Topoisomerase IIα levels and G2 radiosensitivity in T-lymphocytes of women presenting with breast cancer

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Previous studies from our laboratory have identified a link between intracellular topoisomerase IIα (topo IIα) levels and chromosomal radiosensitivity, as measured by the frequencies of chromatid breaks in the so-called G2-assay. Lower topo IIα levels were associated with reduced chromosomal radiosensitivity in cultured human cells. These findings supported a model, in which it is proposed that such chromatid breaks are the result of radiation-induced errors made by topoisomerase IIα during decatenation of chromatids. Studies from our and other laboratories, using the G2-assay, have shown that phytohaemagglutinin (PHA)-stimulated peripheral blood T-lymphocytes from 40% of female breast cancer cases show elevated chromatid break frequencies when exposed to a small standard dose of ionizing radiation, i.e. elevated above the 90th percentile of a group of female control samples. In the present study we have used a modified G2-assay to test whether elevated frequency of chromatid breaks in breast cancer cases is linked with elevated intracellular topo IIα level in PHA-stimulated T-lymphocytes, and also whether there is a general correlation between chromosomal radiosensitivity and topo IIα level. Our results confirm previous studies that 40% of breast cancer cases show elevated radiosensitivity as compared with controls. Also, the mean chromatid break frequency in breast cancer cases was significantly higher than in controls ($P = 0.0001$). We found that the mean topo IIα level in the cohort of breast cancer cases studied was significantly raised, as compared with controls ($P = 0.0016$), which could indicate a genetic propensity towards a raised intracellular production of topo IIα in these individuals. There was no direct correlation between chromosomal radiosensitivity and topo IIα level for individual samples either in the breast cancer cohort or in controls. However, a comparison between control and breast cancer samples shows a higher mean topo IIα level in breast cancer samples that correlates with the elevated mean chromatid break frequency seen in these patient samples. We found no meaningful correlations between either chromatid break frequency or topo IIα level and either tumour grade or hormone status. We conclude that elevated intracellular topo IIα level is likely to be a significant factor in determining the chromosomal response of stimulated T-lymphocytes from certain breast cancer cases.

Introduction

Studies from our laboratory (1–3) using human cells in culture have identified a link between intracellular topoisomerase IIα (topo IIα) levels and chromosomal radiosensitivity, as measured by the frequencies of chromatid breaks in the so-called G2-assay. The G2-assay, which can be applied to essentially any type of mammalian cell, involves exposing exponentially dividing cells in culture to a low standard dose of X- or gamma-rays and scoring the frequency of chromatid breaks in chemically blocked metaphase cells following cell fixation at 1.5 h after radiation exposure and staining. Sampling cells at 1.5 h ensures that all metaphases analysed were in the G2-phase of the cell cycle at the time of radiation exposure. In previous work (2,4) we used a modified G2-assay in which both colcemid and calyculin A were used to condense chromosomes. We found that low topo IIα levels were associated with reduced chromosomal radiosensitivity in cultured human cells (1,2). Studies from our and other laboratories (5–14) had shown using the G2-assay that PHA-stimulated peripheral blood T-lymphocytes show elevated chromatid break frequencies when exposed to a small standard dose of ionizing radiation, i.e. elevated above the 90th percentile of a group of control samples in 30–40% of female breast cancer cases. It was inferred that elevated chromosomal radiosensitivity could be indicative of mutations in low penetrance genes that might concomitantly lead to breast cancer susceptibility (5,8). It was also proposed that chromosomal radiosensitivity, which has also been found in a significant proportion of other cancer cases, is linked with abrogated rejoining of DNA double-strand breaks (dsb), and that the same reduced rejoining of dsb could itself contribute to cancer susceptibility (7). Studies from our laboratory have shown that there is a lack of correlation between rejoining of dsb and chromatid breaks (for review see 3). The causes of chromosomal radiosensitivity in lymphocytes of 40% of breast cancer cases are therefore so far unknown. Here we have tested whether chromosomal radiosensitivity in breast cancer and normal controls could be a result of variation in intracellular topo IIα levels in PHA-stimulated T-lymphocytes.

Materials and methods

Peripheral blood samples

Peripheral blood samples were obtained with informed consent and ethical approval (Tayside Committee on Medical Research Ethics B, Research Ethics Office: REC Reference number 07/S1402/10) from 96 normal control individuals and 89 breast cancer cases at Ninewells Hospital, Dundee. The controls (no personal past history or strong family history of breast cancer) were volunteers recruited from the local population via the regional clinical research unit by advertisement. The breast cancer patients were approached for trial consent at a clinic appointment shortly after the surgery for their breast cancer, and the subsequent blood sample was taken before any radiotherapy or chemotherapy treatment started. Patients in our centre are not usually offered two studies at one time, so early-stage cases not taking part in other cancer therapy trials are over-represented in this study population. The age of the cases ranged from 34 to 80 years (mean 61 years); the age of the controls ranged from 30 to 75 years (mean 54 years, but the ages of
Dynabeads CD3 (Dynal, 4 × 10^8 beads/ml in PBS containing 0.1% bovine supernatant was discarded and the cell pellet re-suspended in PBS/BSA to ensure removal of sodium azide (shipping and storage preservative) are coated with a primary monoclonal antibody specific for the CD3 membrane antigen, predominantly expressed on human T-lymphocytes. Dynabeads were shaken to resuspend in the stock vial. 30 ml of for the CD3 membrane antigen, predominantly expressed on human T-lymphocytes. Dynabeads were shaken to resuspend in the stock vial. 30 ml of PBS/BSA and transferred to 9 ml of RPMI containing 10% Fetal Calf Serum (FCS) in a 25 cm^2 culture flask. 150 µl PHA was added and cultures incubated for 3 days.

**Cell lysis and sample preparation**

Cells were pelleted by centrifugation (800 g for 5 min), washed twice by centrifugation in PBS (Gibco) and re-suspended at 10^6 cells per 14 ml of 10% Fetal Calf Serum (FCS) in a 25 cm^2 culture flask. 150 µl PHA was added and cultures incubated for 3 days.

**Protein assay using the QP-BCA assay**

Protein content of samples was determined by the quantiPro-bicinchoninic acid (QP-BCA, Sigma, Missouri, USA) assay. The assay relies on formation of a Cu^2+-protein complex under alkaline conditions, followed by reduction of the Cu^2+ to Cu+. The amount of reduction is proportional to the amount of protein present. The QP-BCA assay (Sigma, Kit 1) gives a linear response from 0.5 to 30 µg of protein. Standard protein samples (Sigma protein Standard Solution 10914) were run in parallel to each batch of samples and the protein content calculated using the slope of the standard graph. Samples (3 µl of lysate) and standards were made up to 125 µl in a 96-well plate, and 125 µl of QP working reagent added and incubated for 2 h at 37°C. The plate was read at 570 nm in a Dynex multiwell plate reader (Dynex Technologies, East Grinstead, UK).

**Metaphase block using colcemid and calyculin A**

Following metaphase 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 (Karyomyex, Gibco/Invitrogen, Paisley, UK) 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 sulfoxide (DMSO); final concentration in medium 50 ng/ml) was added to both cultures (control and irradiated) for the last 15 min of the 1 h incubation with colcemid. Cells were pelleted by centrifugation (800 g for 5 min), washed twice by centrifugation in PBS (Gibco) and re-suspended at 10^6 cells per 14 ml of 10% Fetal Calf Serum (FCS) in a 25 cm^2 culture flask. 150 µl PHA was added and cultures incubated for 3 days.

**Magnetic separation of T-lymphocytes**

Dynabeads CD3 (Dynal, 4 × 10^8 beads/ml in PBS containing 0.1% bovine serum albumin pH 7.4 (BSA) and 0.02% sodium azide as a shipping and storage preservative) are coated with a primary monoclonal antibody specific for the CD3 membrane antigen, predominantly expressed on human T-lymphocytes. Dynabeads were shaken to resuspend in the stock vial. 30 ml of Dynabeads were added to a 15 ml tube containing 5 ml PBS/BSA, mixed and the tube placed in a magnet (Dynal MPCTM) for 10 sec. With the tube in a magnet, the supernatant was removed. This step was repeated with further 5 ml PBS/BSA to ensure removal of sodium azide (shipping and storage preservative). Washed cells and beads were then re-suspended in 1 ml of PBS/BSA.

**Preparation of cells for the topoisomerase II assay**

Mononuclear cells were separated from whole blood using histopaque (Ficoll). 20 ml of histopaque was added to a 50 ml conical centrifuge tube on top of the histopaque layer. Tubes were centrifuged at 1600 revolutions per minute (rpm) for 30 min at 22°C with the brake turned off at the end of the spin. After centrifugation the upper layer and the opaque interface were collected and transferred to a clean 50 ml centrifuge tube. An equal volume of sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and centrifuged at 2000 rpm for 15 min at 22°C. The supernatant was discarded and the cell pellet re-suspended in PBS/BSA and centrifuged again.

**Analysis of data**

The topo IIα values from the DAS-ELISA assay (A400nm values) were corrected for the amount of sample protein extracted by dividing by the amount of protein present in each sample. By this means we obtained a Topo IIα protein ratio, which could be compared with the chromatid break frequency determined by the G2-assay for each sample. We were not able to recover lysates with sufficient amounts of protein from all samples. The reasons for this are unknown. However, it was observed that in a proportion of both controls and breast cancer cultures cells failed to undergo significant cell proliferation, presumably as a result of failed PHA stimulation, possibly due to sensitivity of the breast cancer cultures. Also, 18 control samples and 19 breast cancer samples were scored for chromatid breaks but not analysed for protein and topo IIα levels. However, we were able to score and analyse all samples using the modified G2-assay.
Results

Figure 1 shows the results of the analysis of chromatid breaks in metaphase cells of cells exposed to 0.4Gy of gamma-rays and processed by the modified G2-assay described above (unirradiated control frequencies of chromatid breaks were subtracted). A five-fold range of individual radiosensitivities is apparent in control samples and an overlapping four-fold range of radiosensitivities observed in breast cancer samples. The histogram also shows that 41% of breast cancer samples showed frequencies of chromatid breaks above the 90th percentile of the control cohort, confirming our previous study of Tayside breast cancer patients [10]. Further analysis of the data (figure 2) showed that the overall mean frequency of chromatid breaks in breast cancer cases ($m = 177.8, n = 66$) was significantly higher ($P = 0.0001$) than that for controls ($m = 126.3, n = 72$). In figures 3 and 4 the topo IIα/protein ratio is plotted against frequency of chromatid breaks. There was no significant correlation between these two parameters in either controls (Figure 3) or breast cancer samples (Figure 4).

Fig. 1. Frequency of chromatid breaks in metaphases of stimulated peripheral blood lymphocytes from breast cancer cases and controls.

Fig. 2. A comparison of mean chromatid break frequencies in stimulated peripheral blood lymphocytes from breast cancer cases and controls.

Fig. 3. Chromatid break frequency compared with Topo IIα/protein ratio for control samples.

Fig. 4. Chromatid break frequency compared with Topo IIα/protein ratio for breast cancer samples.
However, we found (Figure 5) an almost three-fold difference ($P = 0.0016$) in the mean topo IIα/protein ratios between breast cancer samples ($m = 0.03798, n = 33$) and controls ($0.01348, n = 29$). Thus, although individual values of chromatid breaks do not correlate with topo IIα ratio (Figures 3 and 4), the mean topo IIα/protein ratio does correlate with an increased mean chromatid break frequency (Figures 5 and 6). This is further illustrated by Figure 7 that shows a plot of mean chromatid break frequency against mean topo IIα ratio for controls and breast cancer cases.

We have examined a possible relationship between chromatid break frequency and either tumour grade or hormone status in 33 breast cancer patient samples. No meaningful correlation was found between these two parameters (data not shown). Similarly, we examined the relationship between topo IIα levels and either tumour grade or hormone status in 33 breast cancer patient samples, and again found no correlation (data not shown).

![Fig. 5](https://example.com/fi5.png)

**Fig. 5.** A comparison of mean topo IIα/protein ratio between controls and breast cancer samples.

![Fig. 6](https://example.com/fi6.png)

**Fig. 6.** A comparison of chromatid break frequencies in gamma-irradiated lymphocytes from controls and breast cancer cases corresponding to the topo IIα samples plotted in Figure 5.

![Fig. 7](https://example.com/fi7.png)

**Fig. 7.** Mean topo IIα ratio plotted against mean chromatid break frequency for controls and breast cancer cases.

**Discussion**

Despite being a common cancer affecting 1 out of 8 of the UK female population, the factors that determine susceptibility to breast cancer and the molecular mechanisms underlying inter-individual variation of tumour and normal tissue response to radiotherapy are poorly understood. We have examined the peripheral blood lymphocytes from patients with newly diagnosed breast cancer to determine whether variation in topo IIα protein levels might account for such variation.

Our data for chromatid break frequencies in breast cancer samples show that 41% are in excess of the 90th percentile of the control group (Figure 1). This result confirms our previous study of Tayside breast cancer cases in which 46% of breast cancer samples showed chromatid break frequencies in excess of the 90th percentile of controls (10), and also agrees with the majority of studies performed on breast cancer patients (5–14, 16). One study of breast cancer patients in London (17) concluded there was no difference between breast cancer and a group of controls. We also found (Figure 2) that the mean frequency of chromatid breaks in breast cancer samples was significantly higher ($P = 0.0001$) than that in our control group, which also agrees with data from other laboratories (9). Studies on patients with other cancer types using the G2-assay have reported similar elevated frequencies of chromatid breaks in the cancer cases as compared with controls (9,13,14,18). Scott and his colleagues (8) showed that the radiosensitivity (as measured by chromatid break frequency) of breast cancer cases is a heritable trait, and a Mendelian pattern of inheritance was inferred, possibly allowing future application of the G2-assay as a predictive tool.

The striking variation in radiosensitivity of breast cancer patients and controls (see Figure 1), which we presume has a genetic basis, led us to question whether an underlying cause could be a variation in the intra-cellular levels of topo IIα in individual samples, since in previous studies we found a strong correlation between topo IIα and the frequency of radiation-induced chromatid breaks (1,2). Both in human HL60 (human promyelocytic leukaemia) variant cell lines expressing reduced levels of topo IIα, and in hTERT transformed retinal pigment epithelial (hTERT-RPE1) cells in which intracellular expression of topo IIα had been silenced by siRNA (2), chromatid break frequencies in response to low doses of gamma-rays were shown to be significantly reduced.

Based on these findings we have tested whether the wide variation in radiation-induced chromatid break frequency observed in previous studies could be due to a genetically determined variation in intracellular expression of topo IIα. We showed (Figure 5) that the mean topo IIα ratio was significantly higher in the breast cancer cohort than in controls ($P \leq 0.0001$), which correlates with mean chromatid break frequency (Figure 6). However, there was no evidence of a direct correlation between individual radiosensitivity of either breast cancer or control samples and topo IIα ratio (Figures 3 and 4), indicating that topo IIα variation cannot be a single determinant of an individual’s chromatid radiosensitivity.

Thus, while it is clear that factors other than topo IIα level will contribute to the chromatid break response in the G2-assay, we conclude that the elevated intracellular topo IIα level found in stimulated T-lymphocytes from breast cancer cases is likely to be a significant factor in determining the chromosomal response in the G2-assay.
Authors contributions

PEB and ACR supervised the laboratory research that was conducted by OS and PEB. DJAA and JAD facilitated and arranged collection of the clinical material and collaborated during preparation of the manuscript.

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Conflict of interest statement: None declared.

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