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Original Articles

Correlation between the radiation responses of fibroblasts cultured from individual patients and the risk of late reaction after breast radiotherapy

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ABSTRACT

Late normal tissue toxicity varies widely between patients and limits breast radiotherapy dose. Here we aimed to determine its relationship to DNA damage responses of fibroblast cultures from individual patients. Thirty-five breast cancer patients, with minimal or marked breast changes after breast-conserving therapy consented to receive a 4 Gy test irradiation to a small skin field of the left buttock and have punch biopsies taken from irradiated and unirradiated skin. Early-passage fibroblast cultures were established by outgrowth and irradiated *in vitro* with 0 or 4 Gy. 53BP1 foci, p53 and p21/CDKN1A were detected by immunofluorescence microscopy. Residual 53BP1 foci counts 24 h after *in vitro* irradiation were significantly higher in fibroblasts from RT-sensitive versus RT-resistant patients. Furthermore, significantly larger fractions of p53- but not p21/CDKN1A-positive fibroblasts were found in cultures from RT-sensitive patients without *in vitro* irradiation, and 2 h and 6 d post-irradiation. Exploratory analysis showed a stronger p53 response 2 h after irradiation of fibroblasts established from patients with severe reaction. These results associate the radiation response of fibroblasts with late reaction of the breast after RT and suggest a correlation with severity.

Introduction

In women treated with radiotherapy for primary breast cancer after local excision of the primary tumour, breast shrinkage, hardness and pain are common consequences [1–3]. A study of telangiectasia by Turesson [4] showed considerable variation in severity between patients treated under the same conditions. Analysis of these data suggested that if all known extrinsic factors are controlled, those intrinsic to the individual may account for ≥80% of clinical complication risk [5,6].

Radiation damage to fibroblasts is considered to be a key factor in the pathogenic pathway leading to fibrosis. However, although initial results suggested that intrinsic radiosensitivity of fibroblasts isolated from individual patients may predict the patients' risk of developing fibrosis after radiotherapy [7–10], correlations were weak and subsequent studies did not confirm a significant correlation [10–16]. This has been explained by the hypothesis that *in vitro* cellular responses correlate poorly with *in vivo* responses due to the modifying influence of tissue environment [17–19]. The number of residual radiation-induced DNA double-strand breaks (DSBs) has received less attention. An early study reported a correlation between the severity of fibrosis and the fraction of DNA released by pulse-field gel electrophoresis (PFGE) after irradiation of early-passage fibroblast strains *in vitro* [20], but this was not confirmed in a validation cohort [21] or in a different study using constant-field gel electrophoresis [11]. However, very high doses (up to 150 Gy) were used to detect residual DSBs by this technique. Since the latter study found significantly higher numbers of lethal chromosome aberrations in lymphocytes irradiated with a more moderate dose of 6 Gy from patients with severe late reaction, there is still a powerful argument for exploring ways of measuring cellular responses to relevant doses of radiotherapy *ex vivo* using the much more sensitive technique of DNA DSB repair foci.

The purpose of the present study was to explore the relationship between the risk of developing late breast shrinkage/hardening after radiotherapy and the DNA damage response in cultures of dermal fibroblasts from individual patients, established from unirradiated skin and skin given a test irradiation dose *in vivo*.

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Materials and methods

Patients, in vivo *irradiation, and establishment of fibroblast cultures*

The manner of patient selection had been previously described [22]. Briefly, patients in this study had taken part in two randomised clinical trials organised by the Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, comparing fractionation schemes and irradiation techniques, respectively, with prospective annual clinical assessments of late adverse effects using standard proformas [23,24]. Thirty-five breast cancer patients who showed no evidence of recurrent cancer 3–10 years after surgical excision of the tumour and post-operative radiotherapy to the whole breast consented to a test irradiation and subsequent biopsies of the skin 24 h and 12 weeks after irradiation. Ethical approval for the study was obtained from the Royal Marsden NHS Research Ethics Committee, and written consent was obtained from the patients prior to participation.

The study group comprised of clinically RT-sensitive patients (n = 20) and RT-resistant (n = 15) patients based on the severity of their reactions versus known clinical risk factors and was enriched for highly sensitive patients. Follow up was 3–24 years for RT-sensitive (median 11 years) and 11–24 years (median 13 years) for RT-resistant patients. Patient characteristics and treatment parameters are shown in Table 1. Overall, RT-sensitive patients had larger surgical deficits but received less tumour-bed boost irradiation and chemotherapy. One RT-sensitive patient withdrew consent prior to the 12-week biopsies, leaving 34 patients for analysis in the present study. For exploratory analysis, RT-sensitive patients were further subdivided into two subgroups of moderate risk (n = 9) and high risk (severe reaction, n = 10).

Test irradiation of the skin was performed as previously described [25]. Patients received a single 4 Gy radiation dose to a small area of skin on the upper outer quadrant of the buttock. A 6 MeV electron beam from a radiotherapy linear accelerator exposed an area of skin 4 × 2 cm, and an 8 mm perspex build-up filter was used to ensure dose homogeneity throughout the epidermis and dermis.

Twelve weeks following irradiation, four 4 mm punch biopsies were obtained from both irradiated and contralateral unirradiated skin. Two of the replicate biopsies were used to establish primary fibroblast cultures for the present study. The skin samples were shipped to Mannheim in serum-free basal medium at ambient temperature where they were cut into smaller pieces, placed in T25 tissue culture flasks and left to adhere for 2 h. After attachment, cells were incubated with cell culture medium (Gibco AmnioMax C100 basal medium; Life Technologies GmbH, Darmstadt, Germany) supplemented with 7.5% AmnioMax C100 supplement (Life Technologies), 7.5% foetal bovine serum (FBS; Biochrom AG, Berlin), 2 mM glutamine, and penicillin/streptomycin. Primary cultures established by outgrowth were expanded by passaging twice and cryopreserved in the 3rd passage. For the present in vitro experiments, frozen vials of fibroblasts in passage 3 were thawed, expanded by passaging twice and used for the present experiments in passage 5.

Fibroblast cultures were successfully established from all patients and showed similar levels of the human proliferation marker Ki-67 (MKI67) in cultures established from skin irradiated *in vivo* (clinically) 12 weeks earlier as well as cultures from unirradiated skin (Supplementary Fig. S1).

Table 1
Patient characteristics and treatment parameters.[a] Radiotherapy (RT) dose to the whole breast, RT technique (3D or standard 2D wedge), tumour bed boost, surgical deficit, and axillary treatment, were significant risk factors.

	RT-sensitive	RT-resistant
Patients (n = 35)	20[b]	15
Median age, years (range)	70 (52–83)	68 (54–78)
Median follow-up, years (range)	11 (3–24)	13 (11–24)
Mean breast RT dose, Gy*	50.0	50.8
Dosimetry techniques		
3D	10	3
2D	10	12
Number patients prescribed boost dose	15	15
Mean tumour bed boost dose, Gy	9.8	12.7
Breast size		
Small	8	2
Medium	10	13
Large	2	0
Surgical deficit		
Small	8	11
Medium	8	3
Large	4 (1 mastectomy[c])	1
Axillary treatment	15	11
Tamoxifen	14	12
Chemotherapy	8	15

[a] Equivalent total dose given in 2 Gy fractions assuming $\alpha/\beta = 3$ Gy.
[b] One RT-sensitive patient (risk score 1) withdrew consent before biopsies were taken at 12 weeks.
[c] Patient had mastectomy and reconstruction before RT; this was the only patient with <5 year follow up.

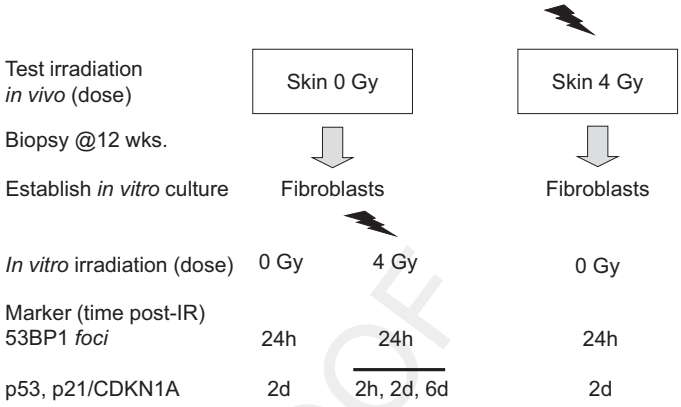

Fig. 1. Experimental design of the present study. Residual foci were also scored in sections of skin biopsies 24 h after irradiation *in vivo* and will be presented elsewhere (Somaiah et al. submitted).

The study design is shown in Fig. 1. Residual 53BP1 (TP53BP1) foci were determined 24 h after *in vitro* irradiation of fibroblasts established from unirradiated skin biopsies taken at the 12-week time point. Furthermore, 53BP1 foci were determined in unirradiated parallel fibroblast cultures established from biopsies taken from unirradiated and *in vivo* irradiated skin 12 weeks after irradiation. p53 (TP53) and p21/CDKN1A were determined 2 h, 2 d and 6 d after irradiation *in vitro* and in unirradiated cultures from unirradiated and *in vivo* (clinically) irradiated skin (in parallel with day 2 samples).

Irradiation and immunofluorescence microscopy of fibroblasts in vitro

5×10^3 cells were seeded per well in chamber slides (BD Falcon), incubated overnight and irradiated the next day with 4 Gy of 6 MV X-rays from a linear accelerator (Synergy, Elekta, Crawley, UK) at a dose rate of 6 Gy/min. After irradiation, cells were incubated at 37 °C under CO_2 and fixed at different time points. The cells were rinsed with PBS, fixed for 15 minutes at room temperature with 3.7% paraformaldehyde and permeabilised for 5 minutes with 0.5% Triton X-100 in PBS at 4 °C. Slides for detection of residual γH2AX and 53BP1 foci 24 h after irradiation were shipped in PBS to the PHE Centre for Radiation, Chemical and Environmental Hazards, Chilton, UK and processed as previously described [26]. For detection of Ki-67, p53 and p21/CDKN1A, fibroblasts were treated and fixed at 2 h, 2 d and 6 d after irradiation using 3.7% formaldehyde in PBS with 0.2% Triton X-100 (PBST) for 10 min. Details of antibodies and staining protocols are given in Supplementary Material.

Statistical analysis

Differences between fibroblasts from RT-sensitive and RT-resistant patients were analysed by the non-parametric Wilcoxon/Mann–Whitney test. Correlations were analysed by linear regression or the non-parametric Spearman's ρ rank correlation test. A linear model was used to test the effect of measured parameters on clinical radiosensitivity. All tests were performed using the JMP.v11 Statistical Discovery software package (SAS Institute GmbH, Boeblingen, Germany). $P < 0.05$ was considered statistically significant for the planned analysis, and $P < 0.01$ for exploratory analysis.

Results

Quantification of residual DSB in skin fibroblasts in vitro

The mean number of residual 53BP1 foci per cell in the nuclei 24 h after *in vitro* irradiation of fibroblasts established from unirradiated skin was significantly increased over the background. The median for all patients was 2.07 [quartiles: 1.73; 2.46] for 4 Gy versus 0.64 [quartiles: 0.51; 0.83] for 0 Gy ($P < 0.0001$). We compared these values with the residual and background numbers of foci in fibroblasts scored *in situ* in sections of skin biopsies 24 h after the clinical test irradiation of the skin *in vivo* (Somaiah et al., submitted). Overall, the two sets of values for irradiation with 4 Gy *in vitro* or *in vivo,* and their corresponding background values without irradiation, compared quite well although the difference in mean foci numbers between irradiated and unirradiated cells was larger