in G_1 could be 'false', resulting from chromosomal aberrations. When cells are irradiated in G_1 following one round of replication in the presence of BrdU, paracentric inversions are visible as double internal or interstitial SCE (Figure 1B and C). The hypothesis of 'false' SCE resulting from aberrations was indirectly supported by the observation that no SCE were observed in human lymphocytes irradiated in the G_0 stage, prior to labelling with BrdU (Littlefield *et al*., 1979). Mühlmann-Diaz and Bedford (1995) analysed interstitial deletions and interstitial SCE in human fibroblasts irradiated in G_1 and found similar frequencies of both. Based on the assumption that ionizing radiation induces equal frequencies of interstitial deletions and inversions, they concluded that low LET radiation induces only 'false' SCE.

Since staining of chromosomes with Giemsa does not allow analysis of inversions, the hypothesis of 'false' SCE is based on theoretical considerations and indirect observations. We have applied a more direct method to correlate SCE with inversions. Human peripheral lymphocytes unifilarily labelled with BrdU were irradiated with X-rays in G_1 and recovered for a further cell cycle in the absence of BrdU. Chromosome preparations were hybridized with an *in situ* hybridization probe specific for the p14 band of chromosome 3. Simultaneously, SCE were detected with anti-BrdU antibodies.

Using this protocol, we analysed those paracentric, double, internal SCE occurring in the p arm of chromosome 3, inside which the probe signal was positioned asymmetrically (Figure 1A and B). In SCE which would have resulted from an inversion the hybridization signal would be shifted in a terminal direction in relation to the centromere (Figure 1B). Pericentric SCE with an asymmetrically positioned centromere which resulted from pericentric inversions would modify the p/q arm ratio (Figure 1E). Our results indicate that only a few radiation-induced SCE observed in the p arm of chromosome 3 are inversions.

Materials and methods

Lymphocyte culture and irradiation

An aliquot of 0.5 ml of whole blood from a healthy, non-smoking, 18-year-old male was cultured in 4.5 ml McCoy's medium supplemented with antibiotics, 20% fetal calf serum (FCS) and 0.125 ml phytohaemagglutinin. Cultures were set up in quadruplicate. Half of the cultures were used as a source of conditioned medium. Twenty hours after culture onset 20 μM BrdU was added to half of the cultures. Following a further 28 h, the BrdU-substituted cells were centrifuged, resuspended in 4.5 ml of pre-warmed McCoy's medium without FCS and irradiated with 0, 1.2, 2.4, 3.6 or 4.8 Gy X-rays (Philips X-ray tube, 180 kV, 13 mA, 3 mm Al filter, dose rate 1.2 Gy/min). Immediately thereafter the cells were centrifuged again, resuspended in the conditioned medium isolated from the cultures not supplemented with BrdU and cultured for another 24 h (total culture time 72 h). Colcemid (0.08 μg/ml) was added for the final 3.5 h and cells were harvested according to a standard cytogenetic protocol.

Preparation of the hybridization probe

A yeast artificial chromosome (YAC) containing DNA sequences specific for the 3p14 region of human chromosome 3 was prepared as previously described (Bardenheuer *et al*., 1996). The YAC was labelled with biotin by nick translation with the BioNick Labelling System (Gibco) according to the manufacturer. The labelled YAC was precipitated with ethanol, diluted in distilled water and kept at –20°C. Before hybridization 3 μl YAC DNA (~120 ng) were mixed with 3 μl Cot-1 DNA (3 μg, Gibco) and 8 μl hybridization mix (2 g dextran sulphate, 10 ml formamide, 2 ml 20× SSC, distilled water to 14 ml). The mixture was denatured at 70°C for 10 min,

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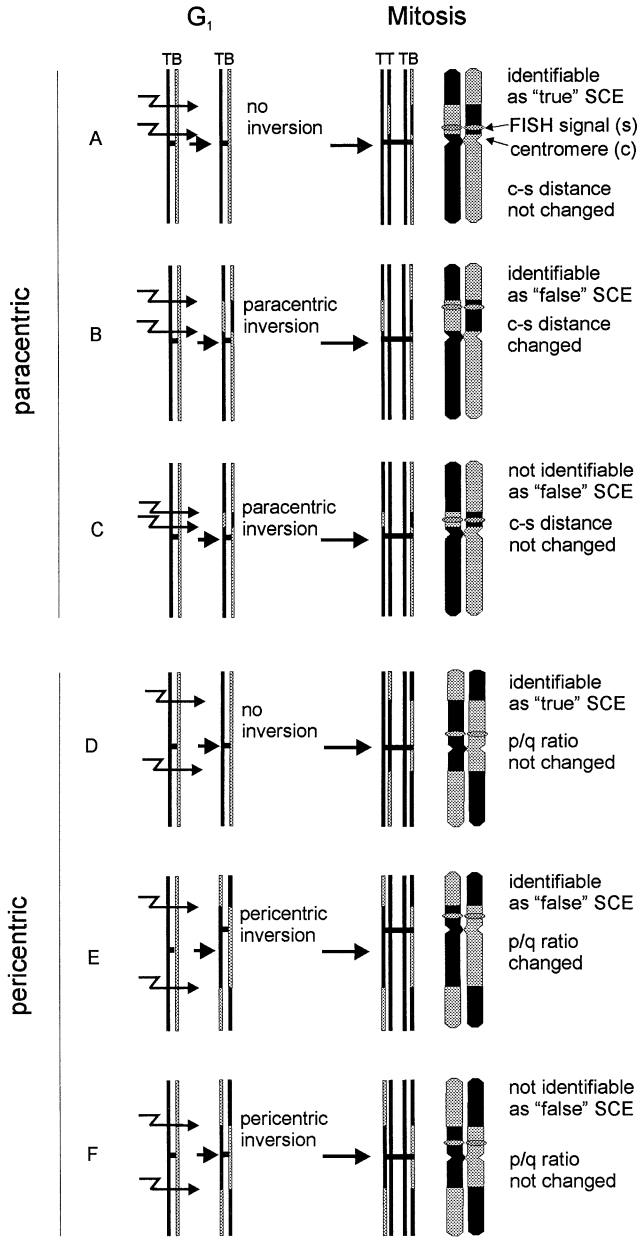

Fig. 1. Scheme of the labelling pattern of chromosome 3 and the types of SCE which could potentially be identified as inversions (**A**, **B**, **D** and **E**) and those where an identification was not possible (**C** and **F**).

pre-annealed at 37°C for 75 min and applied to microscope slides as described below.

In situ hybridization and FPG staining

Slides for *in situ* hybridization were treated with RNase (100 μg/ml 2× SSC at 37°C for 60 min), pepsin (50 μg/ml 0.01 M HCl at 37°C for 10 min) and a 1% solution of formaldehyde [in phosphate-buffered saline (PBS) with 50 mM $MgCl_2$, 10 min at room temperature]. Following dehydration in an alcohol series, slides were denatured for 30 min at 70°C in 90% formamide, 2× SSC (pH 7.0) and dehydrated in an ice-cold alcohol series. An aliquot of 14 μl of denatured YAC probe was applied to each slide, covered with 24×48 mm coverslips, sealed with rubber cement and the slides kept at 37°C in a humidified chamber overnight. Next day the slides were washed at 37°C with 50% formamide, 1× SSC (2×5 min) and 0.2× SSC (3×5 min). Detection of the hybridization signal was performed by incubation for 30 min each with (i) TRITC-labelled avidin (Vector), (ii) biotin-labelled anti-avidin (Vector), (iii) TRITC-labelled avidin (Vector). Avidin and anti-avidin were diluted in 4× SSC, 5% non-fat dry milk. Between incubations the slides were washed with a 4× SSC, 0.05% Tween 20 buffer and pre-incubated with 4× SSC, 5% non-fat dried milk for 5 min. Following incubation (iii), slides were washed

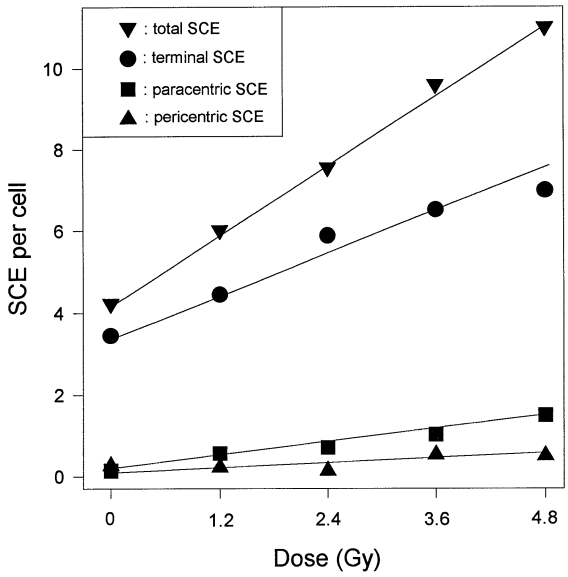

Fig. 2. Frequencies of SCE in all chromosomes stained by the FPG technique. For the total number of SCE, terminal SCE count as one and para- as well as pericentric SCE count as two.

three times with PBS and the BrdU was detected by incubation with mouse-anti-BrdU (CLB, The Netherlands) and FITC-labelled goat anti-mouse, 60 min each, diluted in PBS containing 0.1% bovine serum albumin. Following the final wash, slides were mounted in Dabco Antifade containing 1.5 μl/ml DAPI counterstain. Slides were analysed under a fluorescence microscope equipped with a CCD camera and filters for FITC, TRITC and DAPI. Computer images were obtained and analysed with the ISIS software of MetaSystems GmbH (Germany). The software enables visualization and enlargement of the images both in overlayed and separate colours and on-screen length measurements. The unit of measurement is the number of pixels.

Two slides from each treatment group were differentially stained by a modification of the fluorescence-plus-Giemsa (FPG) method of Perry and Wolff (1974). Slides were treated with 4.5 μg/ml 33258 Hoechst for 20 min, mounted with PBS, exposed to black fluorescent light on a 60°C hotplate for 15 min and stained with 5% Giemsa.

Classification of SCE and inversions

SCE were classified as terminal or interstitial. The latter were further subclassified as paracentric or pericentric. For analysis of SCE in chromosome 3, computer images of metaphases containing both chromosomes were saved. SCE which could result from a dicentric or a translocation were not considered. The analyses of SCE were performed on the computer screen. In addition to the total number of SCE in chromosomes 3, a record was kept of those SCE which could result from a detectable inversion, namely: (i) paracentric SCE with the hybridization signal positioned asymmetrically inside the SCE (Figure 1A and B); (ii) pericentric SCE with the centromere positioned asymmetrically inside the SCE (Figure 2E). In the case of paracentric SCE the distance between the centromere (c) and the hybridization signal (s) (c–s distance) was determined and in the case of pericentric SCE the arm ratio (p/q ratio) was calculated. Respective values were estimated as numbers of pixels and expressed as relative values in relation to the chromosome length. These were compared with the mean values measured in 50 non-irradiated chromosomes. An SCE was classified as an inversion when the appropriate value was outside the range of values estimated in the control chromosomes.

Fifty metaphases per treatment were scored for SCE on FPG stained slides. Metaphases were selected for evaluation on the basis of morphology and differential staining of chromatids without regard to structural aberrations. The classification of SCE was same as for analysis of chromosome 3.

Results

The frequencies of all types of SCE both in whole metaphases and in chromosome 3 increased linearly with dose (Figures 2 and 3). Terminal SCE were most frequent, followed by paracentric and pericentric SCE. The slope of the dose–response curve for terminal SCE was steeper when all chromo-

somes were analysed (Figure 2) than in chromosomes 3 (Figure 3).

The relative distance between the centromere and the FISH signal (c–s distance) in chromosome 3 as well as the arm ratio of chromosome 3 was determined in 50 unirradiated chromosomes. The mean relative c–s distance was 0.117 ± 0.025 (maximal value 0.161, minimal value 0.063). The mean p/q ratio was 0.934 ± 0.077 (maximal value 1.118, minimal value 0.787). The relative size of the FISH signal was 0.063 ± 0.006 (maximal value 0.07, minimal value 0.055).

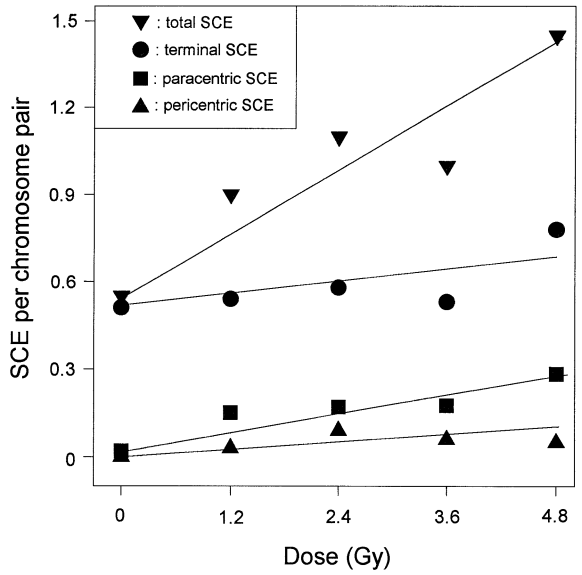

Fig. 3. Frequencies of SCE in chromosome 3 analysed with anti-BrdU antibodies. For the total number of SCE, terminal SCE count as one and para- as well as pericentric SCE count as two.

Based on these values we classified an SCE as a paracentric inversion when the c–s distance exceeded the maximal observed value of 0.161 (Figures 1B and 4). Similarly, a pericentric SCE was considered as an inversion when the p/q ratio was larger than 1.118 or smaller than 0.787 (Figures 1E and 4).

The results of the analysis of those SCE which could potentially be identified as inversions are presented in Table I. In total, 21 paracentric SCE were found which could result from a detectable inversion. Of these, only three SCE were accompanied by a modified c–s distance indicative of an inversion. In all three cases, the c–s distance exceeded the value of 0.2 and was therefore clearly larger than the maximum distance of 0.161 found in the controls. Similarly, out of 21 pericentric SCE with an asymmetrically positioned centromere, only two were found to be pericentric inversions with a modified p/q ratio. These results indicate that the majority of

Table I. Frequencies of para- and pericentric SCE and the appropriate frequencies of inversions in chromosome 3

Dose (Gy)	Paracentric		Pericentric		Cells scored
	SCE	Inversions	SCE	Inversions	
0	1	1	0	0	100
1.2	1	0	3	0	100
2.4	4	0	7	0	100
3.6	6	1	6	1	200
4.8	9	1	5	1	200

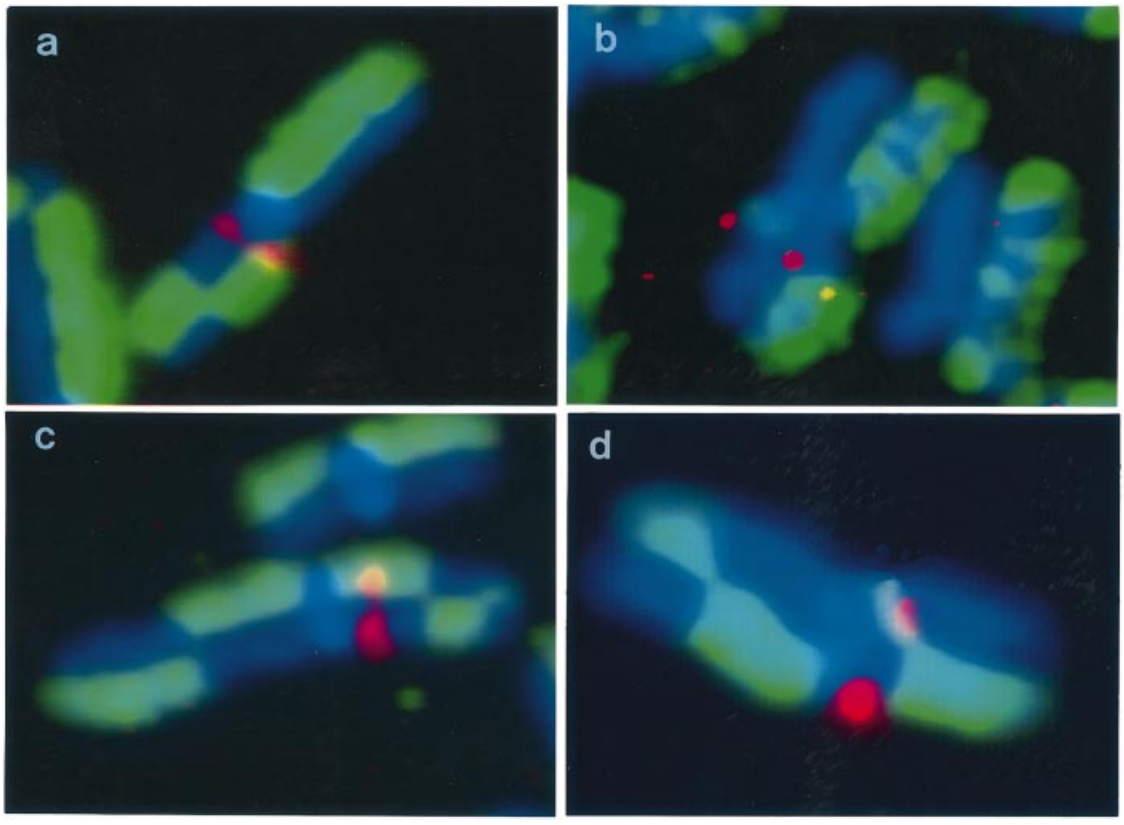

Fig. 4. Examples of SCE in chromosome 3. (**a**) 'True' paracentric SCE. (**b**) 'False' paracentric SCE. (**c**) 'True' pericentric SCE. (**d**) Paracentric SCE with a symmetrically positioned signal not identifiable as 'true' or 'false' SCE.

radiation-induced SCE, both para- and pericentric, are not inversions.

Discussion

Several models of SCE formation assume that the lesion responsible for an SCE is either base damage (Ishii and Bender, 1980; Wolff and Afzal, 1996) or a DNA–DNA crosslink (Shafer, 1977). Although a DNA double-strand break is obviously required for the formation of an SCE it is formed secondarily during S phase when the cell processes the damage (Painter, 1980). The finding that ionizing radiation, a primarily DNA strand break-inducing agent, is a poor inducer of SCE appears to support this. A straightforward explanation of SCE induced by irradiation of cells in G_1 which are unifilarily substituted with BrdU is that they are chromosomal aberrations (Mühlmann-Diaz and Bedford, 1995). These authors suggested that X- or γ-rays produce only 'false' SCE resulting from chromosomal exchange aberrations. We have tested this hypothesis by analysing SCE in the p arm of human chromosome 3. The fact that 37 out of 42 observed SCE were not inversions indicates that X-rays can induce 'true' SCE.

The applied method of analysis allowed discrimination between 'true' and 'false' paracentric SCE in a small region of chromosome 3. An unequivocal identification of paracentric inversions was only possible when the signal was positioned inside a SCE assymmetrically (Figure 1A and B, but not C). This had an impact on the minimal size of a SCE which could be identified as an inversion: firstly, it must have been larger than the size of the signal and, secondly, it had to be larger than the range of estimated c–s distances. Due to this the frequency of small 'false' SCE (i.e. small inversions) is probably underestimated. However, this does not weaken our conclusion that ionizing radiation induces 'true' SCE. With respect to pericentric SCE the minimal size of an SCE which could be identified as an inversion had to exceed double the c–s distance. Given the fact that chromosome 3 is nearly metacentric (p/q ratio 0.934), only those SCE inside which the centromere was positioned asymmetrically could be identified as inversions, because only then could a modified p/q ratio be detected (Figure 1D and E, but not F).

In accordance with our data, the results of several studies indicate that SCE and chromosomal aberrations arise by different mechanisms. Uggla and Natarajan (1983) found no enhancing effect of oxygen on radiation-induced SCE although a distinct effect was seen for aberrations. Morgan *et al*. (1983) observed no enhancing effect of radiation on SCE in cells irradiated in the presence of 3-aminobenzamide (3-AB), an inhibitor of poly(ADP-ribose) polymerase, although an enhancing effect of 3-AB is generally reported for radiation-induced chromosomal aberrations (Althaus and Richter, 1987).

We have shown recently that at least a certain proportion of radiation-induced SCE result from interaction of radiation with BrdU (Bruckmann *et al*., 1999). When incorporated in DNA, BrdU gives rise to alkali-labile sites and strand breaks by debromination and radical reactions and this process may be augmented by X-irradiation (Morris, 1991). This idea is in accordance with the fact that when human lymphocytes are irradiated in G_0 prior to addition of BrdU no SCE are observed (Littlefield *et al*., 1979). It is well known that when cells are irradiated in the G_0 or G_1 phase of the cell cycle without BrdU only chromosome-type aberrations are observed. Natarajan *et al*. (1980) reported that irradiation of cells following one round of replication with BrdU also leads to chromatid-type aberrations. This implies that in G_1 cells unifilarily substituted with BrdU, radiation-induced lesions of BrdU moieties persist until S phase, giving rise both to aberrations and SCE in a typical, S phase-dependent manner.

In conclusion, our results show that X-rays are capable of inducing 'true' SCE. Inversions which could give rise to 'false' SCE are also induced by radiation. Under our experimental conditions, the frequency of SCE is clearly higher than that of inversions.

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