An improved assay for radiation-induced chromatid breaks using a colcemid block and calyculin-induced PCC combination

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We report on a new method for the study of radiation-induced chromatid breaks in stimulated human peripheral blood T lymphocytes, involving a combination of a 1-h colcemid block and a short (15 min) calyculin A treatment. We find that this procedure eliminates the problem of centromere splitting when calyculin A is used alone for a longer period and produces metaphase spreads with superior quality. By this procedure, the chromosomes and the chromatid breaks are expanded and thereby make for improved break scoring. In a comparison of the new technique with the conventional colcemid block method, we show a close proportionality between the frequencies of chromatid breaks scored with the two methods. The frequency of chromatid breaks with the new method was found to be significantly higher than that with colcemid alone, adding a higher sensitivity to the assay as an additional advantage.

Introduction

The frequency of ionizing radiation-induced chromatid breaks in metaphases of phytohaemagglutinin (PHA)-stimulated human peripheral blood T lymphocytes (PBLs) has been shown to vary widely in different apparently normal individuals when the cells are sampled at 1.5 h following exposure. This short sampling time, including a 30-min incubation at 37°C to allow any mitotic cells to re-enter the G1 phase and a 1-h treatment with colcemid, ensures that the metaphase cells sampled by the colcemid block were in the G2 phase of the cell cycle at the time of radiation exposure. Further interest in this so-called ‘G2 assay’ of chromatid breaks was generated by the finding that the frequency of chromatid breaks is highly variable between different normal individuals and also significantly higher in PBL from over 40% of breast and other sporadic cancer cases (1–9). PBL derived from several different types (but not all types) of sporadic cancer cases also showed elevated frequencies of radiation-induced chromatid breaks (5,10). Earlier reports (e.g. 3,11) had claimed that most, if not all, cancer cells would show elevated chromatid break frequencies. However, such a universal hypothesis was not substantiated by other studies (e.g. 12,13).

Elevated chromatid breakage (sometimes referred to as ‘chromatid radiosensitivity’) has been interpreted in terms of the presence of mutations in low-penetrance genes that confer both chromatid radiosensitivity and susceptibility to breast cancer (1,4,6). These putative susceptibility or predisposing genes may be more penetrant in individuals with early onset cancers, although the evidence for this is controversial (14–16).

The concept of low-penetrance susceptibility genes in chromatin radiosensitivity has been strengthened by the demonstration of heritability of G2 chromatid radiosensitivity among families of breast cancer sufferers (4,17). Thus, further study and understanding of the mechanisms underlying the formation of chromatid breaks from radiation-induced DNA double-strand breaks could help clarify the reasons for breast cancer susceptibility.

In all these studies, conventional colcemid-induced mitotic block was used to collect metaphases, and as a consequence, the frequency of chromatid breaks scored could have been influenced by the activation of cycle arrest at the G2 checkpoint, allowing cells time to rejoin their chromatid breaks, and so possibly introducing some uncertainty into the interpretation of elevated chromatid radiosensitivity. Controversy surrounds this hypothesis. Some studies, e.g. of tumour cell lines in vitro (18), show a clear inverse relationship between mitotic inhibition and chromatid break frequency, whereas other studies using PBL from normal or breast cancer patients show only a very weak or no correlation at radiation doses normally used in the ‘G2 assay’ of chromatid breaks (19,20), suggesting that while cell cycle checkpoint delay may have a small influence on G2 chromatid break scores in stimulated PBL, it is not a major determinant of chromatid break frequency.

Recently, it has become possible to use the powerful phosphatase inhibitor calyculin A to efficiently cause premature chromosome condensation (PCC) of G2 cells so that chromatid breaks can be scored more directly following irradiation without the requirement of cells to progress to ‘normal’ mitosis (21–27). Unfortunately, in lymphocytes, calyculin induces not only chromosome condensation but also causes widespread centromere splitting (e.g. 24,28) making scoring more difficult due to the lack of close proximity of the sister chromatids and consequent possible displacement or loss of some chromatid fragments.

To circumvent centromere splitting in lymphocyte PCC, the duration of calyculin treatment can be reduced, and here we describe a technique that combines colcemid block and a short duration calyculin-induced PCC treatment, which gives improved chromosome visualization wider chromatid breaks and an enhanced sensitivity, almost entirely without the unwelcome side effect of centromere splitting. We have made a comparison between the chromatid break scores with colcemid block alone and colcemid plus calyculin treatments.

Materials and methods

Peripheral blood samples

Peripheral blood samples were obtained with informed consent and ethical approval from five normal control individuals and six breast cancer cases at...
Ninewells Hospital, Dundee. The blood samples were taken by venipuncture into heparinized vacutubes (Becton Dickinson, Plymouth, UK) and transported within 24 h to the Bute Medical School where experimental procedures were carried out.

**Cell culture and irradiation**

Blood samples were diluted 1:10 into pre-warmed RPMI medium (Gibco/Invitrogen, Paisley, UK) in 25-cm² tissue culture flasks (Nunc, Roskilde, Denmark) and 150 μl of PHA (HA16; Remel Europe, Dartford, UK) was added. Cultures were maintained at 37°C and in 5% CO₂ for 72 h. For each sample of blood, four 10 ml cultures were set up: two control cultures (sham irradiated) and two irradiated cultures (given 0.4 Gy of gamma rays in a CIS-UK gamma irradiator operating at a dose rate of ~3 Gy/min).

**Colcemid and calyculin treatment**

Following irradiation, cultures were allowed to incubate for 30 min at 37°C to allow any mitotic cells present during irradiation to progress into the next G₁ phase. Colcemid (KaryoMax; Gibco/Invitrogen) was then added at a concentration of 0.1 μg/ml for 1 h. Calyculin A (Sigma, Basingstoke, UK; stock solution 50 μg/ml in dimethyl sulphoxide; final concentration in medium 50 ng/ml) was added to two cultures (control and irradiated) for the last 15 min of the 1-h incubation with colcemid.

**Cell fixation and chromosome preparation**

Following incubation, cultures were centrifuged and the medium was aspirated. Pellets were vortexed and 5 ml of hypotonic solution (75 mM KCl; 4°C) was added while vortexing and samples were then held at room temperature for 10 min before centrifuging again. Hypotonic solution was aspirated, pellets vortexed again and 5 ml of fixative (methanol:acetic acid, 3:1, made up freshly) was added during vortexing. Tubes were allowed to stand for 10 min before centrifuging again, fixative aspirated and 5 ml of fixative again added during vortexing. This procedure was repeated four times, and cells were transferred to 1.5 ml eppendorf tubes in 0.5 ml of fixative. Ten microlitres of cell suspension was dropped on to ethanol-washed slides in a Hanabi humidifier apparatus (drying index below 8.0). Once dry, slides were stained in 5% Giemsa in Gurr’s buffer (pH 6.8) for 5 min, rinsed briefly in Gurr’s buffer and blotted dry on fibre-free paper.

**Chromatid break scoring**

Chromatid breaks were defined as any clear chromatid discontinuity (29). One hundred metaphases were scored for chromatid breaks using a metaphase finder (Applied Imaging, Newcastle on Tyne, UK) coupled with a Zeiss Axioplan 2 microscope using an oil immersion ×63 objective.

**Results**

Figure 1 (panels a and b) show examples of metaphases of irradiated cells using the colcemid plus calyculin method. It was found that the use of a combination of colcemid for 1 h, including calyculin for 15 min (Figure 1a and b), significantly reduced the centromere splitting (or loss of centromeric constriction) as observed with longer (30 min) calyculin treatment alone (Figure 1c and d). Although some chromosomes in metaphase (b) show a tendency for split centromeres, the chromatids still show centromeric constriction and remain closely adherent, thus allowing confident scoring of breaks. A further advantage of the colcemid plus calyculin method was the enlargement and clarity of breaks as compared to those seen following condensation with colcemid alone (see examples in Figure 1a and b). Since with the addition of calyculin to the colcemid block a proportion of G2 cells are condensed, the combination of the two agents substantially increases the number of scorable condensed chromosome figures.

Figure 2 shows a plot of chromatid break frequencies obtained with colcemid block alone against colcemid plus calyculin for the 11 individuals (five controls and six breast cancer cases). There was a clear linear relationship between the two end-points with a high degree of correlation (r² = 0.929). The overall mean chromatid break values for colcemid plus calyculin treatments (196 breaks/100 cells; SEM = 14) were significantly higher (P = 0.0001) than that for colcemid alone (155 breaks/100 cells; SEM = 11), thus making the combined assay more sensitive.

The mean chromatid break value (Figure 3) for the colcemid

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**Fig. 1.** Examples of PBLs chromosome spreads using (a and b) colcemid plus calyculin (15 min prior to harvesting) and using (c and d) calyculin alone for 30 min. Note the marked loss of centromeres and chromatid cohesion in cells (c) and (d), when using calyculin alone.
Data breaks have been subtracted. The coefficient of determination for the combined procedure is 0.929. Error bars represent standard deviations. 

G2 phase of the cell cycle. In a recent study (28) of ataxia telangiectasia (AT) cells, a combination of colcemid and calyculin was also used. However, in contrast to our procedure, these authors used a longer (30 min) calyculin treatment, purposely to yield a fraction of cells (PCC) with split centromeres that were assumed to be G2 cells. Terzoudi et al. showed that when scoring these cells with split centromeres, no enhancement of chromatid break frequency was found for AT cells as compared with normal controls.

However, for our data, the close proportionality of chromatid break frequencies between the colcemid plus calyculin procedure and the standard colcemid block technique for samples from both breast cancer cases and normal individuals (Figure 2) give us confidence to propose that the new procedure is not altering the ‘relative’ radiosensitivity of cells in the assays. The proportionality between the two different procedures also suggests that the radiosensitivity of cells as determined by the two assays is not significantly influenced by the G2 checkpoint, although some of the metaphases scored with the new calyculin procedure will arise from cells in the G2 phase. This could be a real difference between our assay and those used by others (23,28) where a longer calyculin treatment was used. It is possible that in our procedure, the short (15 min) additional calyculin treatment immediately prior to fixation tends to favour those G2 cells that are close to mitosis and initiating chromosome condensation. Hence, our technique could be preferentially sampling cells that are already beyond the G2 checkpoint. A further difference between our technique and that of Terzoudi et al. (28) is the radiation dose used. Terzoudi et al. used 1 Gy, a dose more than twice our standard dose of 0.4 Gy. The higher radiation dose could therefore significantly enhance the effect of the G2 checkpoint.

Thus, when coupled with the fact that we registered very few cells containing split centromeres, we suggest that our combined colcemid plus calyculin procedure has advantages over both the conventional colcemid block technique and the calyculin-alone method when scoring chromatid breaks in stimulated irradiated T lymphocytes.

Discussion

The new method described here using a combination of colcemid and calyculin was found to be superior to colcemid alone, in that scoring is made easier due to the enlargement of chromatid breaks. Also, the new method was found to have a higher sensitivity, giving significantly higher frequencies of chromatid breaks (Figures 2 and 3) for a standard dose of gamma rays (0.4 Gy) without a reduction in mitotic index which would be the case if dose was increased significantly to achieve the same frequency of chromatid breaks using the standard colcemid technique. Our data (Figure 2) show that the frequency of chromatid breaks in samples treated with the new method (colcemid and calyculin) are proportional to those with colcemid block alone ($r^2 = 0.929$), and their frequency is significantly higher, presumably as a result of including prematurely condensed G2 chromosomes (PCC) in the analyses.

Calyculin is a phosphatase inhibitor that causes condensation of cells in the G2 phase of the cell cycle, removing the need for cells to progress to mitosis (21–26). Thus, the metaphases scored following combined treatment with colcemid and calyculin will comprise cells from both mitosis and from the G2 phase of the cell cycle. In a recent study (28) of ataxia telangiectasia (AT) cells, a combination of colcemid and calyculin was also used. However, in contrast to our procedure, these authors used a longer (30 min) calyculin treatment, purposely to yield a fraction of cells (PCC) with split centromeres that were assumed to be G2 cells. Terzoudi et al. showed that when scoring these cells with split centromeres, no enhancement of chromatid break frequency was found for AT cells as compared with normal controls.

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