

## Clinical radiobiology

# Wound healing morbidity in STS patients treated with preoperative radiotherapy in relation to in vitro skin fibroblast radiosensitivity, proliferative capacity and TGF- $\beta$ activity

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### Abstract

**Background and purpose:** In a recent study, we demonstrated that the ability of dermal fibroblasts, obtained from soft tissue sarcoma (STS) patients, to undergo initial division in vitro following radiation exposure correlated with the development of wound healing morbidity in the patients following their treatment with preoperative radiotherapy. Transforming growth factor beta (TGF- $\beta$ ) is thought to play an important role in fibroblast proliferation and radiosensitivity both of which may impact on wound healing. Thus, in this study we examined the interrelationship between TGF- $\beta$  activity, radiosensitivity and proliferation of cultured fibroblasts and the wound healing response of STS patients after preoperative radiotherapy to provide a validation cohort for our previous study and to investigate mechanisms.

**Patients and methods:** Skin fibroblasts were established from skin biopsies of 46 STS patients. The treatment group consisted of 28 patients who received preoperative radiotherapy. Eighteen patients constituted a control group who were either irradiated postoperatively or did not receive radiation treatment. Fibroblast cultures were subjected to the colony forming and cytokinesis-blocked binucleation assays (low dose rate:  $\sim 0.02$  Gy/min) and TGF- $\beta$  assays (high dose-rate:  $\sim 1.06$  Gy/min) following  $\gamma$ -irradiation. Fibroblast radiosensitivity and initial proliferative ability were represented by the surviving fraction at 2.4 Gy (SF<sub>2.4</sub>) and binucleation index (BNI), respectively. Active and total TGF- $\beta$  levels in fibroblast cultures were determined using a biological assay. Wound healing complication (WHC), defined as the requirement for further surgery or prolonged deep wound packing, was the clinical endpoint examined.

**Results:** Of the 28 patients treated with preoperative radiotherapy, 8 (29%) had wound healing difficulties. Fibroblasts from patients who developed WHC showed a trend to retain a significantly higher initial proliferative ability after irradiation compared with those from individuals in the treatment group with normal wound healing, consistent with the results of our previous study. No link was observed between fibroblast radiosensitivity and WHC. Neither active nor total TGF- $\beta$  levels in cultures were significantly affected by irradiation. Fibroblast proliferation in unirradiated and irradiated cultures, as well as radiosensitivity, was not influenced by TGF- $\beta$  content. TGF- $\beta$  expression in fibroblast cultures did not reflect wound healing morbidity.

**Conclusions:** These data are consistent with our previous study and combined the results suggest that in vitro fibroblast proliferation after irradiation may be a useful predictor of wound healing morbidity in STS patients treated with preoperative radiotherapy. TGF- $\beta$  levels in culture do not predict WHC, suggesting that the role of TGF- $\beta$  in wound healing is likely controlled by other in vivo factors.

© 2005 Published by Elsevier Ireland Ltd. Radiotherapy and Oncology 78 (2006) 17-26.

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**Keywords:** Fibroblasts; Radiosensitivity; Proliferation; TGF- $\beta$ ; Wound healing

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Preoperative irradiation is known to result in wound healing complications (WHC) [22,49]. This treatment modality is the standard for most patients with large, deep, high-grade soft tissue sarcoma (STS) treated at the Princess Margaret Hospital/Mount Sinai Hospital, and a significant proportion of patients treated with this procedure develop WHC [7,31,39]. Methods for predicting patient-specific potential for WHC following radiotherapy would be valuable in therapy selection for STS patients. Attempts to establish in vitro predictive assays for normal tissue response to radiotherapy have been based on fibroblast clonogenic survival, differentiation and DNA damage [3,5,6,11,12,37,38,40,46]. Results of these studies have been mixed with findings ranging from relationships between in vitro endpoints and normal tissue reactions [3,6,40] to no useful associations [5,11,12,46].

It has been suggested that radiation-induced premature terminal differentiation of fibroblasts may lead to the accumulation of postmitotic fibrocytes and increased synthesis and deposition of extracellular collagen, an important factor for wound healing [4,28,30,40,43,44]. Since postmitotic fibrocytes produce larger quantities of collagen than mitotic fibroblasts [28,42], the ability of fibroblasts to differentiate after irradiation may play an important role in the effects of radiation on wound healing. Other studies have suggested that the effects of radiation on the clonogenic capacity of fibroblasts may be responsible for the delay in wound healing following radiotherapy and surgery [9,15,20-22].

Radiation leads to reduced fibroblast proliferative capability and function and this would be expected to impact negatively on the normal tissue response [13,49]. We demonstrated in a previous study that skin fibroblasts from individuals who developed wound healing abnormalities following pre-operative radiotherapy tended to show a smaller reduction in early proliferation after irradiation [2]. Increased proliferative potential may render the fibroblasts less capable of differentiating to produce the collagen necessary for effective wound healing.

Irradiation of fibroblasts in vitro has been reported to lead to a strain-specific induction of TGF- $\beta$  [4,42,50], which in turn plays a central role in the induction of terminal differentiation [7,26,43]. This premature terminal differentiation has been proposed to be responsible for the accumulation of postmitotic fibrocytes and increased synthesis and deposition of extracellular collagen [4,25,28,30,32,33,35,43,44,52]. Investigation of the involvement of TGF- $\beta$  in modulating fibroblast proliferation, radiosensitivity and differentiation in culture, and the corresponding associations between these in vitro parameters and normal tissue responses to irradiation is warranted.

In the current study, we used fibroblasts from 46 STS patients (28 treated with preoperative radiotherapy; 18 untreated or irradiated postoperatively constituting a control group) to validate our earlier finding that radiation-induced fibroblast proliferative arrest in vitro, but

not radiosensitivity, predicts for wound healing complications [2]. Further, we assessed WHC in a total of 72 STS patients (26 from the initial cohort [2] and 46 from this validation cohort) in relation to intrinsic TGF- $\beta$  expression, proliferative arrest and radiosensitivity of cultured fibroblasts.

## Materials and methods

### Patients

Fifty-four patients were treated with preoperative radiotherapy (tumour dose of 50 Gy in 25 fractions) followed by resection of their tumours ~6 weeks later. Of these, 26 were from the initial cohort (11 showing wound healing complications (WHC)) and 28 were from the validation cohort (8 showing WHC). WHC was defined as the requirement of further surgery, re-admission to hospital for intravenous antibiotics, aspiration of a seroma in a clinic, or prolonged deep wound packing. Patients with no detectable wound healing complications were designated as NoWHC. Eighteen patients who were either not irradiated or had received post-operative radiotherapy constituted a control group. No patient in the control group developed WHC. In all, there were 27 females and 45 males with a mean age of 53 years (range: 18-85 years).

### Fibroblast strain, MELC and culture maintenance

Fibroblast strains were established from normal skin biopsies obtained with informed consent from 72 STS patients treated at the Princess Margaret Hospital/Mount Sinai Hospital. Biopsies were taken from normal skin distant to the site of surgery and/or radiation treatment as described previously [2]. Of these, 26 were from the initial cohort [2] and 46 (28 treated with preoperative radiotherapy; 18 untreated or irradiated postoperatively and constituting a control group) were from the validation cohort. Mink lung epithelial cells (MLEC-PAI) stably transfected with an expression construct containing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene [1] were kindly provided by Dr M.H. Barcellos-Hoff (Lawrence Berkeley National Laboratory, CA, USA). Fibroblasts and MLEC were cultured in alpha minimal essential medium (Gibco BRL, Burlington, Ont, Canada) supplemented with 10% foetal bovine serum, FBS (Cansera, Rexdale, Ont, Canada), 10  $\mu$ g/ml streptomycin and 10  $\mu$ g/ml penicillin and were incubated at 37 °C in a 5% carbon dioxide humidified atmosphere in air.

### Determination of radiosensitivity

For colony forming experiments, confluent fibroblasts cultures were irradiated at a low dose rate of ~2.0 cGy/min using <sup>60</sup>Co  $\gamma$ -rays to doses ranging from 0 to 13.6 Gy as described previously [2]. Irradiated and control cultures were trypsinized into single-cell suspensions, plated into 100 cm<sup>2</sup> tissue culture dishes and incubated for 7-10 days. Colonies were simultaneously fixed and stained with

1% methylene blue in 50% ethanol for 15 min, washed with tap water and air dried for bright field phase microscopy and counting. Based on our previous work [2], mitotic, mixed and postmitotic colonies with greater than 10 cells were scored and the surviving fraction at 2.4 Gy ( $SF_{2.4}$ ) was determined by fitting the survival data to the linear-quadratic model. Data were expressed as the mean ( $\pm$ SE) of three independent experiments.

### Determination of proliferative capacity

The proportion of cells undergoing one cell division (binucleation index, BNI) was assessed as described previously [2]. Aliquots of the single-cell suspensions used for the colony forming assay corresponding to 0 and 2.4 Gy were plated ( $\sim 3\text{--}5 \times 10^4$  cells per plate) into 35 mm plastic petri dishes (Corning, New York, USA) containing a  $22 \times 22$  mm<sup>2</sup> glass coverslip (VWR, West Chester, PA, USA) in a final medium volume of 2 ml. Three hours after plating, when the cells had attached, the culture medium was replaced with medium containing cytochalasin-B (Sigma, St Louis, MI, USA). The final concentration of cytochalasin-B, previously dissolved in DMSO, in culture medium was 2  $\mu$ g/ml. Forty hours after incubation with cytochalasin-B, samples were fixed in Carnoy's fixative (3:1, methanol:acetic acid), air-dried and stained with acridine orange and the coverslips mounted on glass microscope slides for fluorescence microscopy. A minimum of 1000 binucleated cells were evaluated per sample of each fibroblast strain per dose point, and the BNI (% of cells that are binucleated) was determined. Data were expressed as the mean ( $\pm$ SE) of three independent experiments. The percent reduction in BNI was calculated and used as an indicator of radiation-induced change in the proportion of cells progressing through one cell division. The error in the percent reduction in BNI was determined using appropriate error propagation formulae.

### TGF- $\beta$ assay

For TGF- $\beta$  measurements, confluent fibroblast cultures were trypsinized, aliquoted into four samples (two for active and total TGF- $\beta$  in unirradiated cultures; two for active and total TGF- $\beta$  in irradiated cultures). The single-cell suspensions were irradiated to 2.4 Gy using a high dose rate ( $\sim 1.06$  Gy/min) gamma-cell. Immediately after irradiation, a batch of unirradiated and irradiated samples was left on ice (for active TGF- $\beta$ ) and the other was heated at 70 °C for 10 min to activate latent TGF- $\beta$  (for total TGF- $\beta$ ). The heated samples were immediately placed on ice for 15 min before cells were plated.

TGF- $\beta$  activity was determined based on its ability to induce PAI-1 expression in the MELC-PAI cells. Binding of TGF- $\beta$  to the receptors of the PAI-1/L transfected MELC results in a dose-dependent luciferase activity [1]. This assay measures activity of all the various TGF beta isoforms. The assay for quantifying TGF- $\beta$  at the wound site in vivo as described by Yang et al. [53] was modified to measure active and total (active plus latent) TGF- $\beta$  in skin fibroblast suspensions (Fig. 1). MELC were plated into 24-well culture dishes ( $3 \times 10^5$ /ml, 500  $\mu$ l/well) in high glucose DMEM supplemented with 0.5% FBS and incubated for 3-4 h. When

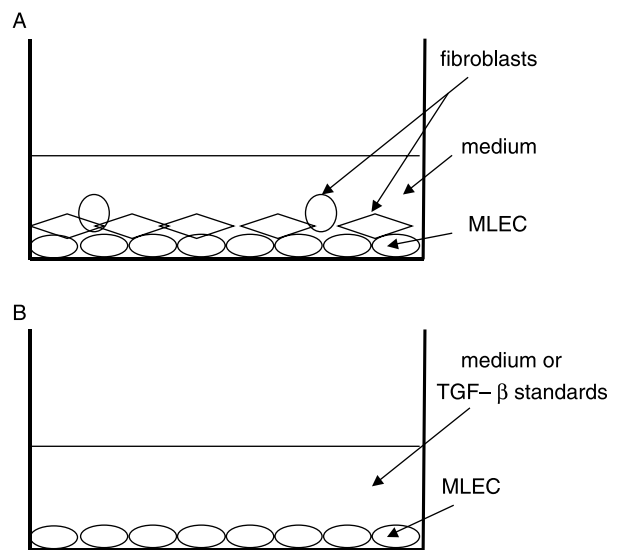


Fig. 1. A schematic of the PAI/L assay for human skin fibroblasts. MELC were plated and incubated for 4 h to attach, and then further incubated overnight with dermal fibroblasts (A). To generate TGF- $\beta$  standard curves, MELC were incubated with serial dilutions of recombinant human TGF- $\beta$ 1 (B).

the MELC had attached, the wells were washing with PBS and the cultures were re-incubated in 500  $\mu$ l of serum-free DMEM/BSA medium (DMEM with 0.1% pyrogen-poor BSA and 1  $\mu$ g/ml aprotinin) containing either fibroblasts ( $3 \times 10^5$ /ml) or recombinant human TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN, USA; cat. # 240-B) serially diluted (0-500 pg/ml) to generate standard curves for TGF- $\beta$  activity. Cell-free DMEM/BSA media on ice (blank for standards) and heated (then cooled on ice) were used to correct for background TGF- $\beta$  activity in the unheated and heated samples, respectively.

The 24-well culture plates containing samples and TGF- $\beta$  standards were incubated for 20 h, washed with PBS and then lysed with 200  $\mu$ l of luciferase cell lysis buffer (tris base, 25 mM; *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 2 mM; DL-dithiothreitol, 2 mM; glycerol, 10%; triton X-100, 1%; pH 7.8) for 20 min at room temperature with gentle agitation. The lysates were transferred in triplicate to white 96-well plates (Nunclon, Roskilde, Denmark). Luciferase activity in lysates was determined using a Luminoskan Ascent Luminometer (Thermo Labsystems, Helsinki, Finland; cat. # 1507520), and then converted into TGF- $\beta$  activity using the TGF- $\beta$ 1 standard curves. The luciferase substrate consisted of tricine (20 mM), magnesium carbonate hydroxide pentahydrate (1.07 mM), magnesium sulphate heptahydrate (2.67 mM), ethylenediaminetetraacetic acid disodium salt (0.1 mM), DL-dithiothreitol (33.3 mM), luciferin (470  $\mu$ M), adenosine 5'-triphosphate disodium salt (530  $\mu$ M) and coenzyme-A (270  $\mu$ M) at pH 7.8. Data were expressed as the mean ( $\pm$  SE) of three independent experiments.

Treatment of MELC cultures containing 500 pg/ml of recombinant human TGF- $\beta$ 1 with 5-100  $\mu$ g/ml of anti-TGF- $\beta$  (Sigma, St Louis, MI, USA; cat. # T9429) for 30 min at room

temperature (22 °C) prior to addition to MLEC cultures revealed that 80 µg/ml of antibody almost completely neutralized TGF-β activity (this antibody is stated to neutralise both TGF-β1 and TGF-β3). This anti-TGF-β concentration was then used on samples from randomly selected fibroblast strains to verify that the detected luciferase activity in the MLEC cultures was solely due to TGF-β expression in the fibroblasts. Unheated and heated (then cooled on ice) media were used to reconstitute the antibody for the unheated and heated cell suspensions, respectively.

### Statistical analysis and curve fitting

Linear regression analyses were used to test associations. Standard equations were used to fit nonlinear relationships. To compare two samples, the Mann-Whitney rank sum test was used. For multi-group analyses, the Kruskal-Wallis and Dunn's multiple comparison tests were used. *P*-values were from two-sided tests.

## Results

### Fibroblast radiosensitivity and proliferation in relation to wound healing morbidity

In Fig. 2(a), the data for fibroblasts from the validation cohort show no association between WHC and fibroblast radiosensitivity based on the surviving fraction at 2.4 Gy ( $P=0.33$ ). Also, no link was observed between  $SF_{2.4}$  and WHC ( $P=0.83$ ) when data for the test and validation groups were combined (Fig. 2b). The median  $SF_{2.4}$ -values for the control group, preoperative radiotherapy patients without wound healing difficulties and with WHC were 0.18 (95% CI: 0.12-0.33), 0.21 (95% CI: 0.17-0.31) and 0.19 (95% CI: 0.13-0.28), respectively, and were not significantly different ( $P>0.05$ ).

In the validation cohort, there was a trend towards fibroblasts from patients with WHC showing smaller reduction in BNI than those from patient with normal wound healing but this did not reach statistical significance ( $P=0.09$ ) (Fig. 2c). When data for the test and validation groups were combined, fibroblast strains from patients with

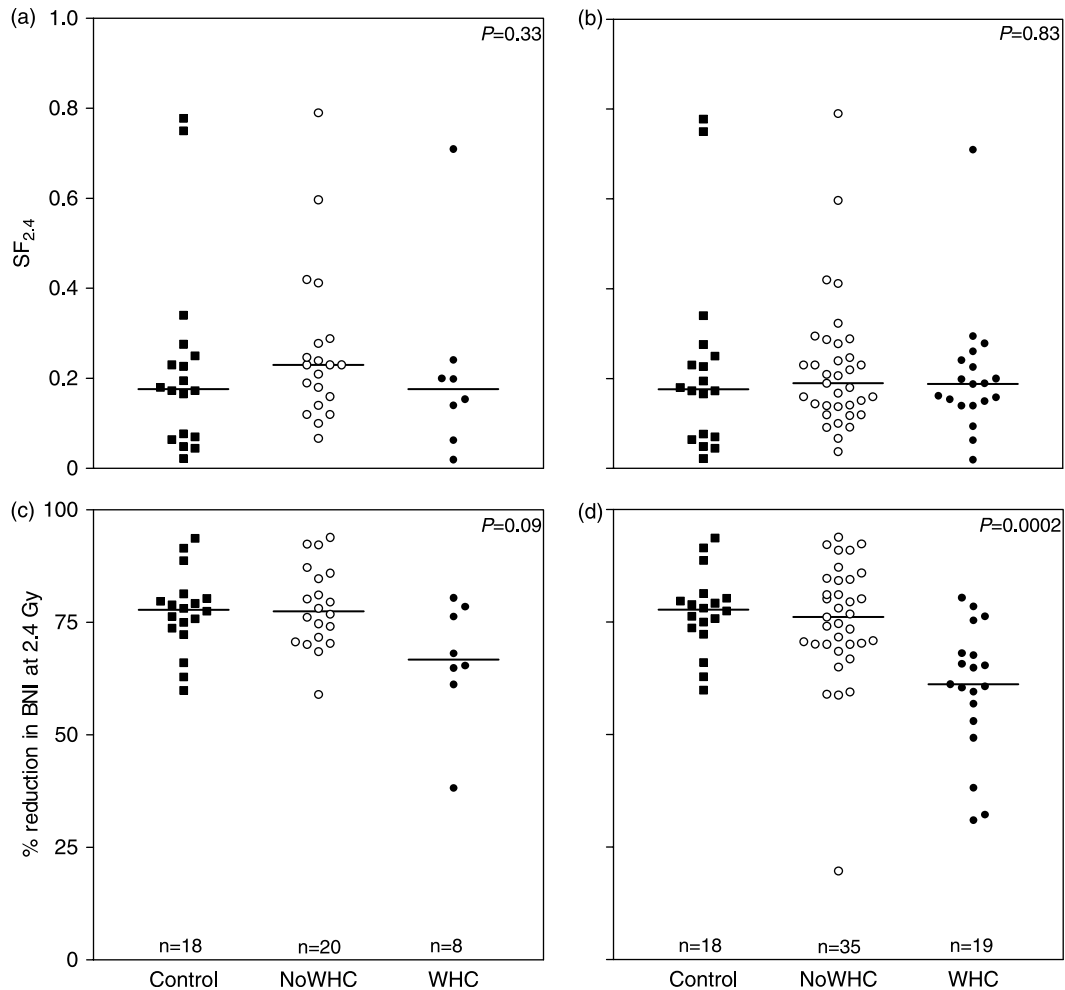


Fig. 2. Association of wound healing complications in 72 STS patients (a test group of 26 treated with preoperative radiotherapy [2]; a validation group of 46 (28 treated with preoperative radiotherapy and 18 treated with postoperative or received no irradiation constituting a control group)) with fibroblast radiosensitivity based on the survival fraction at 2.4 Gy (a) validation data, (b) overall data; percent reduction in binucleation index in fibroblast cultures following a dose of 2.4 Gy (c) validation data, (d) overall data. The data for the test group of 26 patients is taken from our previous paper [2]. The bars represent the median  $SF_{2.4}$  and percent reduction in BNI for each group.

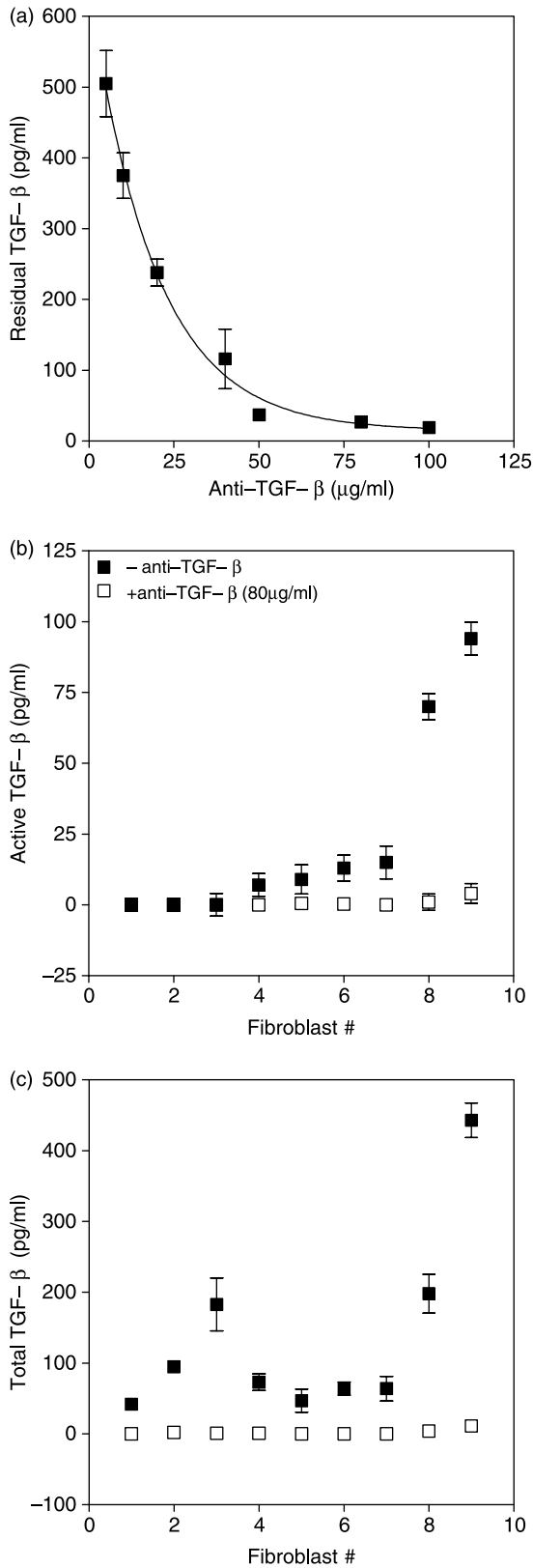


Fig. 3. (a) Residual TGF-β activity after 20 h incubation of MLEC in 500 pg/ml of recombinant human TGF-β1 and 5-100 μg/ml of anti-TGF-β. (b) Active and (c) total TGF-β expression in randomly selected fibroblast strains in the absence (solid symbols) and presence (open symbols) of 80 μg/ml of anti-TGF-β. Points represent the mean ± SE.

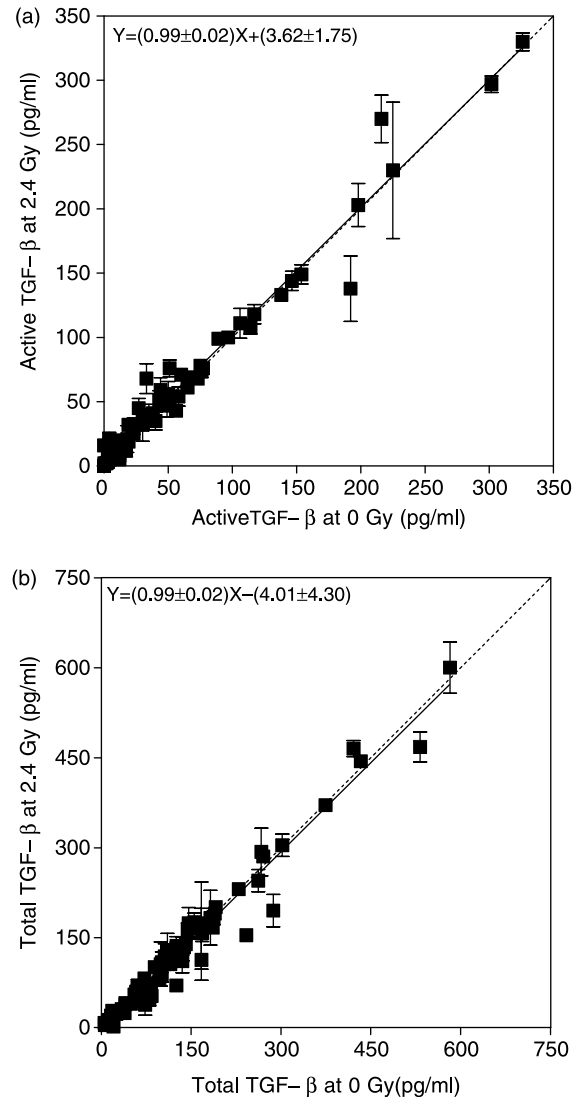


Fig. 4. Effect of irradiation on TGF-β expression in 72 skin fibroblast cultures: (a) active TGF-β after 2.4 Gy versus active TGF-β at 0 Gy; (b) total TGF-β after 2.4 Gy versus total TGF-β at 0 Gy. Points represent the mean ± SE.

WHC exhibited a significantly smaller reduction in binucleation after 2.4 Gy than those from the control population or patients who received preoperative radiotherapy but showed normal wound healing ( $P=0.0002$ ) (Fig. 2d). The median radiation-induced reduction in binucleation for fibroblasts from patients in the control, NoWHC and WHC groups were found to be 77.8% (95% CI: 72.9-81.7%), 76.1% (95% CI: 70.6-79.9%) and 61.2% (95% CI: 53.3-67.2%), respectively. An analysis of the data for reduction in binucleation shown in Fig. 2d to generate an ROC curve gave a result very similar to that shown in our previous paper [2]. Based on this analysis the optimum cut-off value for percentage reduction in BNI occurs at about 69% and this gives a predictive test for wound healing complications with a sensitivity and specificity of about 79%.

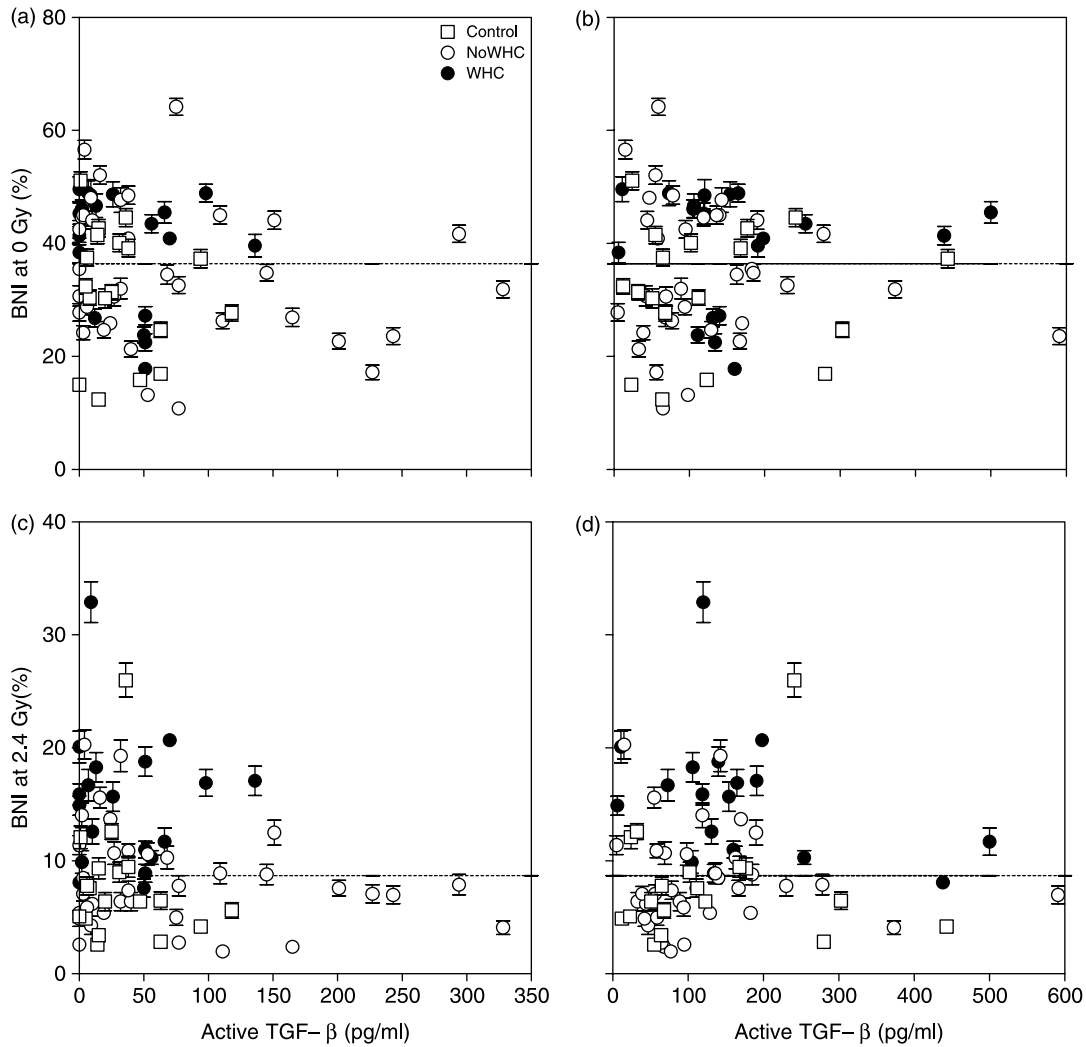


Fig. 5. Relationship between binucleation index in 72 skin fibroblast cultures at: (a,b) 0 Gy; (c,d) 2.4 Gy and active or total TGF- $\beta$ . Dotted lines represent the median binucleation index in unirradiated and irradiated cultures, respectively. Points represent the mean  $\pm$  SE.

### Measurement of TGF- $\beta$ expression in fibroblast cultures

Pretreatment of recombinant human TGF- $\beta$ 1 (500 pg/ml) with anti-TGF- $\beta$  (5-100  $\mu$ g/ml) resulted in an exponential decrease in residual TGF- $\beta$ 1 activity (Fig. 3a). A concentration of 80  $\mu$ g/ml of anti-TGF- $\beta$  could reduce the bioactivity of recombinant human TGF- $\beta$ 1 to  $\sim$ 5% of its original level. Incubation of selected fibroblast suspensions with anti-TGF- $\beta$  (80  $\mu$ g/ml) for 30 min at room temperature prior to plating onto MLEC resulted in a complete inhibition of TGF- $\beta$  activity (Fig. 3b and c), indicating that the induction of luciferase activity detected in the MLEC assay is primarily attributable to TGF- $\beta$ 1 (and TGF- $\beta$ 3) activity.

Active TGF- $\beta$  levels in unirradiated and irradiated suspensions were found to range from 0-225 to 0-230 pg/ml, respectively, and did not differ significantly (Fig. 4a). Similarly, irradiation did not affect the level of total TGF- $\beta$ , which ranged from 5-582 to 2-600 pg/ml at 0 and 2.4 Gy, respectively (Fig. 4b). Therefore, data for

unirradiated and irradiated cultures were pooled for subsequent analyses.

### Influence of TGF- $\beta$ on fibroblast proliferation and radiosensitivity

Proliferation based on the binucleation index in unirradiated and irradiated cultures of fibroblast strains expressing low levels of active and total TGF- $\beta$  varied widely (Fig. 5). The observed variation was independent of age, sex, wound healing morbidity or treatment received by the patients and BNI was found to range from 10.9 to 64.2% and 2.6 to 32.9% in unirradiated and irradiated cultures, respectively. Interestingly, the variation in unirradiated cultures diminished at higher levels of TGF- $\beta$  as fibroblast proliferation tended to converge towards the overall median of 36.4% (95% CI: 32.5-38.1%) (Fig. 5a and b). In irradiated cultures with high levels of TGF- $\beta$ , initial proliferative ability tended to fall below the overall median of 8.7% (95% CI: 8.6-11.4%) (Fig. 5c and d).

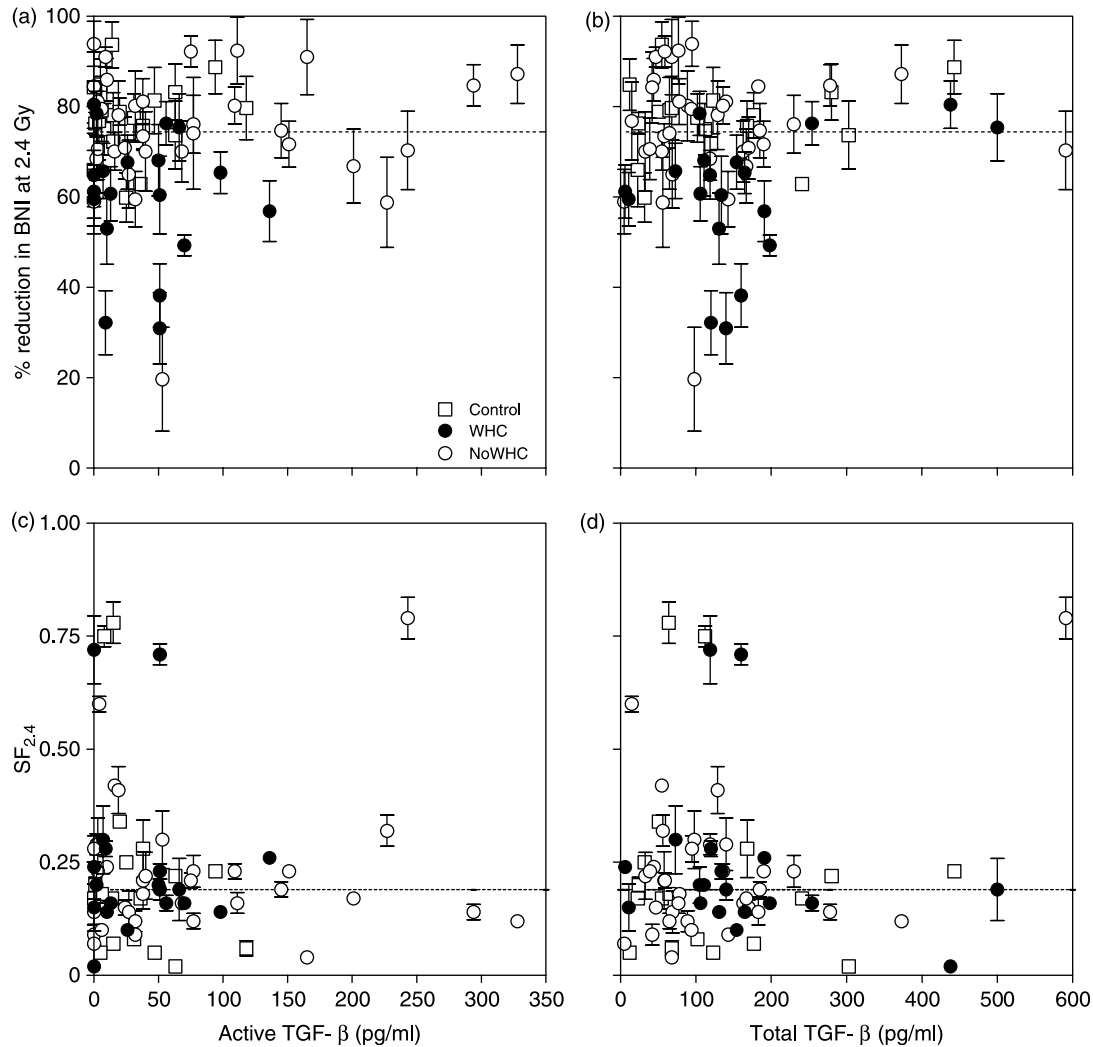


Fig. 6. Plots of: percent reduction in binucleation index after 2.4 Gy against (a) active and (b) total TGF- $\beta$ ; surviving fraction at 2.4 Gy against (c) active and (d) total TGF- $\beta$  in 72 skin fibroblast cultures. Dotted lines represent the median reduction in binucleation and surviving fraction at 2.4 Gy, respectively. Points represent the (a,b) percent reduction in BNI and the corresponding error calculated from the data in Fig. 5; (c,d) mean  $\pm$  SE.

Similarly, radiation-induced fibroblast proliferative arrest and radiosensitivity varied widely at lower levels of TGF- $\beta$ , ranging from 19.7 to 93.9% and from 0.02 to 0.78, respectively (Fig. 6). In fibroblast cultures expressing higher levels of TGF- $\beta$  the reduction in binucleation and radiosensitivity appeared to converge towards the overall median values of 74.4% (95% CI: 68.4-75.1%) and 0.19 (95% CI: 0.19-0.27), respectively.

#### Association of TGF- $\beta$ activity in vitro and wound healing morbidity

No differences were observed in the level of active ( $P=0.36$ ) and total ( $P=0.25$ ) TGF- $\beta$  expression when data for control group, preoperative radiotherapy patients without wound healing difficulties and with WHC were compared (Fig. 7), indicating that differences in wound healing response after preoperative radiotherapy is unlikely to be explained by disparities in TGF- $\beta$  expression in the fibroblasts unless this is affected by in vivo conditions.

#### Discussion

The aim of the current study was to validate our earlier finding that skin fibroblasts from STS patients who developed WHC following preoperative radiotherapy tended to show higher levels of initial proliferative ability when irradiated in culture compared to their counterparts from patients with normal wound healing responses [2]. Consistent with our previous results [2], no relationship was observed between radiosensitivity of the dermal fibroblasts, as assessed by clonogenic survival, and WHC (Fig. 2a and b). The data for the validation group in Fig. 2c supports our earlier finding that fibroblasts from patients with WHC do not undergo as much radiation-induced proliferative arrest as those from individuals exhibiting normal wound healing [2], but this comparison did not reach statistical significance based on the Kruskal-Wallis multi-group analysis. This is likely due to the small number of patients with WHC in the validation group because of limited availability of patients in the time frame of the study. The combined data in Fig. 2d further

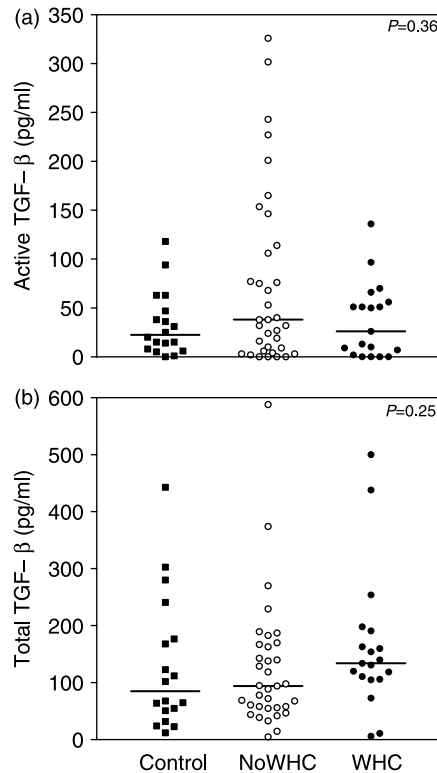


Fig. 7. Association of wound healing complications in 54 STS patients after preoperative radiotherapy and 18 STS patients receiving postoperative or no irradiation considered as the control group with: (a) active and (b) total TGF- $\beta$  expression. The bars represent the median TGF- $\beta$  activity for each group.

strengthen our initial observation [2] and contradict the idea that radiation-induced WHC is due to reduced fibroblast proliferative ability [45,48].

TGF- $\beta$  is a key player in the wound healing process and it is strongly involved in fibroblast proliferation and differentiation [10,16-19,23,25,34,36,51,52]. Fibroblast radiosensitivity is also reported to be affected by TGF- $\beta$  [10,16,26]. Tissue response to TGF- $\beta$  exposure is complex and multifaceted. Depending on the cell type and stage, TGF- $\beta$  may induce either growth stimulation [26,31,36,43,47] or inhibition [19,23,36], and some cell systems have been found to be unresponsive to TGF- $\beta$  activity [27]. Although extensive evidence exists for a significant induction of TGF- $\beta$  in tissue following irradiation [4,14,34], the irradiation of fibroblasts in vitro has not yielded significant levels of TGF- $\beta$ . Our data in Fig. 4 show no significant effect of radiation on TGF- $\beta$  levels in the fibroblast cultures and are consistent with other reports [29,41]. Irradiation of subconfluent rat lung fibroblasts cultures with 4 Gy resulted in only a modest elevation in the level of total TGF- $\beta$ , even after 48 h [26]. In the present study, the levels of active and total TGF- $\beta$  varied significantly over ranges of 0-1.5 fg/cell and 0.01-4.0 fg/cell, respectively, and are comparable with other published data [26,29,41]. Interestingly, using a pneumocyte/fibroblast co-culture system Burger et al. demonstrated that irradiation resulted in a rapid and significant induction of TGF- $\beta$  [8]. They suggested that the increase in proliferation and clonogenicity of the co-cultured fibroblasts was

attributable to the TGF- $\beta$  released by the pneumocytes. It is worth noting that the radiation-induced TGF- $\beta$  levels in the pneumocyte system are 200-2000-fold greater than those found in the 'pure' fibroblast cultures in the present study and elsewhere [26,29,41].

Our data in Figs. 5 and 6 constitute a significant addition to this literature and may help explain the varied conclusions of other studies. To date, studies linking TGF- $\beta$  expression to clonogenicity and radiosensitivity are controversial [8,19,23,26,31,36,41,43,47]. While high TGF- $\beta$  levels have been shown to induce increased proliferation and clonogenicity [8], other investigators observed elevated TGF- $\beta$  expression in radiosensitive fibroblasts compared to their radioresistant counterparts [41]. TGF- $\beta$  is known to inhibit proliferation [4,14,19,23,36]. It is possible that in some fibroblasts with low levels of TGF- $\beta$  the inhibitory effect is absent and will result in high proliferation. Another possibility is that the fibroblasts expressing low levels of TGF- $\beta$  lack the growth stimulation attributable to TGF- $\beta$  [26,31,36,43,47], and thus will exhibit low proliferation. High TGF- $\beta$  expression is also known to lead to growth inhibition [19,23,36] or stimulation [26,31,36,43,47], or even no effect on proliferation [27]. It is therefore not surprising that our data show a complex relationship between TGF- $\beta$  expression in fibroblast cultures and proliferation or radiosensitivity.

No relationship was seen between TGF- $\beta$  expression in our fibroblast strains and wound healing morbidity in the corresponding patients (Fig. 7). As illustrated by the reports from the in vitro studies, intrinsic TGF- $\beta$  levels in fibroblast cultures may be too low to reflect any impact of TGF- $\beta$  on fibroblast responses to irradiation in vivo. This suggests that radiation-induced changes in fibroblast proliferation and clonogenicity, and the subsequent impact of these changes on wound healing, require the high levels of TGF- $\beta$  as observed in vivo or in co-cultures [4,8,14,34]. In general, our investigations are consistent with the multicellular models proposed for the interactive response of fibroblasts in irradiated tissue and their possible involvement in normal tissue reaction [24,42].

In conclusion, these data add to our previous observation that cultured fibroblasts from individuals who developed WHC after preoperative radiotherapy show a significantly lower radiation-induced proliferative arrest compared to their counterparts from patients with normal wound healing. Any link between TGF- $\beta$  in fibroblast cultures and radiation-induced changes in proliferation or clonogenic survival, as well as with wound healing response remains largely inconclusive. The data demonstrate that the expression of TGF- $\beta$  is highly fibroblast strain specific and independent of age and sex of the patient from whom the strain was derived. Studies involving the treatment of fibroblasts with high levels of TGF- $\beta$  similar to those observed in vivo or the use of co-cultures consisting of fibroblasts and other cell types known to express significant amounts of TGF- $\beta$  upon irradiation may provide useful patient-specific information regarding normal tissue reaction. We are currently examining whether plasma TGF- $\beta$  levels in patients may be linked to the development of WHC.



## Acknowledgements

This study was supported by an IHRT grant from the Canadian Institutes of Health Research. Aileen Davis is supported by a Health Career Award from the Canadian Institutes of Health Research. The authors acknowledge Can Gong, Bob Kuba and Pavel Kaspler in helping to isolate and maintain the fibroblast strains, Patrick Subarsky and Jeffrey Wong for technical assistance and Dr M.H. Barcellos-Hoff for providing the MLEC and assistance in setting up the assay.

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Received 16 December 2004; received in revised form 4 November 2005; accepted 2 December 2005; available online 27 December 2005

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