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Tempol prevents impairment of the endothelial cell wound healing response caused by ionising radiation

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Summary It is known that radiation therapy results in some form of damage to the microcirculation. In support of this view, we found that capillary endothelial cells (EC) treated with X-rays (8 Gy) were defective in their ability to recover a denuded area. A scrape wound of 2 mm width was produced in monolayers 30 min after X-ray or sham treatment. After 48 h, the number of cells migrating into each of five successive 125 μm zones from both sides of the original wound were determined. Greater numbers of sham-treated EC entered zones 3 and 4, compared with irradiated cultures, and only sham-treated EC entered the most distant zone 5. We examined actin fibre orientation within migrating irradiated and sham-treated EC using 2-(D-2-aminobutanoic acid)-7-(*N*6-((((3,6-bis(dimethylamino)xanthylium-9-yl) carboxyphenyl) amino)thioxomethyl)-L-lysine), chloride (NBD)-phalloidin, immunofluorescent microscopy and computer image analysis. After 48 h, sham-treated, but not irradiated EC, contained actin which was orientated perpendicular to the original wound edge. After 6-9 days, only sham-treated EC closed the wounds. Tempol (4 hydroxy-2,2,6,6-tetra methylpiperidine-1-oxyl)(0.5 or 2 mM)), included in the media during irradiation, prevented this wound healing delay, when measured within the first 24 h. In conclusion, radiation treatment of capillary EC results in a wound healing defect. This defect appears to be related to the EC's inability to realign actin. Tempol protects EC from exhibiting a wound healing delay.

Keywords: actin filaments; nitroxide; superoxide; microcirculation

It has long been appreciated that radiation therapy causes damage to normal vasculature (Hopewell, 1986). In particular, injury of the microcirculation is thought to be involved in several late effects of radiation that are poorly understood (Jordan *et al.*, 1978; Law, 1981). Normally, the endothelium is maintained as a quiescent monolayer of cells lining the blood vessels. The turnover rate of normal endothelial cells (ECs) along straight aspects of the vessel is very low (Denekamp and Hobson, 1982), estimated to be of the order of once every three years. This low rate of division facilitates the essential function of the endothelium, which is to act as a selective barrier to the movement of blood elements from plasma to interstitial spaces. Higher rates of EC division are detected, however, at vessel bifurcations as measured by an increased rate of thymidine incorporation at these branch points (Engerman *et al.*, 1967). Turbulent and chaotic flow patterns are experienced by the endothelium at these locations and EC are required to migrate and proliferate to replace cells damaged and lost by shear stresses. If breaches of the monolayer occur and are not repaired, this could lead to haemorrhage and altered permeability, vascular tone and pressure at these regions of vessel. These are all changes noted *in vivo* following treatments with ionising radiation (Fajardo *et al.*, 1976; Adamson and Bowden, 1983; Altman and Gerber, 1983). As one example, haemorrhage following radiation has been noted to be most pronounced at these vessel branch points (Lauk and Trott, 1990), where we propose a wound healing demand exists. These observations led us to suspect that irradiated EC had an impaired ability to migrate into and recover a denuded area. We therefore tested *in vitro* the ability of irradiated EC to heal a scrape wound. An established *in vitro* model system of EC wound healing has previously been described (Sato and Rifkin, 1989; Pepper *et al.*, 1993). Within 24 h of wounding a monolayer of ECs, cells can be observed actively entering the denuded area. The original wound edge is distinct and closure of the wound can be monitored over the next 7 to 10 days (Sato and Rifkin, 1989; Pepper *et al.*, 1993). Normal EC have been shown to divide (Sato and Rifkin, 1989), realign their actin fibres (Lampugnani *et al.*, 1991) and migrate in response to wounding. All three functions are required to recover a wound of 2 mm dimensions. Actin is the major cytoskeletal element involved in EC migration. The randomly arrayed filamentous meshwork in a quiescent cell undergoes dramatic redistribution upon wounding, and is reorganised into longitudinal filaments projecting into the leading lamellipodial extension (Fishkind and Wang, 1993). These filaments are orientated perpendicular to the wound edge for maximum bipolar flow of organelles and allowing the interaction of motor proteins. This rearrangement of actin facilitates the immediate spreading and directional movement of EC into the denuded area. Once ECs achieve closure of the wound, they again become quiescent.

Radiation is known to have several immediate and prolonged effects on EC morphology, F-actin organisation and monolayer integrity. Kantak *et al.* (1993) demonstrated that monolayers of pulmonary microvascular EC, subjected to 12.5 to 100 cGy doses of radiation, exhibited cellular retraction and developed large gaps between 50-60% of the cells within 4-8 h following radiation treatment. These investigators detected a depolymerisation of actin fibres within retracting cells and complete loss of contact between adjacent cells. In these studies, 24 to 48 h were required to reverse these changes. These and previous studies have not addressed the mechanism by which radiation causes these changes in cytoskeletal elements within EC. The radiolysis products of water and other free radical species produced following ionising radiation, for example, have not been evaluated for their role in causing this form of EC injury.

Tempol represents a new family of nitroxides that act as radioprotectors and are effective in scavenging superoxide anions produced by radiation. Capillary EC had not been previously examined for their ability to heal a scrape wound following radiation exposure.

Materials and methods

Cell culture

Microvascular cells were a gift from Dr Judah Folkman and Ms Katherine Butterfield (Children's Hospital, Boston, USA)

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and were originally derived from bovine adrenals. ECs were used up to passage 12. Stock cultures were grown in gelatin-coated tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) containing 5% calf serum (CS) supplemented with basic fibroblast growth factor (bFGF) (3 ng ml^{-1}), 2 mM glutamine, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. Recombinant bFGF was a gift from the Takeda Pharmaceutical Co., Osaka, Japan. All tissue culture reagents were acquired from Grand Island Biological Company (Gaithersburg, MD, USA), tissue culture plastics from Costar Corporation (Cambridge, MA, USA) and biochemical reagents were from Sigma (St Louis, MO, USA). EC cultures were examined for contaminating cell types by their homogeneous expression of von Willebrand factor (vWF) using immunofluorescence methods.

Radiation and Tempol treatment

Confluent (75 000 cm^{-2}) cultures of capillary EC were prepared. The medium, with or without Tempol(4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl), was changed 10 min before and 1 h following irradiation. Tempol was solubilised with ethanol and diluted in culture media 1:1000 to achieve concentrations of 0.5 or 2 mM Tempol. Control cells received vehicle alone. Irradiation (8 Gy) was performed with a 250 kVp X-ray machine. A scrape wound of uniform width (2 mm) was produced in monolayers 30 min after irradiation or sham treatment. After 24 and 48 h, numbers of cells entering the denuded area were determined. The denuded area (2 mm) was partitioned into five zones of 125 μm increments from the original wound edge on each side of the wound and in a blind study, two independent observers counted the numbers of cells in each zone of 20 separate fields (10 fields per side of the wound) per treatment group using an ocular grid (125× magnification). Observers' data were pooled per zone per treatment group. In each experiment at least duplicate wells per treatment were performed, and each experiment was repeated at least three times. Student's *t*-tests using the SAS statistical software package (version 2.0) were performed comparing the mean numbers of cells detected in each zone. In some experiments, at T_o and various times after wounding, cells were fixed and stained with Commassie blue for computer image analysis, as described previously (Braunhut and Palomares, 1991). The wound edge was outlined permitting the total area of each well that remained denuded to be determined. In parallel dishes, the degree (or per cent) of wound closure was calculated as the denuded area at 24 h/the denuded area at $T_o \times 100$.

Measurement of actin orientation

At 24 and 48 h, some wounded monolayers were fixed in 3.75% formaldehyde, permeabilised with acetone at −20°C and stained for F-actin using a fluorescence phallotoxin solution 2-(D-2-aminobutanoicacid)-7-(N6-((((3,6-bis(dimethylamino) xanthylium-9-yl) carboxyphenyl) amino) thioxomethyl)-L-lysine), chloride (NBD)-phalloidin,(NBD-phalloidin) (Fishkind and Wang, 1993). Cells were viewed under epifluorescence, the image digitised and analysed using a Quantimet 570 digital image analyser. Images were rotated such that the original wound edge was always defined as 0°. At least 16 images were collected for each treatment condition and data collected, pooled and analysed.

Results

After 48 h, increased numbers of sham-treated EC (lower five bars) were detected within the denuded area when compared with the number of irradiated EC that migrated in response to equivalent wounding (upper five bars) (Figure 1). In addition, only sham-treated EC penetrated to more distant zones (open bar, zone 5). These studies reveal that irradiated EC exhibit a defect in wound healing ability, measured by the appearance of fewer numbers of cells detected in zones of the denuded area, and by the ability of control cells to penetrate further into the denuded area compared to irradiated cells over the same time period. We noted that sham-treated EC entering the wound were elongated perpendicular to the wound edge and appeared to move as a continuous sheet of cells. In contrast, the wound edge of irradiated cultures appeared to be jagged, with some actively migrating ECs frequently orientated parallel, rather than perpendicular, to the wound edge. These observations made by phase microscopy suggested to us that irradiated EC were impaired in their ability to reorganise cytoskeletal components used for cell movement. We therefore investigated the orientation of actin microfilaments within these cells.

We found that irradiated EC at the original wound edge did not orientate their actin perpendicular to the original wound edge in preparation for migration (Figure 2b) to the same extent as control, non-irradiated EC (Figure 2a). In sham-treated EC, actively migrating cells at the wound's edge contained actin fibres that were orientated perpendicular, between 60 and 70 degrees, relative to the wound edge (Figure 2). In contrast, actin fibres in irradiated cultures were randomly orientated, ranging from 10 to 140 degrees relative to the wound edge. After 6–9 days, sham-treated EC wounds had achieved total closure whereas irradiated wounds still had areas of denuding.

Tempol-treated irradiated ECs achieved coverage of 45–50% of a denuded area within the first 24 h, a level that was indistinguishable from that achieved by non-irradiated ECs (Figure 3). In contrast, this was statistically distinguishable from the degree of recoverage achieved by irradiated ECs, tested in parallel, in the absence of Tempol. These cultures only achieved a 33% recoverage of the denuded area in the same period of time. Thus, it appears that inclusion of Tempol in the media during irradiation eliminated the delay in the wound healing ability of ECs caused by radiation.

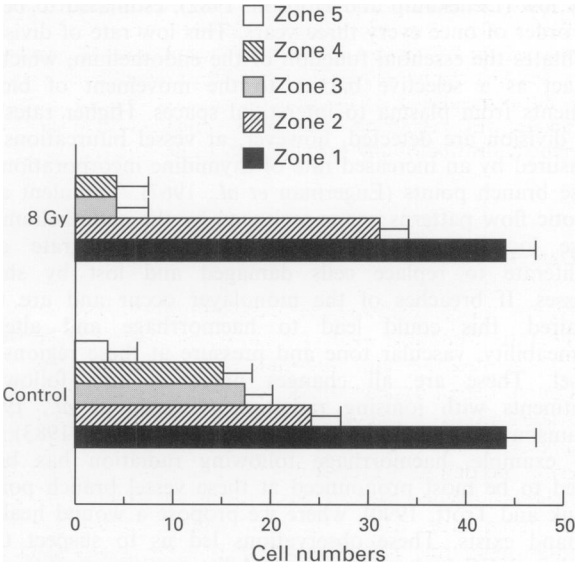

Figure 1 Number of endothelial cells migrating into successive 125μm zones distal to a scrape wound edge. At 48 h, in a representative experiment, the number of sham-treated (lower bars) or irradiated EC (upper bars) was determined in five successive 125 μm zones from the original wound edge (1–5). Equivalent numbers of control (lower bars) and irradiated (upper bars) EC migrated into the first two zones of the denuded area (1 and 2). This is an area immediately adjacent to the original wound. Significantly fewer irradiated EC, however, migrated to further regions of the denuded area (zones 3 and 4) compared with control EC in the same period of time. Finally, control cells were detected in the furthest zone (zone 5, □), 500 μm from the original wound edge, whereas irradiated EC were not detected in this zone. Error bars represent the pooled data from 20 fields counted by two independent observers per data point.

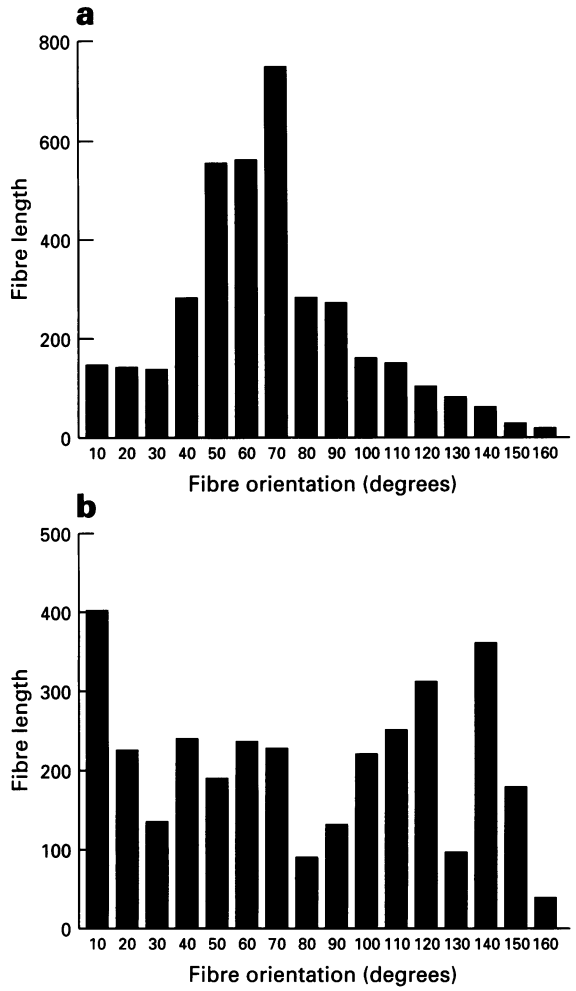

Figure 2 Forty-eight hours after wounding, sham-treated and irradiated EC were fixed, stained for actin and digitised images collected. Actin fibre orientation as calculated for control (**a**) and irradiated (**b**) EC migrating into the denuded area. The analysis revealed that sham-treated EC oriented their actin fibres between 50–80° perpendicular to the wound edge after 48 h. In contrast, irradiated EC exhibited actin fibre orientation which was randomly distributed and ranging from 10 to 150°.

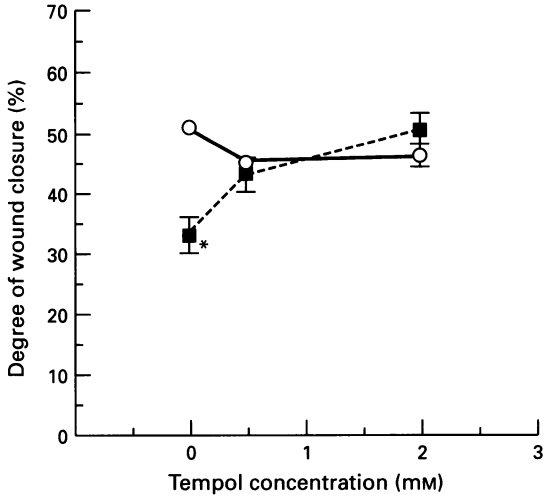

Figure 3 Effect of Tempol on radiation-induced wound healing delay. Monolayer cultures were established and received varying doses of Tempol (0, 0.5 or 2 mM) before sham (○) or radiation (■) treatment. Medium was changed after 1 h, cells fed fresh basal medium and wounded. After 24 h, the cells were fixed and photographed. The area that remained denuded was measured by computer image analysis in triplicate dishes for each condition. Without Tempol present, irradiated EC achieved 35% wound closure whereas sham-treated EC closed equivalent wounds by 53% in the same time period. When Tempol was included in the medium during sham or X-ray treatments, EC achieved similar wound closures of 45%.

Discussion

These studies demonstrate that exposure of intact monolayers of ECs to radiation leads to an impairment of the cells' ability to migrate and repopulate a denuded area. The functional defect appears to be related to the inability of the irradiated EC to reorientate their actin fibres during the act of migrating. After 24 h, we could already detect fewer cells entering the denuded area following radiation exposure (data not shown). When we stained endothelial cells to visualise actin fibres after 24 h, we were unable to analyse fibre orientation in the limited numbers of cells involved in this early response. However, by 48 h, adequate numbers of well spread cells had entered the denuded area and in these studies we were able to analyse and demonstrate that, (1) irradiated EC cultures had significantly fewer cells in the denuded area and (2) these ECs exhibited aberrant actin fibre orientation when compared with control cultures. These studies permitted us to identify a mechanism for the wound healing delay, namely inability of ECs to realign actin cytoskeletal elements.

Wounds of this size, 2 mm, require ECs to proliferate and migrate to achieve full wound closure. However, physiological wounds at bifurcations represent smaller lesions of the monolayer, and as a conservative estimate, consist of an area of roughly 10 to 20 cells deep. We reasoned that to achieve protection, a modifier would simply have to restore the ability of ECs to recover a short distance of denuding. These kind of wounds might not require a replacement or proliferative response to achieve closure. Thus, we conducted modifier studies at 24 h, at a time when ability to orientate and migrate would predominate.

Within the first 24 h, we could distinguish statistically irradiated cultures from control cultures by measuring the area that remained uncovered. We found that Tempol included in the media during the irradiation restored the ability of ECs to cover areas of denuding proximal to the original wound edge. This extent of wound repair resembles more closely the type of response required *in vivo* to repair small areas of denuding at vessel branch points. Other investigators have already shown that Tempol is a potent radioprotector, *in vitro* (Mitchell *et al.*, 1991) and *in vivo* (Hahn *et al.*, 1992). The mechanism of Tempol's radioprotection, however, may not be entirely known (Liebmann *et al.*, 1994). To date Tempol is thought to exert radioprotective effects, in part, through its action as a superoxide dismutase mimetic, and possibly through its ability to interact with carbon-centred free radicals. It has been suggested that Tempol may protect cells from the deleterious effects of ionising radiation by an, as yet, unspecified mechanism. The dose of Tempol found to be effective in our studies (< 2 mM) in averting this form of radiation damage to ECs suggests that the radioprotective mechanism is unrelated to the prevention of radiation-induced DNA damage. Our studies further suggest that if a similar wound healing defect occurs *in vivo*, in vessels of irradiated tissues, this could explain observations of haemorrhage, denuding and alterations of vascular integrity at vessel bifurcations where a wound healing demand exists. It also suggests that Tempol may protect normal tissue from radiation damage, in part, by preserving the integrity of the vessels within those tissues. In conclusion, additional work will be required to determine if the defect in wound healing ability we detect using cultured capillary ECs relates to functional changes noted to occur in irradiated microvessels *in vivo*.

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