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Studies of the *in vivo* radiosensitivity of human skin fibroblasts

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Abstract

Background and Purpose—To examine the radiosensitivity of skin cells obtained directly from the irradiated skin of patients undergoing fractionated radiation treatment prior to surgery for treatment of soft tissue sarcoma (STS) and to determine if there was a relationship with the development of wound healing complications associated with the surgery post-radiotherapy.

Methods—Micronucleus (MN) formation was measured in cells (primarily dermal fibroblasts) obtained from human skin at their first division after being removed from STS patients during post radiotherapy surgery (2-9 weeks after the end of the radiotherapy). At the time of radiotherapy (planned tumor dose - 50 Gy in 25 daily fractions) measurements were made of surface skin dose at predetermined marked sites. Skin from these sites was obtained at surgery and cell suspensions were prepared directly for the cytokinesis-blocked MN assay. Cultured strains of the fibroblasts were also established from skin nominally outside the edge of the radiation beam and DNA damage (MN formation) was examined following irradiation *in vitro* for comparison with the results from the *in situ* irradiations.

Results—Extensive DNA damage (MN) was detectable in fibroblasts from human skin at extended periods after irradiation (2-9 weeks after the end of the 5-week fractionated radiotherapy). Analysis of skin receiving a range of doses demonstrated that the level of damage observed was dose dependent. There was no clear correlation between the level of damage observed after irradiation *in situ* and irradiation of cell strains in culture. Similarly, there was no correlation between the extent

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of MN formation following *in situ* irradiation and the propensity for the patient to develop wound healing complications post surgery.

Conclusions—Despite the presence of DNA damage in dermal fibroblasts weeks after the end of the radiation treatment, there was no relationship between this damage and wound healing complications following surgery post irradiation. These results suggest that factors other than the radiosensitivity of the skin fibroblasts likely also play a role in wound healing in deep wound sites associated with surgery for STS following radiation therapy.

Keywords

fibroblasts; radiosensitivity in situ; micronuclei; wound healing; bystander effects

INTRODUCTION

Preoperative irradiation is the preferred treatment for most patients with large soft tissue sarcomas (STS) treated at the Princess Margaret Hospital/Mount Sinai Hospital in Toronto. However, a significant proportion of patients treated with this procedure develop wound healing complications (WHC) [40]. The alternative of giving the radiation treatment post-surgery has led to a significant incidence of late radiation-induced fibrosis and bone fracture; an incidence that is much reduced in preoperatively-treated patients, presumably because of the smaller total radiation dose ordinarily delivered in the preoperative setting [11,22]. 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 [3,9,42,44]. Results of these studies have been mixed with conclusions ranging from relationships between *in vitro* endpoints and normal tissue reactions [5,25] to no useful associations [42,45]. Some reviews emphasize the importance of analyzing orchestrated response (i.e., cytokines dynamics and genes expression) rather than target-cell approach for prediction of normal tissue reactions [4,44]. It has also been suggested that most normal tissue complications arise in patients with normal radiosensitivity simply because every patient has a certain probability of responding severely [12]. However, animal 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 [10,17].

Radiation leads to reduced fibroblast proliferative capability and function and this might be expected to impact negatively on the normal tissue response [10,17]. In a previous study we demonstrated that skin fibroblasts from individuals who developed wound healing complications following pre-operative radiotherapy had no detectable differences in radiosensitivity *in vitro* but tended to show a smaller reduction in early proliferation after irradiation [2]. A recent ‘validation’ study showed a similar trend although the number of new patients studied was small [1]. We hypothesized that increased proliferative potential may render the fibroblasts less capable of differentiating to produce the collagen necessary for effective wound healing. However, we could not rule out the possibility that the radiosensitivity of the fibroblasts was affected by *in vivo* conditions that were not reflected in our studies done with fibroblast strains irradiated and analyzed in culture. Consequently in the current study we analyzed DNA damage (micronuclei) in fibroblasts obtained directly from the irradiated skin of 31 STS patients and compared the results with the extent of DNA damage induced by irradiation *in vitro* of fibroblast strains derived from the skin of the same patients.

MATERIALS AND METHODS

Patients, clinical background

After Institutional Research Ethics Board approval, human skin biopsies were obtained from 31 patients undergoing treatment (radiation and surgery) for STS at the time of surgery (at 2-9 weeks after the end of radiation therapy). The cohort included 18 males and 13 females, with mean age: 53.2 (range: 19-87 years), 12 cases of upper limb and 17 cases of lower limb STS. Biopsies were obtained from skin being discarded at the time of surgery from regions in the irradiation field adjacent to the wound margin, at the edge of the irradiation field and outside the irradiation field, and stored in cold Hanks salt solution until processing within 2-5 hours. These regions had been predetermined based on the radiation treatment plan and marked on a life-size mold made of the limb involved. Doses received at the skin surface at the marked points were measured directly (see below) and the mold was placed on the patient during the surgical procedure to identify the location of the biopsies. The planned radiation dose (to tumor) was 50 Gy in 25 fractions over 5 weeks. For wound healing morbidity, patients were categorized into two groups namely: WHC—wound healing complications requiring either further surgery or prolonged deep wound packing; and No WHC—no detectable WHC as described previously [2,40]. Although these criteria are objective they inevitably include a degree of subjectivity in the decision of the particular surgeon involved.

Dose measurements at different depths

Radiation treatment planning (conformal, non-IMRT) for each patient was performed jointly by the radiation oncologists and surgeons and in particular the location of the planned incision site was identified. A mold of the treatment site of the patient was prepared prior to CT scan and the surgeon marked the planned surgical incision on the mold. Thermo-Luminescent Dosimeters (TLDs) LiF:Mg,Ti were then used during the radiotherapy treatment to measure in-vivo surface doses on the patient in the region of the planned incision. The TLDs had 3.15mm² square area and 0.14mm thickness and were calibrated on 6 MV X-rays, the same energy used for the radiation treatment of the patient. For in-vivo surface dose measurement seven to eight points of interest were selected on the marked area. Before radiation treatment the TLDs were placed on the patient's skin on each of the selected points. The TLDs were removed after radiation treatment and the doses were read. This process was repeated for three consecutive days of radiation treatment. For a given point an average of the three days readings was recorded as the in vivo dose for that point. Since the TLDs could not be physically inserted several phantoms were used to determine the dose at the near-skin depths of 1mm, 4 mm and 10 mm in relation to the surface dose. One of them was an anthropomorphic leg phantom in which the TLDs were inserted at the near-skin depths and doses were measured. The leg phantom was also used to simulate patient treatment geometry and validate the calculated dose with the delivered dose. The calculated near-skin dose agreed with the near-surface measurements on the leg phantom within $\pm 3\%$. Since the treatment was delivered as a fractionated schedule with an equal number of fractions there were different fraction sizes in different regions of the skin so we also calculated equivalent single (E. S.) doses based on the linear-quadratic relationship assuming values for α/β of 3 or 10 Gy [20].

Isolation of *in situ* fibroblasts and micronucleus assay

Fibroblast isolation from skin biopsies and the cytokinesis-blocked micronucleus (MN) assay were performed as described previously [1,2,26,27], with minor modifications. Briefly each biopsy (about 0.75 cm²) was minced with sterile scissors and treated with an enzyme cocktail of 0.06 mg/ml collagenase and 0.5 mg/ml DNase (Sigma, St Louis, USA) in α MEM with 2000 mg/ml glucose, 100 mg/ml streptomycin and 100 mg/ml penicillin at 37°C for 1.5 hours (mixing every 30 minutes). The digest was then strained into 50 ml tubes containing 5 ml of ice-cold α MEM supplemented with 20% fetal bovine serum (CanSera, Etobicoke, Canada)

(termed fibroblast isolation medium) on ice via a 70 μm nylon strainer (BD Falcon, Bedford, USA). Remaining tissue was further digested in 1 mg/ml trypsin (Sigma, St Louis, USA) at 37°C for 30 minutes (mixing every 10 minutes). Digestion was stopped by adding an equal amount of fibroblast isolation medium plus 200 μl of DNase stock solution (20 mg/ml), and the cells were strained into the same tube. After centrifugation, the cell pellet was resuspended in fibroblast isolation medium and plated into chamber slides (Nunc, Rochester, USA) for the micronucleus assay and/or placed into 25cm² culture flasks (Nunc, Roskilde, Denmark) for primary culture. The chamber slides were washed and fresh medium containing 3.2 $\mu\text{g/ml}$ cytochalasin B (Sigma, St.-Louis, USA) was added after 24 hrs and 72 hrs later the slides were washed with PBS, incubated in hypotonic KCl (5.56 mg/l) for 10 min. and fixed in cold methanol for 1.5 min. Just prior to scoring of MN, the slides were stained with Acridine orange (BD, Sparks, USA) for 2 min., washed in PBS and mounted in PBS. Micronuclei were scored in up to 1000 binucleate cells and were defined as rounded bodies, no more than 1/3 of the size of the nucleus, having staining color and intensity identical to the staining of nuclei and completely detached from nuclei [16]. The small number of binucleate cells apparently containing more than 6 MN were not scored because it is very difficult to confidently interpret them as micronuclei in a single binucleated cell. The total MN score per 1000 binucleated cells as well as per cent of cells containing MN were calculated. Personnel conducting these studies were blinded to the doses received by the skin biopsies knowing only that they had come from regions nominally 'in-field', 'edge of field', or 'out-of-field'.

Establishment of primary culture

Primary cultures were established from patients skin samples that were nominally out of the treatment field. Measured (fractionated) doses given to these skin samples ranged from 0.19 to 14.02 Gy. The cells were grown in the 25cm² culture flasks until confluency and then trypsinized and transferred to 75cm² culture flasks until almost confluent. They were then trypsinized, resuspended in FCS+10% DMSO and stored cryogenically until future use. Cells from the different patients required 16-37 days to establish these frozen cell strains. Of the 31 patients in the study, only 23 provided skin samples that could be used to establish these primary cultures.

Irradiation and Analysis of fibroblasts in vitro

Cultures were thawed and grown in 25cm² culture flasks in αMEM , supplemented with 10% fetal bovine serum until confluent, then trypsinized and divided onto two 25cm² culture flasks. Upon confluence, the flasks were incubated overnight in 15 cc of fresh αMEM . The flasks were sealed, irradiated at room temperature using a Gammacell 40 Exactor ¹³⁷Cs γ -irradiator to a dose of 2.4 Gy (dose rate 0.94 Gy/min), incubated for 4-5 hours at 37°C to allow for recovery, trypsinized and plated onto 12 \times 12 mm cover slips in 35 mm culture dishes. At 3 hours after plating, medium was replaced with fresh medium containing 3.2 $\mu\text{g/ml}$ of cytochalasin B. After 40-42 hours the cells were incubated in hypotonic (5.56 mg/l) KCl for 10 minutes and fixed in cold methanol for 1.5 minutes. This procedure is similar to that described previously [2]. For scoring of MN, the fixed cover slips were stained with Acridine orange for 2 minutes, washed in PBS, mounted in PBS on glass microscopic slides and sealed by Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, USA). Upon scoring of MN, binucleated and mononucleated cells were counted, and BNI was calculated as the percent of binucleated cells in the total population of binucleated and mononucleated cells. Studies were also performed with non confluent fibroblasts plated onto 12 \times 12 mm cover slips in 35 mm culture dishes. The cells were allowed to attach for 3-4 hours and then irradiated on ice using Gammacell 100 Elite ¹³⁷Cs γ -irradiator to doses of 0.25-6 Gy (dose rate 2.7 Gy/min). Immediately after irradiation, medium was replaced with fresh medium containing cytochalasin B, and the micronucleus assay was conducted as described for confluent fibroblasts.

Statistical analysis

For associations, linear and non-linear regression analysis was used. For comparison of two independent samples, the Mann–Whitney rank sum test was used; for comparison of matched samples, Wilcoxon signed-ranks test for matched pairs was used (Prizm 4 for Windows software, version 4.03). Association between wound healing complications and various parameters was assessed using logistic regression for numeric data [23; statpages.org/logistic.html] and Fisher's exact test for nominal data (www.matforsk.no/ola/fisher.htm). P-values were from two-sided tests. The dose response curves were fitted to three different models (linear; $[MN/1000BN = aD + b]$, linear quadratic; $[MN/1000BN = aD^2 + bD + c]$; and exponential; $[MN/1000BN = a e^{kD}]$, where a,b and c are constants). Confidence intervals for correlation coefficients of these models were obtained using Fisher's z' transformation: (<http://icp.giss.nasa.gov/education/statistics/page3.html>), (<http://davidmlane.com/hyperstat/B8544.html>).

For comparison of the fitted models, the Akaike information criterion (AIC) was used (<http://www.theses.ulaval.ca/2004/21842/apa.html>). AIC compares fitting quality while accounting for the different number of coefficients in the models and thus compensating for over-fitting by a model with more coefficients. The difference (delta) between AIC values for different fitting models determines whether one model can be chosen in favor of another. Delta exceeding 10 suggests a strong preference for the model with the lesser AIC value, whereas delta less than 2 suggests no preference for model [6].

RESULTS

Taken together the total MN scores for fibroblasts irradiated *in situ* showed a significant correlation with dose measured at 0 mm and calculated at 1 mm depth (Fig. 1 A,B) but not at 4 mm or 10 mm (data not shown). The dose at 1 mm depth is likely to be the biologically-relevant dose because the depth of the dermis (from where fibroblasts are primarily being analyzed) is approximately 0.1 to 1.5 mm from the surface [14, 18, 24, 30, 41]. Furthermore, a depth of 1-2 mm has been reported in the literature as relevant for skin irradiation [19, 37]. Consequently we have used the dose at 1 mm depth for our further analyses.

Replacing the actual total fractionated dose with calculated equivalent single dose did not change the pattern of the plot (Fig. 1 C,D). The two equivalent single dose calculations for $\alpha/\beta=3$ or 10 Gy demonstrated very similar correlations ($R_{lin}^2=0.483$ compared to 0.481 respectively). Since the α/β value for skin is generally expected to be in the region of 10 for an early responding tissue [20] and wound healing is an early response, the equivalent single dose calculated for $\alpha/\beta=10$ was used for further analysis.

For the total dataset, both the linear fit and the exponential fits were significant ($P<0.01$), (unlike the linear-quadratic fit), but the linear fit was favored by the AIC test (delta AIC >10). For 2 of the 31 patients, the generation of individual dose response curves was not possible because of limited data. For the remaining 29 patients individual dose-response curves were generated (see examples in Fig. 2) and these were also fitted to a linear model. If the fitting produced a negative intercept, making no biological sense, a forced intercept was set equal to average intercept for the total dataset (47.4 MN/1000 BN). Otherwise, actual values of the intercept and the linear slope were used. The intercept (for *in situ* fibroblasts) was subtracted from the response values, and the dose required to achieve 400 MN/1000BN was then determined from the fitted line (see Fig. 3).

The MN burden of *in situ* fibroblasts, that were used for culturing, correlated with the received dose *in situ* ($R_{lin}^2=0.483$, $P<0.01$) (see Fig. 3A), and was considered a part of the overall *in*

situ dose-response curve (Fig. 1). However, background MN for the cultured fibroblasts did not correlate either with MN burden for the corresponding *in situ* fibroblasts (Fig. 3B) or with the dose the skin sample received *in situ*. Also, the irradiation-induced MN in culture did not correlate with the background MN level in culture (Fig. 3C). Finally, no correlation was found between the radiation response in culture (equivalent single dose to induce 150 MN above background estimated by linear fitting of the data) and that *in situ* (equivalent single dose to induce 400 MN determined from the linear fit to the data as described above) (Fig. 3D). This finding was not different for confluent vs non-confluent cultures (data not shown).

Wound healing complications (WHC) following surgery occurred in 8 of the 31 patients (26%) included in this study; a percentage which is similar to that reported by our group previously for similarly treated patients [40] and in a recent retrospective analysis [48]. The median age and sex profile of the patients and median time between irradiation and surgery did not differ between WHC- and WHC+ groups. The previous studies [40,48] identified a higher level of WHC in patients with lower limb sarcomas but the number of patients involved in the current study was too small to detect such a difference. Maximum measured skin dose was also not different between WHC- and WHC+ groups. Importantly, there was no significant difference in dermal fibroblast radiosensitivity between WHC- and WHC+ groups of patients (Fig. 4) even upon removal of the high values where extrapolation of the linear fit was necessary to obtain the dose required to induce 400 MN and may therefore contain more error. Similarly, no significant differences were observed between WHC- and WHC+ groups for other analyzed characteristics of MN and BNI, *in situ* and *in culture* (Table 1). The data was also examined by stratification according to *in situ* radiosensitivity (defined as the dose to induce 400 MN/1000BN cells) consistent with suggestions of Dikomey et al [12]. The frequency of WHC did not differ in patients with *in situ* radiosensitivity above and below the median value (Table 2).

DISCUSSION

The present work follows up our previous studies examining possible relationships between fibroblast radiosensitivity and the development of wound healing complications observed in soft tissue sarcoma patients treated with pre operative radiotherapy [1,2]. Our previous work studied fibroblasts in culture and demonstrated a trend for a relationship with the proliferative capacity of the fibroblasts (as measured by the BNI) following irradiation but found no evidence for a correlation with radiosensitivity measured by clonogenic assay or MN formation. It is possible, however, that the *in situ* microenvironment of the dermal fibroblast might affect their radiosensitivity by a mechanism not present in culture. Recent work has suggested that integrin signaling, such as may occur in interaction between cells and the extracellular matrix can affect radiosensitivity [8]. Thus we were interested to study the radiosensitivity of the dermal fibroblasts irradiated *in situ*.

For this purpose we isolated cells directly from the skin of patients undergoing surgery following radiotherapy and subjected them to a cytokinesis-blocked MN assay within a few hours after removal from the patient, so that we observed the DNA damage present in the cells at their first division in culture. Since this analysis was only possible at the time of surgery (2-9 weeks but mostly 4-8 weeks after the end of the 5-week course of irradiation) it was of considerable interest that we were still able to detect substantial DNA damage in the skin fibroblasts at this time. This result is consistent with our studies in rat skin, which have demonstrated that damage can be detected out to at least 9 months after irradiation (Kaspler and Hill in preparation 2007). This finding suggests that, because skin fibroblasts are largely quiescent, the DNA damage caused by the irradiation has remained in the cells throughout the time between irradiation and assay after surgery and/or that damage is being repaired and regenerated post irradiation. Our previous work using a MN assay with rat lung fibroblasts has indicated that repeated regeneration of DNA damage can occur in lung tissue following

irradiation *in situ* [7,28]. Such results are consistent with work on the bystander effect *in vitro* that suggests that following irradiation there can be chronic production of ROS that could cause cellular DNA damage [35,36]. Recent studies in human skin have demonstrated evidence for prolonged oxidative stress following irradiation [29].

In our study, we had an opportunity to compare MN formation in fibroblasts directly from the patient and in fibroblast strains after culturing the cells from the same patient *in vivo*. No correlation was observed between radiosensitivity *in situ* and in culture (Fig 3D) but we did observe that higher equivalent single doses were required *in situ* to induce MN in the skin than in fibroblasts irradiated in culture. The dose required to induce 150 MN per 1000 BN cells was about 5 times lower for the cultures than for the corresponding *in situ* fibroblasts ($P < 0.0001$). Because the time between irradiation and MN analysis *in situ* and in culture was very different (weeks *in situ* and hours in culture), it is possible that DNA damage repair occurring in the quiescent *in situ* fibroblasts resulted in lower numbers of MN. However, we did not observe any difference in the *in situ* dose-response curves between the patients with short post-irradiation time (14 – 35 days) and those with long post-irradiation time (50 – 65 days) which suggests that MN levels could be stable at least from 2 weeks to 2 months after irradiation. Similarly we have observed in rat skin that the level of DNA damage after irradiation does not decline until at least 1 month after irradiation (Kaspler and Hill, in preparation, 2007). This suggests that comparison between MN irradiation response of *in situ* and cultured fibroblasts can be relevant despite the difference in time between irradiation and analysis. It is possible that the higher MN response in culture than *in situ* at a given dose may be due to higher proliferation of cultured cells following irradiation, in contrast to quiescent cells *in situ*.

The “out-of-field” biopsies, the source of cultured fibroblasts, received some (scattered) dose during radiotherapy (0.19-14.02 Gy fractionated dose or 0.19 – 8.16 Gy₁₀ equivalent single dose), and this generated variability in MN score (burden), which correlated with pre-op dose ($R^2 = 0.4729$, $P < 0.01$). However, a relationship between the background MN in culture and the corresponding MN burden *in situ* was not observed for confluent cultures (Fig. 3C). The effects of the confluent state may be due to the effect of cell synchronization into G0/G1 phase, which occurs with fibroblasts under such conditions. The lack of correlation between *in situ* and in culture values was not due to damage depletion during culturing because the background MN in confluent cultures did not correlate with the time or number of passages needed to establish primary cultures (similar to the studies of O’Driscoll et al. [39]), nor with the time needed to grow thawed fibroblasts for *in culture* studies. In addition, no decrease in background MN burden was observed when five strains of the fibroblasts were cultivated for four passages (up to 4-6.5 weeks of culture) without irradiation (data not shown). These results suggest that genomic damage is persisting during culture consistent with studies by others of surviving descendants of irradiated cells *in vitro* and *in vivo* [13,15,33,34,38,49,50], even after a dose as low as 0.5 Gy [43], although only a fraction of the cell progeny (about 30-60%, depending on cell type and dose) may carry this damage [21,32]. Such persistent instability is also consistent with work on the bystander effect and could be due to factors released into the medium during culturing. [31,35,36,38,47].

We observed no significant correlation between the radiosensitivity of the dermal fibroblasts irradiated *in situ* and the development of WHC in the patients regardless of whether we split the group according to WHC (Fig. 4) or according to median radiosensitivity (Table 2). Since the number of patients (29) contributing to this part of the study was relatively small this negative finding has low power but the overlap of the data indicates that even if a significant correlation did exist the difference in radiosensitivity is too small (or the assay error is too large) for it to be used as a predictive factor. We also observed no relation between WHC and the maximum dose measured on the irradiated skin for the individual patients since these in-field skin samples were taken from skin near the wound margin. However we have no direct

information to indicate whether the doses measured represented the maximum dose received by the skin in the wound site.

One of the reasons of these negative results may be that not all actual DNA damage is translated into micronuclei. Even in ideal populations, the MN frequency always will be less than the frequency of acentric fragments, so MN inevitably under-estimates actual DNA fragmentation damage; this discrepancy grows with dose [46]. In addition, MN score may be affected by different exclusion of DNA fragments from nuclei [46]. Some lethal damage may not be expressed as MN at the first division, only in later ones (though many are eliminated at the first mitosis) [50]. Unfortunately, it was not possible to assess the BNI accurately for fibroblasts being assayed directly from skin samples, thus we could not examine for a relationship between BNI in the *in situ* samples and WHC, however, the BNI for the treated fibroblast strains also did not correlate with WHC in the limited group of 23 patients for whom fibroblast strains were derived.

Another reason may be that healing of the skin may not in itself be sufficient to prevent the development of a wound complication in situations where fluid accumulates in the wound cavity inevitably developing after the removal of a large tumor mass. We have shown previously that injection of non-irradiated autologous fibroblasts (or bone marrow stromal cells) into an irradiated *superficial* wound site in rats can result in improved wound strength but using a deep wound model in rat hind limb we found that such injections are of limited value in improving wound strength [10,17,51]. Overall our results suggest that factors other than the radiosensitivity of the skin fibroblasts must play a critical role in wound healing in deep wound sites associated with surgery for STS.

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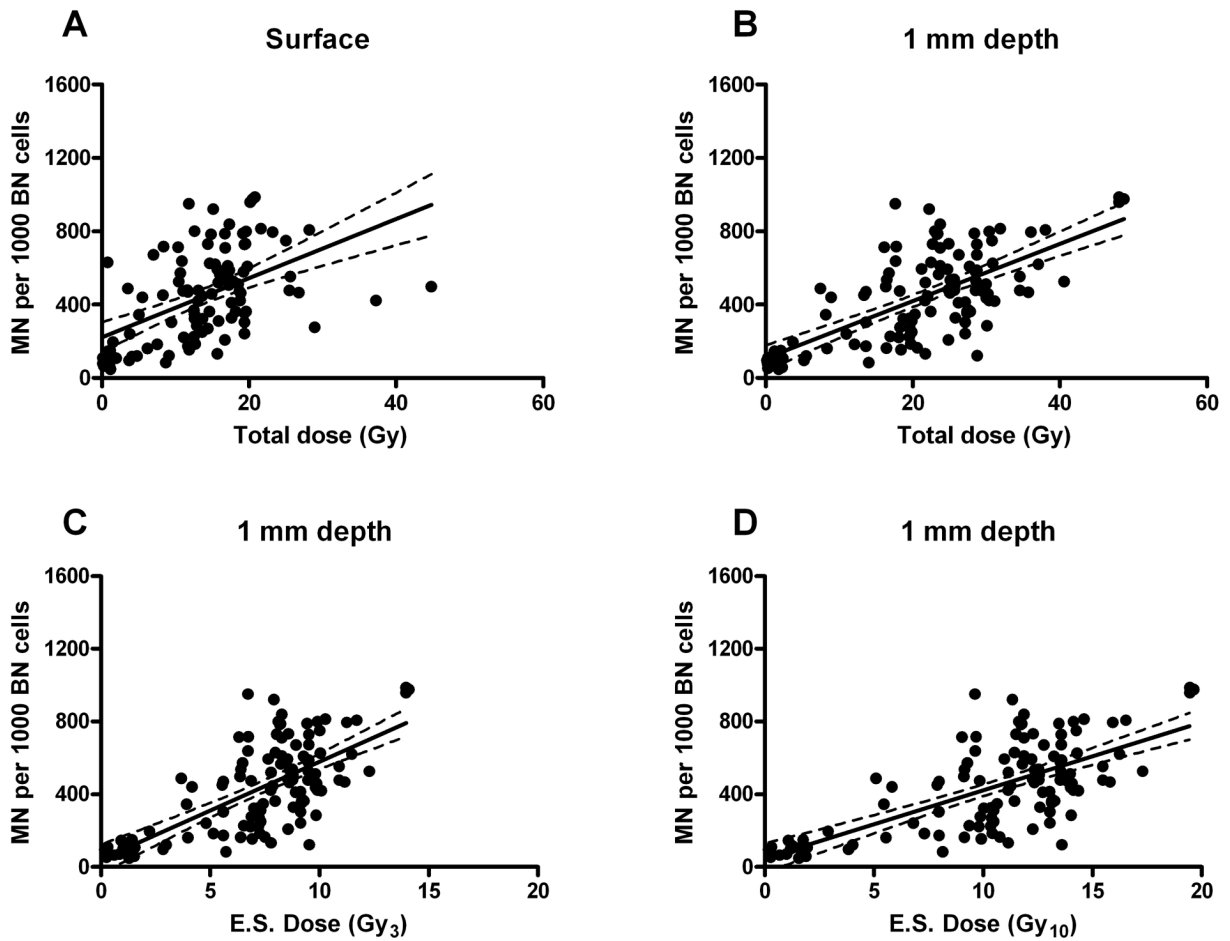


Fig. 1. MN in freshly isolated *in situ* fibroblasts in all available biopsies. MN number per 1000 of BN cells is plotted against fractionated dose at depth of 0 mm (**A**) and 1 mm (**B**) and against equivalent single dose (E.S.) at 1 mm depth for $\alpha/\beta=3$ (**C**) (Gy₃) or $\alpha/\beta=10$ (**D**) (Gy₁₀). The line from the linear fit model with 95% confidence intervals is shown on each plot.

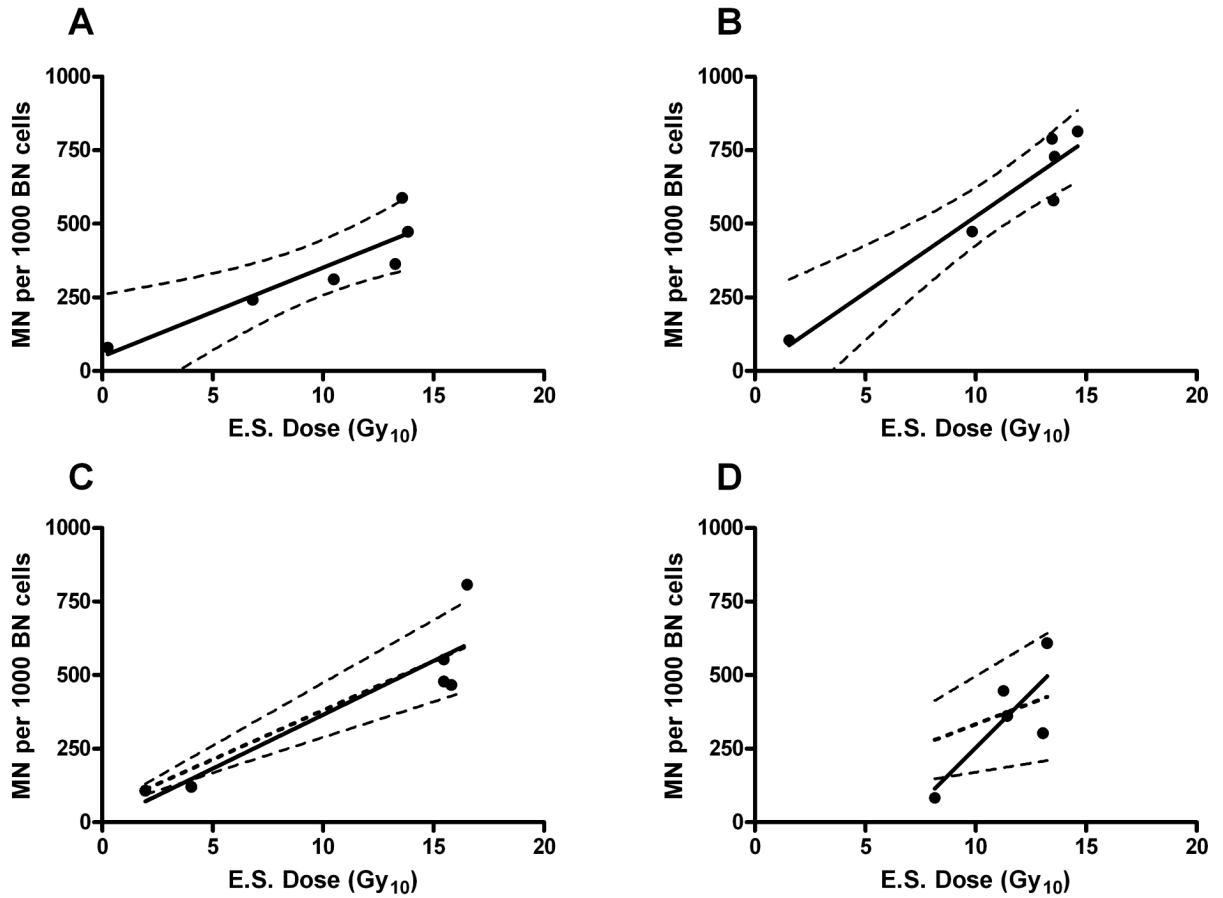


Fig. 2. MN in freshly isolated *in situ* fibroblasts, individual curves for different patients. MN number per 1000 of BN cells is plotted against equivalent single (E.S.) dose (Gy₁₀ for calculation based on $\alpha/\beta=10$). A line from a linear fit model with 95% confidence intervals is shown on each plot. Dashed linear trend on panels C and D shows linear fit using intercept forced to the average value of the whole dataset (Fig 1D).

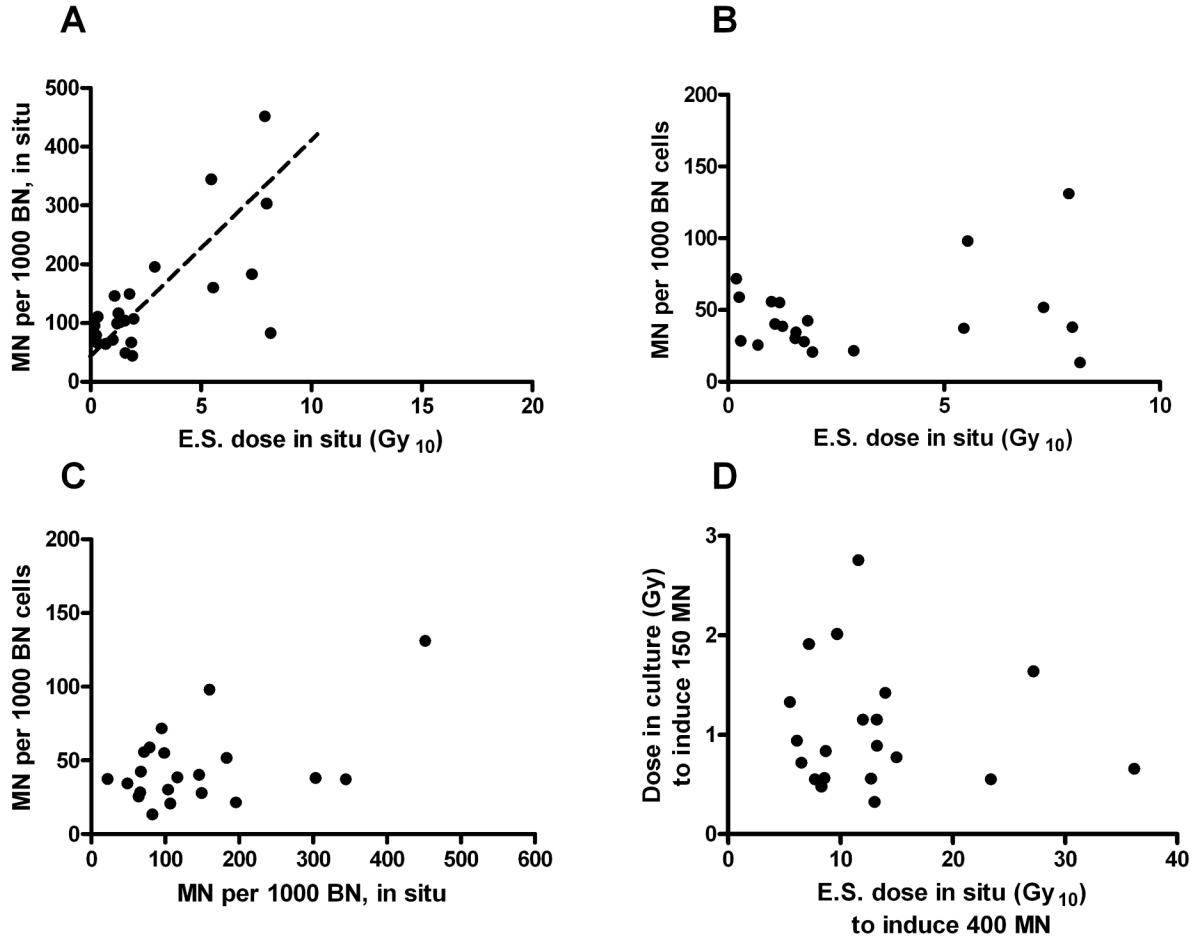


Fig. 3. (A) MN in *in situ* fibroblasts used for culturing plotted against pre-op equivalent single (E.S.) dose (Gy₁₀ for calculation based on $\alpha/\beta=10$) (regression trend from fig 1D for the total dataset *in situ* is shown as a dashed line); (B) background MN in confluent cultured fibroblasts plotted against pre-op equivalent single (E.S.) dose ($\alpha/\beta=10$); (C) background MN in confluent cultured fibroblasts plotted against corresponding values for *in situ* fibroblasts; (D) dose required to induce 150 MN per 1000 BN cells in confluent cultured fibroblasts vs. equivalent single (E.S.) dose ($\alpha/\beta=10$) required to induce 400 MN per 1000 BN cells in *in situ* fibroblasts (in cultured fibroblasts, background was subtracted from the response values).

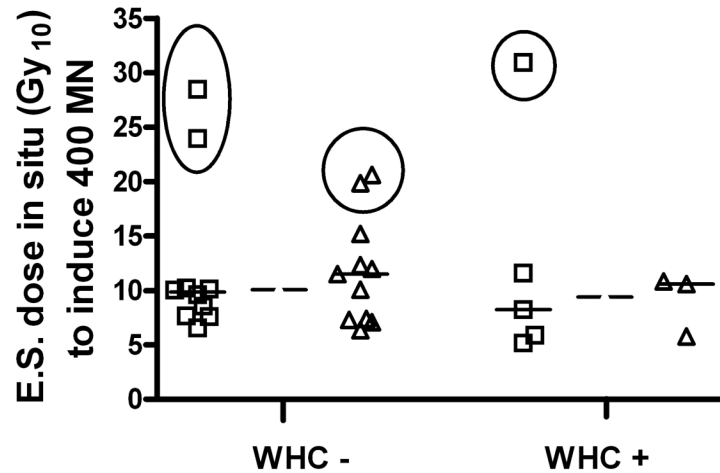


Fig. 4. Relationship between wound healing complications (WHC) and induced MN response in *in situ* fibroblasts. Equivalent single dose (Gy₁₀ for calculation based on $\alpha/\beta=10$) required to induce 400 MN per 1000 BN cells using actual linear fit intercepts (□) and using a forced intercept equal to the average intercept for the total dataset (△) are shown separately. Data in circles are the cases when extrapolation beyond the data points needed to be done to obtain the value. Medians are marked for each group separately (solid lines) and for pooled data for the patients without WHC or with WHC (dashed lines).

Table 1

Relationship between occurrence of wound healing complications (WHC) and micronuclei (MN) and binucleation index (BNI) in fibroblasts *in situ* used for culturing and in cultured fibroblasts

	No WHC	N	WHC	N
MN Burden in <i>in situ</i> fibroblasts used for culturing	99 (22 - 452)	17	93 (44 - 196)	6
¹ Induced MN response in confluent cultures (Gy ₁₀)	0.9 (0.6 - 2.8)	14	0.7 (0.3 - 1.9)	7
² Induced BNI response in confluent cultures (%)	84 (65 - 91)	15	84 (79 - 90)	5
¹ Induced MN response in non-confluent cultures (Gy ₁₀)	0.9 (0.6 - 2.6)	15	1.0 (0.7 - 1.3)	2
² Induced BNI response in non-confluent cultures (%)	59 (31 - 84)	17	65 (47 - 81)	6

Medians are shown (range in brackets).

¹ Induced MN response is expressed as equivalent single dose ($\alpha/\beta=10$, Gy₁₀) required to induce 150 MN per 1000 BN cells for cultured fibroblasts.

² Induced BNI response is expressed as per cent decrease in BNI at 1 Gy in non-confluent cultures and as per cent decrease in BNI at 2.4 Gy in confluent cultures.

Table 2

Relationship between occurrence of wound healing complications (WHC) and stratified micronuclei (MN) response in fibroblasts *in situ*

	No WHC	WHC
Induced MN response (G_{y10}) at or below median	11	4
Induced MN response (G_{y10}) above median	10 (7)	4 (3)

Induced MN response is expressed as equivalent single dose ($\alpha/\beta=10$, G_{y10}) required to induce 400 MN per 1000 BN. Values in brackets represent occurrence of WHC after exclusion of extrapolated cases of MN response.