A comparison of G2 phase radiation-induced chromatid break kinetics using calyculin-PCC with those obtained using colcemid block

Peter E. Bryant* and Hossein Mozdarani1

Bute Medical School, University of St Andrews, St Andrews KY16 9TS, UK and 1Department of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, PO Box:14115-111, Tehran, Islamic Republic of Iran

To study the possible influence of cell-cycle delay on cells reaching mitosis during conventional radiation-induced chromatid break experiments using colcemid as a blocking agent, we have compared the chromatid break kinetics following a single dose of gamma rays (0.75 Gy) in metaphase CHO cells using calyculin-induced premature chromosome condensation (PCC), with those using colcemid block. Calyculin-induced PCC causes very rapid condensation of G2 cell chromosomes without the need for a cell to progress to mitosis, hence eliminating any effect of cell-cycle checkpoint on chromatid break frequency. We found that the kinetics of the exponential first-order decrease in chromatid breaks with time after irradiation was similar (not significantly different) between the two methods of chromosome condensation. However, use of the calyculin-PCC technique resulted in a slightly increased rate of disappearance of chromatid breaks and thus higher frequencies of breaks at 1.5 and 2.5 h following irradiation. We also report on the effect of the nucleoside analogue ara A on chromatid break kinetics using the two chromosome condensation techniques. Ara A treatment of cells abrogated the decrease in chromatid breaks with time, both using the calyculin-PCC and colcemid methods. We conclude that cell-cycle delay may be a factor determining the absolute frequency of chromatid breaks at various times following irradiation of cells in G2 phase but that the first-order disappearance of chromatid breaks with time and its abrogation by ara A are not significantly influenced by the G2 checkpoint.

Introduction

The frequency of chromatid breaks in human and rodent cells irradiated with X or gamma rays in the G2 phase of the cell cycle and sampled using colcemid block decreases with time between irradiation and cell harvest (1–7). The explanation for the observed first-order decline in chromatid breaks with time has been variously interpreted as representing (i) the rejoining of DNA double-strand breaks (DSBs; 1,3), (ii) the completion of a chromatid rearrangement (8,9) and (iii) that the decreasing number of chromatid breaks with time between exposure and harvest results from variation in the radiosensitivity of cells with cell-cycle stage within the G2 phase (10). According to the latter hypothesis, cells near to mitosis at the time of irradiation would have the highest radiosensitivity (i.e. a high frequency of chromatid breaks) and those furthest away from mitosis at irradiation (i.e. those entering G2 from S phase) would have the lowest radiosensitivity. One observation that argues against this hypothesis is that when the length of G2 phase in CHO cells is doubled by lowering and maintaining culture temperature at 33°C, the first-order disappearance of chromatid breaks continued at the same (exponential) rate as at 37°C and the frequency of breaks decreased by a further order of magnitude (5). If radiosensitivity of cells in G2 is cell-cycle stage dependent, the rate of disappearance with time would have changed as G2 phase was extended. The hypothesis (i) that chromatid breaks simply represent expanded DSB also appears untenable, since it was shown that while the decrease in chromatid breaks with time following irradiation is abrogated by treatment of Chinese hamster cells with the nucleoside analogue 9-β-D-arabinofuranosyladenine (ara A;11), the abrogation of chromatid break decline occurs in the absence of an inhibitory effect of ara A on DSB rejoining (9). The lack of inhibition of DSB rejoining by ara A in this system indicates that chromatid breaks, although clearly induced by DSB (e.g. 12,13), cannot be directly equated to this type of DNA damage.

Since the assay of chromatid breaks using colcemid block normally depends on the ability of cells to reach the metaphase stage of mitosis via movement through the G2 checkpoint, it seems logical to assume that the yields of chromatid breaks would depend on the ability of cells to pass the G2 checkpoint. The effect of the checkpoint would increase with dose of radiation. This reasoning is supported by results of experiments using ataxia telangiectasia (AT) cells, which show abrogated G2 cell-cycle checkpoint (14). When calyculin-induced premature chromosome condensation (PCC) was applied to AT cells following irradiation, frequencies of chromatid breaks in G2 were found to be similar in AT and normal control cells (15). Thus, agents such as ionizing radiation, which strongly increase the number of chromatid breaks in G2 phase cells, also potentially affect the passage of these damaged cells through G2 and into mitosis, and hence affect the number of chromatid breaks scored in metaphase cells.

The G2 checkpoint could also influence the kinetics (disappearance of chromatid breaks with time) of chromatid breaks following irradiation, i.e. cells suffering more damage could in theory be preferentially held back by the G2 checkpoint while the less-damaged cells progress on to mitosis. However, this scenario seems unlikely since, if true, the later arriving cells should show more chromatid breaks at metaphase than those (less damaged) escaping a significant block and arriving earlier; the opposite of what is observed experimentally. However, to examine more closely a possible influence of cell-cycle checkpoint delay on chromatid break frequency and kinetics, we have used calyculin A-induced PCC (15–21), where cells can be induced to condense their chromosomes within a few minutes, and are therefore not required to progress through the G2 checkpoint in order to arrive at mitosis for their

*To whom correspondence should be addressed. Tel: +01334 463 510; Fax: +01334 463 482; Email: peb@st-and.ac.uk

© The Author 2007. Published by Oxford University Press on behalf of the UK Environmental Mutagen Society. All rights reserved. For permissions, please e-mail: journals.permissions@oxfordjournals.org.

359
chromosomes to become condensed. We have compared the chromatid break frequencies using calyculin-PCC with kinetics determined using conventional colcemid block. So far as we can determine, there are no previous reports of such a direct comparison between the colcemid block and calyculin-PCC assays measuring the kinetics of chromatid breaks with time following irradiation.

As reported for human cells, in Chinese hamster cells calyculin A causes condensation of chromosomes of G2 cells within 5–10 min, thereby allowing a much more accurate kinetic analysis of chromatid break frequency with time after radiation exposure, without the necessity of cells progressing through the G2 part of the cell cycle to arrive at metaphase. Thus, any influence of G2 checkpoint on cell-cycle progression is removed when using the calyculin-PCC technique.

As mentioned above, ara A is known to abrogate the disappearance of chromatid breaks with time in both human and rodent cells (3,4,11), so we wished to examine also whether chromatid break kinetics in the presence of ara A were altered in the absence of the effect of G2 checkpoint when chromosomes were condensed using calyculin-PCC. We used a concentration of ara A previously shown to produce full abrogation of the decrease in chromatid breaks with time after irradiation but without affecting DSB rejoining (9).

Materials and methods

Cell culture
Chinese hamster CHOK1 cells were routinely cultured in Eagle’s MEM containing 10% foetal calf serum, glutamine and antibiotics in an atmosphere of 5% CO₂. Cells were passaged twice weekly and used for experiments while exponentially growing. For experiments, cells were cultured in 75 cm² flasks set up at 3 × 10⁵cells per flask in 10 ml of MEM and grown for 2 days.

Irradiation and ara A treatment
Cells were irradiated with gamma rays from a CIS-International Irradiator that delivers a dose rate of ~3.5 Gy/min. Irradiations (0.75 Gy; a dose found to be optimal in previous experiments, maintaining an adequate mitotic index for scoring) were performed at room temperature in air/5% CO₂ (in tissue culture flasks). Following irradiation, cells were immediately returned to 37°C in an incubator. Ara A was added to cultures at 100 μM, 30 min before irradiation to allow its phosphorylation, and cells were held in the presence of ara A throughout the time intervals between irradiation and sampling.

Calyculin-induced PCC assay of chromatid breaks
At various times following irradiation (times were chosen to correspond with harvesting times used in the colcemid block assay—see below), cells were trypsinized (trypsin-ethylenediaminetetraacetic acid for 6 min) and then centrifuged (5 min), most of the medium was removed and cells re-suspended in the remaining 1 ml of the medium. Calyculin A (Sigma) was added at a final concentration of 50 nM (1 ml of stock solution of 10 μg of Calyculin A in 200 μl of dimethyl sulfoxide). Cells were incubated at 37°C in a water bath for 5 min. Hypotonic solution (10 ml) (0.075 M KCl) was added for 10 min at room temperature. Cells were then centrifuged and fixed 4 × in 3:1 methanol:acetic acid. Following spreading, cells were stained with 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.

Assay of chromatid breaks using colcemid block
Following irradiation, flasks were incubated for at least 30 min before adding colcemid for 1 h immediately prior to harvest. Cells were then harvested at various times following irradiation. Following incubation, the growth medium was saved and cells were trypsinized and trypsin washes added to saved medium. Cells were centrifuged and re-suspended in 10 ml of hypotonic solution (0.075 M KCl) at room temperature for 10 min. Cells were again centrifuged and fixed 4 × in 3:1 methanol:acetic acid before spreading on slides and staining with giemsa (5% in Gurr’s buffer pH 6.8 for 5 min).

Scoring and analysis
Two hundred metaphases per sample were scored for the number of chromatid breaks. Breaks were defined as clear discontinuities in the chromatid (22).

Results

Figure 1 shows a semi-logarithmic plot of chromatid breaks as a function of time after a gamma ray dose of 0.75 Gy using the colcemid block assay. The first time point represents the yield of chromatid breaks following 30 min incubation without colcemid and 1 h in the presence of colcemid. The 30-min interval before colcemid was scheduled to allow those cells in mitosis at the time of irradiation (that show very few, if any, chromatid breaks) to pass on into the next G1 phase. The other time points in each case represent a 1-h treatment with colcemid following longer incubation times without colcemid. Ara A clearly completely abrogated the exponential decline in chromatid break frequency with time, as reported previously (11).

Figure 2 shows results of an equivalent kinetic experiment as shown in Figure 1, but performed using the calyculin-PCC assay. The frequency of chromatid breaks in the control arm of the experiment (i.e. without ara A treatment) shows a similar exponential (first order) rate of disappearance of breaks with time. However, the absolute frequency of chromatid breaks when using the calyculin-PCC method was found to be higher at the 1.5-h point than with the colcemid block assay by a factor of ~1.7.

![Figure 1](image1.png)

**Figure 1.** A semi-logarithmic plot of chromatid breaks as a function of time following irradiation in the presence (triangles) or absence (circles) of ara A using the colcemid-based assay.

![Figure 2](image2.png)

**Figure 2.** A semi-logarithmic plot of chromatid breaks in the presence (triangles) or absence (circles) of araA using PCC in G2 phase cells following irradiation.
In Figure 3, we have plotted the results for chromatid break kinetics using the two different condensation methods. Panel (a) shows the data from two independent experiments plotted semi-logarithmically to show the exponential (first order) decrease in chromatid breaks with time. Disappearance of chromatid breaks appeared to be more rapid when using the calyculin-PCC technique than with the colcemid method. However, the slopes of the exponentials (panel b) were not significantly different at the 95% level ($t = 3.363$). The frequencies of chromatid breaks and their kinetics during treatment with ara A were almost identical using the two different methods (Figures 1 and 2).

Discussion

A comparison of the results for the frequencies of chromatid breaks measured using the colcemid block assay and calyculin-PCC methods decline exponentially with time following irradiation (Figures 1 and 2) but that the number of chromatid breaks scored in CHO cells when using the calyculin-PCC method was higher, especially at 1.5 h following irradiation. The exponential disappearance of chromatid breaks with time using the calyculin-PCC technique appears more rapid, but was found not to be significantly different from the rate of disappearance of chromatid breaks measured by the colcemid block assay (Figure 3, panels a and b). The exponential rate of decline in chromatid break frequency in CHO cells using the calyculin-PCC assay is similar to that reported for human cells (17). Calyculin A condenses G2 cell chromosomes of CHO cells within 5–10 min (longer incubation with calyculin was found to result in cell lysis and entanglement during fixation), so providing a convenient means of examining the frequency of chromatid breaks in G2 phase without requiring cells to progress through the G2 phase of the cell cycle to mitosis. A possible reason for the higher frequency of chromatid breaks with the calyculin-PCC method at 1.5 and 2.5 h could be that PCC causes a more rapid chromosome condensation, possibly revealing additional narrow chromatid breaks that might otherwise be ‘hidden’ in the complex chromatin structure when using colcemid block with ‘natural’ chromosome condensation.

Another possible reason for the small difference in kinetics of chromatid breaks in samples condensed with colcemid and calyculin-PCC could be influenced by the passage of cells from the S phase into G2 during the post-irradiation period, thereby possibly adding some more radioresistant cells to the samples analysed, especially at longer time intervals.

The results we obtained with ara A treatment during the period between irradiation and sampling (Figures 1 and 2) are very similar when using either technique of condensation; the decline in chromatid breaks with time after irradiation is abrogated in chromosome samples condensed by both methods. The inhibitory action of araA on chromatid break ‘rejoining’ is puzzling; it does not appear to involve the inhibition of rejoining of DSB, since at 100 mM araA the rejoining of bulk DSB in CHO cells is not affected (9). However, we could not rule out the possibility that a very small subset of DSB (a fraction that might be invisible when using the electrophoresis method) was affected by the presence of araA. The abrogation of chromatid break kinetics by ara A are very similar to those we published previously using both human fibroblasts and lymphocytes, as well as CHO cells (3,4,6,11) where we did interpret the abrogation of chromatid break rejoining as inhibition of DSB rejoining. We also noted previously that in human fibroblasts, the frequency of chromatid exchanges increases with time following irradiation in the presence of araA, while the disappearance of chromatid breaks with time is abrogated (3); a further indication that chromatid breaks cannot be simply equated with DSB (i.e. assuming that DSBs are the lesions interacting and leading to exchanges).

It could be argued that because araA (a DNA synthesis inhibitor) acts by blocking cells in S phase, thereby preventing them (or restricting them) from entering G2, the abrogation of the decline in chromatid breaks with time by araA could be interpreted as the uniform radio response of a ‘pure’ cohort of G2 cells. However, as noted above, the entry of (more radioresistant) S phase cells into the G2 compartment would not be a significant factor in determining chromatid break kinetics in the case of cells collected by colcemid-induced mitotic block. Nevertheless, the abrogation of the disappearance of chromatid breaks by ara A in these (colcemid block) experiments is the same as in cells sampled by calyculin-PCC, suggesting that a different mechanism is most likely responsible for the abrogation.

The assumed lack of correspondence between DSB rejoining and disappearance of chromatid breaks with time (9) has, together with other evidence, led us to the conclusion that an
indirect mechanism must exist for the formation of chromatid breaks from DSB (8,9). Our hypothesis is that most (if not all) chromatid breaks are the result of chromatin rearrangements, which are only ‘initiated’ by the presence of a DSB within a megabase pair looped chromatin domain, which would subsequently be rejoined either by non-homologous end joining or homologous recombinational rejoining. Chromatid breaks disappear (rejoin) at the same rate following irradiation in xrs5 cells as in wild type CHO cells although the rejoicing of DSB in xrs5 is severely reduced (23). Thus, as with the model of Revell (24) on which the signal model is partly based (for a fuller explanation, see Bryant et al. 2004 [9]), our model proposes that all chromatid breaks are the result of chromatin rearrangements rather than simply ‘expanded’ DSB as is proposed by the ‘breakage first’ or ‘breakage and reunion’ model, modified by Bender et al. (25) to be based on DSB. Thus, under the ‘signal’ model, the disappearance of chromatid breaks with time (Figures 1, 2 and 3) therefore does not directly represent DSB rejoining, but represents the completion of chromatin rearrangements at the crossover points of large (Mbp) looped chromatin domains.

In summary, we show that the assay of chromatid break kinetics using colcemid block and the calyculin-PCC assay, both result in an exponential decrease of chromatid breaks with time. The rates of disappearance of chromatid breaks with time were not significantly different with the two condensation techniques. We conclude that the small differences in the kinetics revealed by the two techniques could be attributed to the effects of cell-cycle checkpoint block. However, the first-order exponential decline in chromatid breaks with time observed with calyculin-PCC cannot itself be attributed to the influence of cell-cycle block. We propose that the chromatid break kinetics represents the completion of chromatin rearrangements rather than the rejoicing of DSB.

Funding
Bute Medical School, University of St Andrews.

Acknowledgements
The authors wish to thank Mary Wilson and John Macintyre for excellent technical assistance and Dr Christie Marr for advice on statistical analyses.

References

Received on March 16, 2007; revised on May 22, 2007; accepted on May 30, 2007