Radiation-Induced Impaired Surgical Skin Wound Healing: Pathophysiology and Prevention

by

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Abstract

We investigated the pathophysiology of radiation-induced impaired surgical wound healing using a rat skin flap wound receiving pre-operative single dose or fractionated irradiation, and the efficacy of the radioprotector Amifostine in mitigating the adverse effects of irradiation on wound healing. We examined wound breaking strength (WBS), wound hydroxyproline content, wound densities of macrophages, fibroblasts and capillaries, and wound protein expression of transforming growth factor β_1 (TGF-β_1) and vascular endothelial growth factor (VEGF). Both single dose and fractionated irradiation impaired wound healing as indicated by decreased WBS and wound hydroxyproline content compared to the control ($p<0.05$). Wound densities of macrophages, fibroblasts and capillaries were decreased in irradiated wounds compared to the control ($p<0.05$). Western blot analysis demonstrated down-regulation of VEGF in irradiated wound tissue ($p<0.05$). Finally, Amifostine prophylaxis significantly ($p<0.05$) attenuated the adverse effects of pre-operative irradiation on WBS, wound tissue hydroxyproline content, and wound tissue densities of fibroblasts and capillaries.

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Abbreviations

bFGF	Basic fibroblast growth factor
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
Gy	Gray (unit of radiation)
h	Hour
HBO	Hyperbaric Oxygen
HCl	Hydrochloric acid
IGF-1	Insulin-like growth fator-1
ILs	Interleukins
IL-1	Interleukin-1
IL-2	Interleukin-2
i.m.	Intra-muscular
i.p.	Intra-peritoneal
IU	International units
Kda	Kilodaltons
KGF	Keratinocyte growth factor
LTs	Leukotrienes
MCP-1	Monocyte chemoattractant protein-1
mm	Millimeter
MMP-1	Matrix metalloproteinase-1
Min	Minutes
[OH•]	Hydroxy free radical
PDGF	Platelet derived growth factor
PGs	Prostaglandins
TBI	Total body irradiation
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-α
μm	Micrometer
VEGF	Vascular endothelial growth factor
WBS	Wound breaking strength

1.0 Introduction

1.1 Overview of the Present Research Project

A combination of surgery and pre- or post-operative radiotherapy is the current standard of treatment for many forms of cancer (158). The addition of radiotherapy to surgery alone is beneficial because the combination of radiotherapy and surgery minimizes the need for radical dissection, reduces the rate of limb amputation, and improves local tumor control (4, 20, 212, 214). Irradiating patients pre-operatively has some advantaged over post-operative irradiation, but unfortunately, exposing patients to pre-operative instead of post-operative ionizing radiation is known to cause a higher incidence of impaired surgical skin wound healing (28, 162), which can result in wound healing complications (43, 166, 178, 180). The mechanism by which pre-operative irradiation impairs skin wound healing is unclear, and no therapeutic interventions are currently available for clinical use to prevent or treat the problem. Understanding the pathophysiology of radiation-induced impaired surgical wound healing will most likely lead to identification of modalities for the prevention and treatment of radiation-induced impaired surgical wound healing.

This research project used a clinically relevant, in vivo skin flap model to examine the mechanism by which pre-operative single-dose and fractionated local irradiation of skin impairs surgical wound healing. Specific emphasis was placed on the effects of irradiation on wound breaking strength (WBS), on the wound inflammatory cell, fibroblast and capillary densities during wound healing, and on wound content of growth factors which may play key roles in wound healing. Furthermore, the putative radioprotective agent Amifostine was used for the first time as a probe to investigate both the mechanism underlying radiation-induced impaired surgical wound healing, and the efficacy of prevention of radiation induced impaired skin wound healing by prophylactic Amifostine treatment.

1.2 The Clinical Wound Healing Problem Associated with Pre-Operative Radiotherapy

As stated earlier, the combination of ionizing radiation therapy and surgical excision has become standard therapy for many forms of cancer. The rationale behind using radiation therapy to treat cancer is that ionizing radiation can inflict relatively more damage to cancer cells than to the normal surrounding tissue, with resulting destruction of the tumor, and relative, but not complete sparing of the surrounding normal tissue.

There are several advantages to the combination of radiation and surgery for the treatment of cancer. Because radiation can potentially sterilize the tissue immediately surrounding a tumor, the need for a more radical surgical resection is minimized. In some instances, this can, for example, result in salvage of a limb that would otherwise require amputation. Local tumor control is also improved by combining radiotherapy and surgery (4, 20, 212, 214).

Radiotherapy may be delivered prior to or after surgical intervention. In the clinical setting, post-operative radiation is usually delayed until the surgical wounds have healed. Typically, pre-operative radiotherapy precedes surgical ablation and wound reconstruction by 3-8 weeks, thus allowing the initial tissue reaction to settle prior to surgery (72,178).

Compared to post-operative radiation therapy, the advantages of pre-operative irradiation are that it allows for the use of a smaller dose of radiation delivered to a smaller target volume, giving a better functional and less deforming result (168,181,213). Pre-operative radiation therapy may also shrink a tumour, sometimes rendering an

inoperable tumor operable, and often making tumor resection technically easier (181). Pre-operative radiation therapy may also eradicate sub-clinical disease beyond the surgical resection margin, diminish tumor cell implantation at the time of surgery by decreasing the number of viable tumor cells within the operative field, and sterilize lymph node metastases outside the surgical field (168).

An important disadvantage of pre-operative radiation is a greatly increased incidence of post-operative wound complications, including wound dehiscence and skin necrosis, exposure of vital structures, infection, fistula formation and pain (110, 134, 135, 166). In breast cancer patients, pre-operative radiation is associated with delayed wound healing in 29-35% of cases (5, 98, 191). The reported incidence of wound complications in sarcoma patients irradiated pre-operatively ranges from 16-37% (35,166). For head and neck cancers, the incidence of wound complications after surgery in irradiated tissues can be as high as 50% in cases where high dose radiation is employed (134,135). In contrast, when radiation is given post-operatively the incidence of wound complications is as low as 8% for soft tissue sarcomas, and 15% for head and neck cancers (44,134).

These wound complications associated with pre-operative irradiation often lead to secondary operations, higher patient morbidity, longer hospital stays, higher costs, diminished function and more disfigurement (109,179). It is crucial to understand the mechanisms underlying the effects of pre-operative radiotherapy on surgical skin wound healing if reasonable strategies for preventing and treating this problem are to be established. It is currently unclear why some irradiated wounds fail to heal. Furthermore, there are no clinically proven therapeutic interventions for prevention/treatment of radiation-induced impaired surgical wound healing.

1.3 Effects of Irradiation on Surgical Wound healing in Experimental Animals

Pre-Operative Irradiation: As early as 1939 Dobbs, using a rat abdominal skin incision model and a single 18 Gy radiation dose, showed that the tensile strength of wounds irradiated 1 or 3 weeks pre-operatively was diminished by 50% compared to controls, when measured 8 days post-operatively (69). Since then, others have shown diminished incisional wound breaking strength (WBS) resulting from single doses of pre-operative radiation. For example, Bernstein used a single radiation dose of 18 Gy to demonstrate a reduction of WBS of 62% in guinea pig incisional skin wounds made two days after irradiation (25). Using a mouse skin model, Gorodetsky outlined a single dose radiation threshold of 8 Gy to reduce WBS, a plateau of 20 Gy and maximal WBS reductions to 30-50% of normal were achieved with 18 Gy (89). Several other studies examining the effects of pre-operative irradiation on wound healing in rat skin used doses in the range of 15-25 Gy with similar reductions in WBS, in the order of 50%-60% of the control value (56,79,153,155). These observations confirmed the clinical impression that pre-operative radiotherapy impairs surgical wound healing.

Post-operative Irradiation: The timing of post-operative radiation is vital in determining the effect on wound healing. In terms of WBS, one study, using a rat skin incision model, demonstrated that a 20 Gy dose of radiation delivered 7 days post-operatively did not affect WBS compared to the control when measured 2 weeks after wounding (65). A similar study using 18 Gy delivered 12 days after wounding of mouse skin also found no significant deficit in WBS when measured 2 weeks after wounding. However, the same study did find that wounds irradiated within 5 days after wounding showed a decease in WBS of approximately 50% of the control (89). The author

suggested that the impaired wound healing seen with radiation delivered 5 days post-operatively and the diminished radiation effect on WBS seen with radiation delivered 12 days post-operatively simply reflected the extent of wound healing already completed. Another study found that radiation of skin wounds in mice could actually transiently enhance WBS if done 7, 9 or 14 days post-wounding (228).

1.4 Pathophysiology of Normal Wound Healing

Before analyzing the problem of radiation-induced impaired surgical wound healing, one needs a clear understanding of the normal wound healing process. The following section reviews the pathophysiology of normal wound healing, and provides the background to understand impaired wound healing.

Wound healing in normal tissues occurs in three overlapping yet distinct phases, as depicted in Figure 1: The hemostasis and inflammation phase, the wound repair and regeneration phase, and the wound remodeling phase (124,239). These three phases of wound healing and the important cell types and cell functions of each phase are discussed below.

1.4.1 Hemostasis and Inflammation Phase (Days 0-5 Post Wounding)

The hemostasis and inflammatory stage of wound healing accomplishes two goals: The arrest of bleeding at the wound site and migration of inflammatory cells required for wound healing into the wound site. There are several important inflammatory cell types involved in this phase: Platelets, mast cells, neutrophils, and macrophages.

Platelets: Immediately upon tissue injury, local platelets aggregate and adhere to

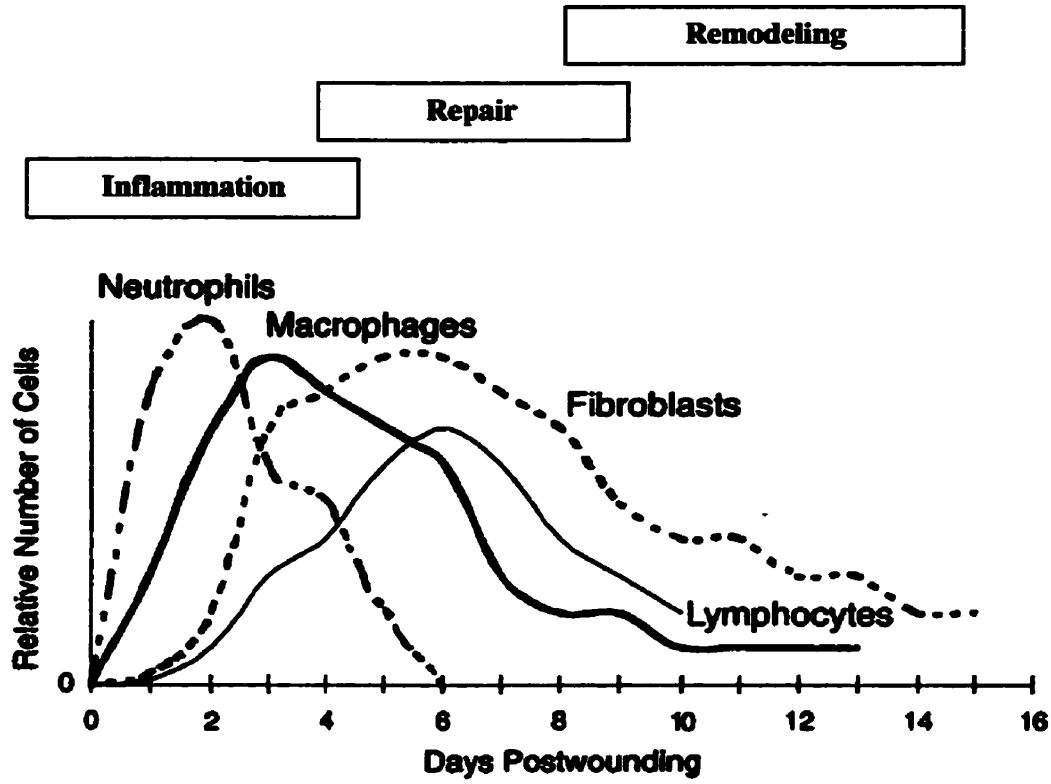

Figure 1. Outline of the three major phases of normal wound healing in the skin and the time course of inflammatory cell and fibroblast infiltration into the skin wound (239).

exposed sub-endothelial collagen and intertwine with fibrin to form a platelet plug, thus stopping active bleeding (124). Platelets also release several cytokines from intracellular alpha granules. These cytokines include vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF). These cytokines attract inflammatory cells, and stimulate collagen synthesis by fibroblasts and angiogenesis in vascular endothelial cells, which are all processes needed for wound healing. The release of these cytokines from platelets is a vital first step in initiating the wound healing process (74,124,132,150). In addition to the platelet plug, circulating norepinephrine and prostaglandins (PGs) from injured cells cause local vasoconstriction which also facilitates hemostasis (124).

The accumulation of wound fibrin, the end product of the coagulation cascade, occurs simultaneously with the formation of the platelet plug to achieve hemostasis. Fibrin facilitates the attachment of migrating inflammatory cells such as neutrophils and monocytes, and mesenchymal cells such as fibroblasts. A fibrin-fibronectin lattice acts as a reservoir for cytokines involved in the healing process (124).

Mast Cells: Mast cells are increasingly considered to be an important source of mediators during the wound healing process (225). Within mast cells, there are dense granules containing a multitude of mediators including TGF-β, PDGF, VEGF, tumor necrosis factor-α (TNF-α), interleukins (ILs), histamine and heparin among many others (10). At the time of wounding, regional mast cells are attracted to the wound area by products of the coagulation cascade as well as by TGF-β. Mast cell degranulation is triggered initially by C3a and C5a from the coagulation cascade (10), and thus the

influence of mast cells on wound healing begins as early as the onset of the coagulation process. The contents of mast cell granules have been found to be chemotactic and mitogenic for fibroblasts and endothelial cells, thus influencing the processes of matrix production and angiogenesis (10). Mast cells may also influence angiogenesis through their production of VEGF, and bFGF (10). In addition, histamine and leukotrienes (LTs) from mast cells, along with prostaglandins (PGs) derived from the cell membranes of injured cells cause vasodilatation and increased vascular permeability. Mast cell induced vasodilatation and vascular permeability thus facilitate the entry of plasma, leukocytes and proteins into the wound region, bringing the components of the wound healing process to the injured area. (124,132,202)

Neutrophils: In response to the chemo-attractant stimulus of C3a, C5a from the coagulation cascade, and platelet activating factor and platelet factor-4, neutrophils leave the circulation and enter the wound region through a multi-staged process (124). The first step, called margination, involves the settling of neutrophils out of the central column of flowing blood. This occurs due to vasodilatation and a decreased rate of blood flow. Once the neutrophils fall out of the central column of flowing blood, they roll along the endothelium (55). The next step is neutrophil adhesion to and transmigration through the endothelial wall. Adhesion is achieved by selectins on the endothelial cell surface, and integrin receptors on the leukocyte surface (55). Transmigration occurs by the process of diapedesis. Finally, neutrophils migrate through interstitial tissues towards a chemotactic stimulus such as complement components or ILs (55). Leukocyte enzymes facilitate migration between endothelial cells by dissolving the basement membrane (55). Wound neutrophils peak in number within 24-48 h (124), and digest foreign materials in

the wound (194).

Macrophages: In response to specific factors such as collagen and fibronectin fragments, elastin from damaged matrix, complement, thrombin and TGF-β_1, local tissue macrophages enter the wound using an adhesion and migration process analogous to that used by neutrophils (195, 239). Once in the tissues, macrophages are activated by IL-2 derived from T-lymphocytes, and PDGF (124). Macrophages outnumber neutrophils by 48 h after wounding and their numbers remain high for several days (239).

Activated macrophages phagocytose bacteria and dead tissue, break down damaged wound matrix and regulate tissue breakdown and wound remodeling (124, 239). They also secrete VEGF, TGF-β, TNF-α, PDGF, IL-1, insulin-like growth factor-1 (IGF-1), TGF-α, and bFGF. Fibroblast proliferation is stimulated by PDGF, TGF-β, IL-1 and IGF-1. Collagen production is induced by PDGF, TGF-β and bFGF. VEGF and TNF-α stimulate angiogenesis (29, 124). In the absence of macrophages, normal wound healing cannot occur. This phenomenon was demonstrated in experiments which used anti-macrophage serum to impair wound healing in a guinea pig linear incision model (126, 127).

1.4.2 Wound Repair and Regeneration Phase (Days 1-21 post wounding)

The repair and regeneration phase starts approximately 1 day after wounding and continues up to 3 weeks after wounding. During this phase, it is epidermal repair, collagen production and angiogenesis that play the important roles in wound healing.

Epidermal Repair: Under the influence of epidermal growth factor (EGF) and TGF-α, epithelial cells re-establish the barrier function of skin, a process usually

completed within 48 h of wounding (124, 152). Keratinocytes have also been shown to be directly involved in the re-establishment of the epithelial layer in human burn scars, and in the maturation of the final scar, due to their production of TGF-β_1, β_2, and β_3, as well as bFGF, and VEGF (99).

Collagen Production: Collagen is the most abundant protein found in all mammals, constituting approximately 25% of the total body protein (3). The role of collagen is to provide strength and integrity for all body tissues including skin, and it therefore plays a particularly large role in wound repair (96). There are several different forms of collagen known, with the differences based on the composition of the constituent α chains making up the triple helical structure of collagen. The types of collagen predominantly found in skin are types I and III. The initial type produced early in wound healing is type III, with type I ultimately replacing it as the wound matures (96). The difference between types I and III collagen is that type I is composed of two α-1 chains and one α-2 chain, whereas type III is composed of three α-1 chains (3).

Macrophages produce PDGF and TGF-β to attract fibroblasts to the wound (124). Fibroblasts from regional connective tissue and perivascular adventitia appear in the wound by day 2-3, and are the dominant cell in the wound by day 7 (195). Fibroblasts migrate into the wound on a scaffolding of hyaluronate and fibronectin, aided by enzymes such as matrix metalloproteinase-1 (MMP-1), gelatinase and stromolysin that facilitate their movement through the provisional wound matrix (124). Fibroblasts proliferate under the influence of TGF-β_1, PDGF, TNF, IL-1 and EGF (124). Results from in-vitro studies also suggest a role for fibroblasts in the wound re-vascularization process by secretion of VEGF (205).

When stimulated by TGF-β_1, FGF and EGF, fibroblasts begin producing collagen 2-3 days after wounding (124). Collagen production peaks approximately 7 days after wounding. Wound collagen levels and tensile strength rise for 3-4 weeks then plateau as collagen degradation equals production (203). Collagen deposition correlates with increasing wound tensile strength (131). The rate-limiting step in the increase of wound tensile strength is the rate of pro-collagen production (173). Approximately 1 week after wounding, myofibroblasts, a specialized form of fibroblast, contract, and thus reduce the overall surface area of the wound.

Wound Angiogenesis (Capillary Formation): The healing wound tissue requires delivery of oxygen and nutrients and as such the vascular supply needs to be re-established. The process of angiogenesis is defined as the growth, expansion and remodeling of existing regional blood vessels, and it is this process which replaces vasculature damaged by wounding (52, 244). Angiogenesis occurs under the influence of a large array of growth factors, including VEGF, bFGF, PDGF, and indirectly, TGF-β_1 (8). In addition, the process involves multiple steps, including vascular dilatation, increased vascular permeability, and disintegration of extracellular matrix to allow for endothelial cell migration. Endothelial cells from the existing vessels and from the bone marrow proliferate and migrate towards the source of the angiogenic growth factor stimulus. The new endothelial cells assemble, and acquire lumens enabling them to become functional capillaries (8). bFGF and VEGF, play a key role in angiogenesis (159, 160), and in their absence, angiogenesis does not occur (124, 244). Once established, the new vessels are stabilized in several ways. Angiopoietin-1 (ANG-1) tightens the interactions between endothelial smooth muscle cells, solidifying the structure and inhibiting permeability (52). Endoglin, a TGF-β1 binding protein found

on the surface of endothelial cells, stabilizes new vessels by stimulating the production of ECM, and plasminogen activator inhibitor-1 prevents ECM dissolution (52).

1.4.3 Wound Remodeling Phase (Days 10-18 months post wounding)

Approximately 21 days after injury, the net accumulation of collagen stabilizes, and wound remodeling becomes the dominant process. Collagen cross-linking and re-arrangement of collagen into dense bundles increases wound strength. After 6 weeks, the wound will have achieved 80% to 90% of its eventual strength and by 6 months the scar will have 80% of the tensile strength of normal skin, which is the strongest it will ever be (124). With scar maturation, type III collagen decreases, and is replaced by type I. The process finally comes to completion 12 to 18 months after wounding.

No direct comparisons of the phases of wound healing rats and humans were found in the literature. However, one study using rats did demonstrate that wound inflammatory cells (neutrophils, monocytes and macrophages) peak on day 3-4 after incisional wounding, and wound fibroblasts peak on day 7 after incisional wounding (169). In other studies of rat wound healing, researchers examined wound macrophages 3 days after wounding (154), and wound fibroblasts 7 days after wound healing (17), suggesting that the time-frame of wound healing applied to humans is applicable to rats as well.

1.5 Pathophysiology and Pharmacological Intervention of Impaired Chronic Wound healing

Pathophysiology

There is a significant amount of literature exploring the pathophysiology of impaired wound healing in chronic wound tissues. Many different mechanisms underlie

impaired wound healing seen in chronic wounds, including an altered inflammatory response, a tendency towards infection, tissue hypoxia, chronic venous stasis, metabolic disorders, malignancy, steroid use, poor nutrition and advanced age (17, 161, 201, 215).

Traditionally, management for these types of chronic wounds has included strategies to eliminate the underlying cause. For example, arterial bypass surgery is used for patients with chronic lower limb wounds secondary to arterial insufficiency. Debridement of necrotic tissue along with administration of antibiotics may be effective at eliminating chronic wounds resulting from infection. Appropriate blood sugar management can help control the complex wounds seen in diabetic patients. Nutritional support may be useful in patients whose wounds are results of a specific dietary deficiency.

The study of chronic wounds has also stimulated much research on different types of dressings and dressing techniques, including such novel approaches as using artificial skin or vacuum assisted wound closure (14,111,164,175).

More recently, attention has been turned to the role of growth factors such as VEGF, TGF-β, PDGF, bFGF, IGF and EGF in the pathophysiology and treatment of non-irradiated chronic wounds (53). Inadequate amount or function of these growth factors and/or their receptors has been implicated in animal models of chronic wound healing. Several representative examples of these studies are described below.

Studies using aged and diabetic mice demonstrated decreased VEGF production by wound macrophages (215, 245). Diabetic and steroid treated mice were found to be deficient in PDGF ligand and receptors both in unwounded and in newly wounded skin (19). In in-vivo studies using mice, and in in-vitro studies using mouse keratinocytes,

steroids have also been shown to reduce keratinocyte growth factor (KGF) and TGF-β expression after wounding (33,80).

The above experiments have led to the idea that applying exogenous growth factors may improve healing in chronic wounds, and this area of research is discussed below. To date, the exact role of growth factors in accelerating chronic wound repair remains unclear, due to equivocal clinical results seen thus far (170).

Pharmacological Intervention Using Growth Factors

TGF-β: This is a mammalian protein consisting of two 12.5 kDa polypeptide chains. TGF-β is abundant in platelets, as well as in macrophages, lymphocytes and fibroblasts (24, 56, 115, 155, 196). It is known to stimulate the production of collagen, fibronectin, and glycosaminoglycans, as well as inhibit their breakdown by down-regulating the proteins that degrade them (155). TGF-β is also known to be an extremely potent stimulator of chemotaxis for the migration of macrophages, lymphocytes neutrophils and fibroblasts (118, 143).

TGF-β has three isoforms, TGF-β_1, β_2 and β_3, which share 64-85% amino acid homology (80). TGF-β_1 is the most abundant isoform in most tissues, the only form found in human platelets, and the predominant form secreted by wound fibroblasts and macrophages (118). Of the three TGF-β isoforms, TGF-β_1 and TGF-β_2 are the ones most involved in scar formation, while TGF-β_3 seems to reduce scarring (193). TGF-β_2 and TGF-β_3 are also prevalent early after excisional wounding in the migrating epidermis (128), suggesting that they are more important for re-establishing the epithelial barrier of skin than for strengthening the dermis and providing wound strength. In fact, recent work

using TGF-β_1 knockout mice suggests that TGF-β_1 may actually inhibit wound re-epithelialization and wound closure (118). This supports the notion that TGF-β_1 is inhibits proliferation of epithelial cells, and stimulates proliferation of mesenchymal cells such as fibroblasts (136).

TGF-β activity is mediated by signaling through receptors having intrinsic serine-threonine kinase activity. This signaling mechanism is somewhat unique among growth factors involved in wound healing, which usually employ tyrosine kinase activity (11). There are two cell surface receptors employed by the TGF-β proteins, TGF-βRI and TGF-βRII. TGF-βRII binds TGF-β and presents it to the TGF-βRI receptor. The two receptors then form a heterodimeric complex, which is the active form of the receptor (136). This active receptor form then initiates a series of intracellular signals mediated by the recently described SMAD proteins (11).

TGF-β has been used to reverse impaired wound healing in several non-irradiated models of impaired healing. Pierce et al. demonstrated that single applications of TGF-β (0.25-1 μg/wound) applied locally at the time of wounding reversed the incisional skin wound healing deficit in steroid treated rats (172). Slavin et al. used pig intestinal wounds to demonstrate a similar effect of TGF-β (198). Also in steroid treated rats, Beck et al. showed a reversal of impaired healing using a single intravenous administration of TGF-β_1 (100 or 500 μg/kg), and also showed a similar wound healing improvement in aged rats using a topical application of TGF-β_1 at the time of wounding (18). Others have shown that TGF-β1 can improve incisional wound healing in diabetic animal models (21, 31). TGF-β1 has not been uniformly successful in improving chronic wound healing.

Wu at al. found no improvement in healing in an ischemic dermal ulcer model in aged rabbits when using a single topical application of TGF-β1 at the time of wounding (1μg/wound) (232).

VEGF: This is a 45 Kd homodimeric protein whose main action is to stimulate angiogenesis and promote vascular permeability (157). While VEGF is produced by many different cell types, including platelets, neutrophils, macrophages, lymphocytes and arterial smooth muscle cells, it acts specifically on vascular endothelial cells (15,49,81,86,106,241). It is accepted that VEGF plays a key role in the normal wound healing process (222), a conclusion resulting from a multitude of studies done in normal and impaired wound healing protocols in both human and animal studies (54,107,160).

VEGF has multiple effects on the formation of new blood vessels. In addition to increasing vascular permeability and stimulating extravasation of proteins, VEGF also influences the angiogenic sprouting pattern (52). In addition, VEGF determines the lumen diameter of the newly formed vessels, and is involved in giving the new endothelial cells the specific properties they need to function in their physiologic environment (52).

In animal studies, local VEGF, produced through intravascular cDNA gene therapy, was observed to increase survival of ischemic abdominal skin flaps in rats due to neovascularization (220, 221). In a rabbit ischemic dermal ulcer model, topical VEGF (30 μg/wound) was found to increase granulation tissue compared to saline treated controls (54). Another study using a rabbit ischemic skin flap model demonstrated accelerated wound neovascularization compared to saline treated controls in wounds treated with VEGF (0.1μg/ml) (206).

PDGF: This is a 30 kDa dimeric protein consisting of disulfide linked polypeptide chains called A and B (147). The existence of the two chains results in three possible isoforms, PDGF-AA, AB and BB. PDGF binds two distinct receptors, α and β. The α receptor binds all three of the PDGF isoforms, while the β receptor binds BB best, AB poorly, and AA not at all.

Endothelial cells, vascular smooth muscle cells, activated monocytes, macrophages and of course platelets produce PDGF, which is important in the processes of normal wound healing (147). PDGF was the first growth factor known to attract neutrophils and monocytes, and accounts for at least 50% of the mitogenic activity caused by factors released from platelets (147,173). PDGF was also observed to attract fibroblasts to the healing wound, stimulate them to divide, and sustain them by paracrine and autocrine mechanisms (64,197). PDGF stimulated monocytes and macrophages to produce TGF-β_1, which in turn stimulated collagen and matrix production during wound healing (171). In response to PDGF, fibroblasts were shown to transcribe genes that encode proteins important for intercellular communication, cellular migration and subsequent wound healing (64).

Currently, recombinant PDGF-BB (Regranex ^{TM} 0.01% topical), is the only recombinant growth factor licensed by the United States Food and Drug Administration for use to improve wound healing, although its use is restricted to diabetic foot ulcers (182). Regranex ^{TM} has recently become available in Canada as well. In clinical trials, recombinant PDGF-BB gels, (100 μg/g), applied once a day to clean, non-infected diabetic foot ulcers, was found to accelerate wound healing (75, 237).

The utility of PDGF in treating diabetic wounds is not without controversy.

PDGF did not result in significantly accelerated wound healing in diabetic neuropathic ulcers in a study by Young et al. (242). As well, PDGF did not show a benefit compared to good ulcer care alone in a study done by the Beclapermin (PDGF) Gel studies group (237).

1.6 Pathophysiology of Radiation-Induced Injury and Impaired Wound Healing

1.6.1 Cellular Damage Caused by Ionizing Radiation

When high-energy photons known as ionizing radiation interact with a target atom and have enough energy to displace an electron from its orbit around that target atom, the process is called ionization. This is because the target atom is left with a net positive charge and is therefore an ion. (34). The displaced electrons may collide with other electrons in tissues, resulting in the initial kinetic energy from the radiation source being lost and the energy dissipated into the surrounding tissues. The radiation dose delivered is measured in terms of the amount of energy (joules) absorbed per unit mass (kg) of tissue, and is expressed in Grays (1 Gy = 1 joule/kg) (34).

The cellular damage caused by ionizing radiation is classically described as direct or indirect. Direct damage refers to the direct interaction of electrons with macromolecules causing breakage of bonds and new radical formation. Indirect damage is mediated through the production of free-radicals (218), as shown in figure 2 on page 34.

Free-radicals are generated by the interaction of energized electrons and intracellular water. Since most of the target tissue is made up of water, the generation of free-radicals is the predominat process (238). Free radicals are chemical species that have a single unpaired electron in an outer orbital, rendering them extremely reactive and

unstable. They also initiate autocatalytic reactions, meaning that the molecules they react with are themselves converted into free radicals, thus propagating the cycle of molecular damage (55).

The most important free-radical in the process of tissue damage is the free hydroxy radical (OH•), which damages various target molecules including cell membranes, microtubules and DNA (235). Once generated, (OH•) radicals lead to cell death through peroxidation of cell membrane lipids, oxidative modification of cellular proteins, and breaking DNA strands (55). It has been suggested that agents that can scavenge (OH•) radicals can protect against 50-70% of the damage caused by x or γ rays, since it is the (OH•) radicals that cause most of the damage induced by these forms of radiation (100) as shown in figure 2 on page 34.

Cellular DNA is recognized as the most important target of ionizing radiation (162). Multiple single and double stranded DNA breaks occur in each irradiated cell in a dose dependant manner, with about 1000 single strand and 25-40 double strand breaks occurring per diploid cell per Gy (162). As a result of direct interaction between the excited electrons and cellular DNA, complex breaks occur in the DNA sugar-phosphate backbone, causing alteration or loss of DNA bases and formation of cross-links between DNA strands (34).

Radiation damage to cells has been classified as lethal, sub-lethal and potentially lethal. Lethal damage is so severe that it is 100% fatal to cells. Sub-lethal damage can be repaired by the cell within hours under normal circumstances. Potentially lethal damage is a theoretical state in which DNA damage can be modified by changing the cellular environment after irradiation (28). It is this DNA damage and the inability of the cell to

adequately repair it before entering mitosis that ultimately leads to the death of the cell and the diminished function of the tissue as a unit (238). The sensitivity of a given tissue to the effects of ionizing radiation is largely dependent on the ability of individual cells to repair the damage, and the ability of the cell population as a whole to regenerate itself into a functional unit (103).

An irradiated cell, which has sustained DNA damage, can remain viable despite the destruction of its reproductive capabilities (238). Irradiated cells are most at risk of dying from the effects of ionizing radiation during mitosis, when they need an intact DNA template for accurate cellular reproduction. Ordinarily, tissues that are quiescent for long periods of time, such as connective tissue cells and endothelial cells, would not immediately manifest the effects of radiation damage. Wounding initiates the process of tissue repair, which forces cellular mitosis and proliferation to occur. Therefore, wounding can accelerate the rate at which tissues manifest radiation damage (25,90,94).

Another form of cell death caused by radiation is apoptosis. Apoptosis is a form of programmed cell death characterized by a non-inflammatory process involving nuclear fragmentation and chromatin condensation followed by loss of membrane integrity. The central biochemical event in the apoptotic process is the activation of a calcium/magnesium dependant, zinc sensitive endonuclease that cuts DNA (231). The resulting nucleic acid fragmentation is identified as apoptotic cell death by a characteristic 180-200 base pair ladder sequence seen on gel electrophoresis (67,167).

The initiation of apoptosis following irradiation can originate from the nucleus, cytosol, or plasma membrane. Nuclear DNA damage may initiate p53 dependant apoptosis, which involves the regulation of the Bcl-2 family of genes, and CD95

dependant initiation of caspase activity (229). Apoptotic signals originating from the plasma membrane involve ceramide derived signals stimulating the stress activated protein kinase or c-Jun N-terminal kinase signalling pathway (SAPK/JNK) resulting in activation of the caspase pathway. The IL-1β convertase gene has also been identified as a possible activator of apoptosis, since the gene product is thought to initiate caspase activity which fragments DNA (231). It is the caspases, which are cysteine proteases, which have been labeled the "executioners of apoptosis", since their activation is the final common pathway resulting from various apoptotic signals (229). It is also believed that the p53 gene may facilitate apoptosis, since mice homzygous for mutation or deletion of p53 are resistant to apoptosis (231). The likelihood of a cell undergoing radiation-induced apoptosis depends on the cell type. Cells of the lymphoid and myeloid lineage, as well as cells of the intestinal crypts and salivary glands are more likely to undergo early apoptosis than other cell types, such as mesenchymal cells (229).

1.6.2 Skin Tissue Damage Caused by Ionizing Radiation

Acute Effects: The acute effects of ionizing radiation on skin are defined as those effects occurring within six months of radiotherapy. The acute effects include erythema, due to injured dermal blood vessels, dry desquamation due to destruction of superficial keratinocytes with enough surviving basal keratinocytes to regenerate the epidermis, and moist desquamation resulting from the destruction of the basal epidermal layer leaving the dermis exposed (28, 223).

Chronic Effects: The chronic effects of ionizing radiation on the skin are defined as those effects occurring 6 months or more after radiotherapy. The chronic effects of

radiation include the following: Pigmentation changes due to melanocytes producing and transferring more pigment, thickening and fibrosis of skin and subcutaneous tissue secondary to dense and irregular collagen deposition, telangectasia due to dilated vessels in the dermis, decreased number and progressive obliteration of capillaries, sebaceous and sweat gland dysfunction, hair loss, skin necrosis, and tumorigenesis (142, 192).

The factors determining the onset of the effects of irradiation include the rate of cell proliferation, the cell cycle position, and the number of stem cells in the tissue. Rapidly proliferating cells will manifest the effects of irradiation sooner because the effets of irradiation are manifested during cell division. Mitotic cells are most sensitive to radiation effects. Having fewer stem cells minimizes the ability of tissues to tolerate loss of a population of constituent cells. The dermal components of skin, including fibroblasts and capillary endothelial cells, are generally slowly proliferating cells, with relatively fewer stem cells. This explains why the effects on the dermis such as thickening and fibrosis, and the progressive loss of capillaries are late effects. This is in contrast to the effects on the epidermal keratinocytes, which proliferate more rapidly to re-supply the shedding corneified skin layer, and thus manifest the effects of radiation more quickly (168, 238).

1.6.3 Impaired Wound Healing Caused by Ionizing Radiation

The effects of pre-operative ionizing radiation on the wound healing process have been investigated in experimental animal models using single dose irradiation. The following section summarizes what is known to date with respect to the detrimental effects of ionizing radiation on the three phases of wound healing.

Hemostasis and Inflammatory Phase:

Platelets: Reports on the effects of radiation on platelet function in terms of aggregation, clot retraction and cytokine release in the early wounding period are contradictory and unclear. Werner used human platelets and a single 12 Gy dose of x-rays to show that radiation impaired clot retraction in-vitro (236). Using in-vitro human platelet-rich plasma and a single 100 Gy dose of radiation, Hartman et al. showed that radiation did not impair clot retraction (101). Kalovidouris et al. also using human in-vitro platelet rich plasma, showed that single radiation doses of 1 to 50 Gy had no effect on clot retraction, ADP release, or platelet factor 3 availability (112). To date, there have been no studies examining the effect of ionizing radiation on platelet production of growth factors.

Inflammatory Cell Density: In 1939, Dobbs, using histological assessments, reported a non-specific diminished inflammatory cell reaction in the deeper skin layers after a single 18 Gy radiation dose (69), but made no mention of changes in population of any particular cell types. More recently, using histological sections, Wang et al. reported finding fewer inflammatory cells in the dermis 1 day after incisional wounding in rat skin exposed to a 10 Gy radiation dose 7 days pre-operatively **(233)**, but again, no mention of the particular cell types was made. Wang et al. did demonstrate diminished fibroblast infiltration on days 3, 7 and 14 after wounding.

Inflammatory Cell Function: Decreased phagocytic activity of mouse peritoneal macrophages was observed after exposure to single doses of ionizing radiation ranging from 1 to 20 Gy (22). Neutrophils collected from wound cylinders 7 days after surgical implantation in a rabbit model had decreased phagocytic activity, as well as decreased

superoxide anion and MAC-1 integrin expression following radiation doses of 10, 20 or 30 Gy (82). The effects of irradiation on neutrophils may not be a major factor given the study by Simpson and Ross suggesting that neutrophils are not essential components of normal wound healing (194). In their study, Simpson and Ross used anti-neutrophil serum to block the activity of neutrophils in a guinea pig dorsal skin incisional wound model, and found no difference in the rate of wound debridement or the extent of wound repair in the neutropenic animals compared to the normal controls (194).

Wound Blood Flow: There is the suggestion in the literature that ionizing radiation can damage small blood vessels by occluding them in a process called obliterative endarteritis (187). This process is thought to lead to skin hypoxia and impaired surgical wound healing (140,141). Using histological methods, Wolbach et al. found occluded microvessels in radiation-scarred human skin (240). More recently, Marx et al. found decreased transcutaneous oxygen pressure in patients with osteoradionecrosis, and attributed the findings to obliterative endarteritis (140,141). Patterson irradiated pig flanks, and found decreased skin flap viability when skin flap surgery was done later than 6 weeks after irradiation. He attributed the decrease in skin flap viability to changes in the skin vasculature (165).

In contrast, however, fluorescein perfusion studies of radiation ulcers by Rudolph et al. showed that the ulcers fully fluoresce, which would not be the case if the microcirculation to the region was not functional (186). Rudolph et al. also measured transcutaneous oxygen pressure in human skin and found no evidence of skin hypoxia, even many years after irradiation (187). Aitasalo et al. measured subcutaneous oxygen tension in the hind limbs of x-ray irradiated (5-30 Gy) rabbits. He found a transient

decrease subcutaneous oxygen tension measured 1 day and 5 weeks after irradiation, but no significant decrease 11 weeks after irradiation (2).

Baker et al. reported that early after irradiation, the skin microvasculature may become more permeable, likely as a result of histamine and serotonin activity (13). Wang et al., however, found diminished inflammatory fluid in rat incisional wounds after a single radiation dose of 10 Gy, and concluded that capillary permeability was inhibited (233). A recent study using human cervical arteries from head and neck cancer patients subjected to pre-operative fractionated irradiation demonstrated a diminished ability of arteries to relax. The impairment was attributed to impaired endothelial cell reaction to nitric oxide, and it was suggested that this impaired relaxation response may lead to decreased arterial blood flow and arterial thrombosis (211). Other work, using single radiation doses of 15-30 Gy, demonstrated increased leukocyte-vessel wall interactions to explain reduced or lost microvascular flow (1). Overall, any pathophysiologic role of altered skin blood flow causing impaired wound healing following ionizing radiation remains unclear.

Wound Capillary Density: Dimitrievich et al. suggested that capillary density in non-wounded rabbit ears dropped several days after single-dose irradiation, ranging from 2-20 Gy, with inter-capillary distances increasing from 150 μm to 300 μm (68). Roth, using a hamster model, showed decreased red blood cell velocity, and decreased capillary surface area in muscle blood vessels treated with a single 10 Gy dose of radiation 3, 7 and 30 days prior to evaluation (185).

The above studies demonstrate that exposure to ionizing radiation may adversely affect blood vessels, however the exact effect of radiation damage to the skin

microvasculature, the timing of these effects and the role these effects play in the wound healing process is currently unclear.

Wound Collagen Production: As the following studies demonstrate, proposed mechanisms for the observed decrease in WBS include diminished collagen production or the deposition of poor quality collagen (25). Bernstein et al. showed a decrease in the expression of alpha-1(I) chain of type I collagen in guinea pig incisional wounds made 2 days after exposure to a single dose of 18 Gy of radiation, and biopsied 7 days post-operatively (25). Another study using a rat dorsal skin incision model demonstrated a decrease in type III collagen after a single 15 Gy radiation dose (97). In a study of ^{3}H-hydroxyproline content to examine new collagen production in incisional wounds, rats irradiated with a single 20 Gy dose of x-rays showed lower ^{3}H-hydroxyproline content measured 21 days after wounding, than did the control rats, (65). In experiments using rat skin grafts exposed to a single 10 Gy dose of radiation and followed over a 3 week period, wound collagen bundles themselves were shown to be thinner, which may detract from their inherent strength and stability (234).

Fibroblast specific changes due to radiation may be responsible for the alterations in collagen content and quality. After radiation, dermal fibroblasts displayed phenotypic changes including increased size, basophilia, elongated cytoplasm, and nuclear atypia (78). The ability of cultured human fibroblasts irradiated 2-18 years prior to investigation to proliferate was also hindered by radiation (188). It was suggested that the human irradiated fibroblasts were arrested in the G1 phase of the cell cycle, and were thus incapable of proliferating in response to signals produced by surgical wounding (183).

1.7 Potential Treatment for Radiation-Induced Impaired Surgical Wound Healing

The pathophysiologic role of inadequate growth factor protein expression in radiation-induced impaired surgical wound healing has been indirectly investigated. The studies done to date assessed the healing of irradiated wounds following the application of exogenous growth factors. The growth factors investigated in this way included TGF-β_1 (24,56,155) and PDGF-BB (153). No studies have actually examined endogenous growth factor expression in an in-vivo irradiated wound model, despite ongoing speculation that one mechanism by which radiation impairs wound healing is through alterations in wound growth factor levels (233). There are also no studies examining growth factor receptor population and function in inflammatory cells, endothelial cells or fibroblasts in irradiated skin wounds. It may, therefore, be important to study the wound tissue content and function of growth factors as well as the population and function of their receptors.

It should be noted that TGF-β_1 levels in mouse skin were examined following local irradiation, however, the study was not done in a wound healing model. In this study, biopsies of intact skin taken after radiation exposure revealed that radiation up-regulated TGF-β_1 expression (24,56,155,176,177).

Despite the lack of a documented down-regulation of growth factors in irradiated wounds, the use of recombinant growth factors to reverse the effects of radiation damage on wound healing has received much attention (182). The following section will briefly outline the important experimental information relating to growth factor treatment in

irradiated wounds.

PDGF-BB: In a 25 Gy, single dose, surface irradiated rat dorsal linear incision model, a single application of PDGF-BB (10μg/wound) increased WBS by 50% (153). In the case of total body radiation, PDGF-BB had no significant effect on wound healing. The authors suggested a mechanism that requires functioning bone marrow components, and they speculated that these important marrow components were macrophages (153). Unfortunately, they did not directly demonstrate the role of macrophages in PDGF-BB induced improvement of wound healing. The rats in this study were irradiated 2 days prior to wounding.

TGF-β: Guinea pigs wounded with a linear incision 2 days after a single radiation dose of 15 Gy showed improved WBS 7 days after wounding, using topically applied exogenous TGF-β_1 at doses of 1 or 5μg per wound, compared to irradiated control wounds. The TGF-β_1 treated group also showed increased α(I) collagen mRNA expression 7 days after wounding (24). In a different study, a single topical application of TGF-β_1 (2μg/wound in a methylcellulose vehicle), applied at the time of wounding partially reversed the effects of total body radiation on wound healing in a rat linear incision wound model (56). A third study, also in an irradiated rat model (15 Gy single dose), used a topical TGF-β_1 dose of 4 μg/wound with a gelatin sponge pledget as a vehicle at the time of wounding to significantly improve flap viability by 10% and wound tensile strength by 25% (155).

The use of recombinant growth factor therapy to treat impaired wound healing has several disadvantages, including the large amounts of purified protein needed, the short half-life of the proteins, potential toxicity of the growth factors when given repeatedly,

and the method of protein delivery (59). Many questions remain unresolved with regard to the application of growth factors to healing wounds in irradiated skin. The effects of multiple doses at different stages of wound healing are not known. The optimal doses of different factors are also unclear. The utility of combinations of growth factors has not been investigated. The timing of administration, and the exact physiological characteristics of the factors themselves all still need to be clarified. Finally, in no experiment was the use of exogenously applied growth factors able to completely reverse the effects of radiation on wound healing, suggesting that other factors besides a simple single growth factor deficit are involved in the pathogenesis of impaired wound healing.

Implanted Fibroblasts: Krueger et al. showed that injecting non-irradiated embryonic fibroblasts grown in cell culture into an irradiated wound in a guinea pig could significantly improve wound breaking strength (119). Similar work by Gorodetsky et al. using a mouse model (91) and Ferguson using a rat model (79) showed improved WBS using non-irradiated intradermal fibroblast injection. In the study by Gorodetsky et al, cultured fibroblast implants were compared to fresh ones, and the fresh ones showed a more enhanced improvement in WBS. The authors conceded that this may have been because the fresh implants were mixed with macrophages, which could have enhanced healing through added inflammatory signals (91). There is the possibility of inadvertent transferring of growth factors along with the fibroblast implants in these studies. Like the growth factor application studies, the fibroblast injection studies only partially restored WBS to normal levels, suggesting that other factors besides decreased population and function of fibroblasts are also responsible for the impaired healing of irradiated wounds.

Vitamins: Taren et al. demonstrated an increased incisional WBS in rats

supplemented with intraperitoneal vitamin E (30-90 IU) prior to exposure to localized radiation. The suggested mechanism of action is through the anti-oxidant effects of vitamin E (219). Vetrugno reported using vitamin E to speed corneal re-epithelialization after cataract surgery in humans (230)

Hyperbaric Oxygen: Hyperbaric Oxygen (HBO) has been reported to increase bactericidal efficiency, collagen synthesis, angiogenesis in guinea pigs (133), the take of skin grafts, and flap survival (156) in irradiated skin (114). According to one published report, the use of hyperbaric oxygen has not gained widespread popularity as a wound healing therapy due to a lack of prospective, randomized studies clearly quantifying the benefits of hyperbaric oxygen therapy (152). The availability of hyperbaric oxygen is another problem limiting its use, especially in smaller medical centers, since very specialized and expensive apparatus is needed to deliver this therapy.

Electric charge: Galiano et al. used a 25 Gy dose of surface radiation to study impaired wound healing in a rat model, and showed a 47% increase in WBS over control wounds after applying 0.1 ml of vehicle with charged beads immediately after wounding. The suggested mechanism is via recruitment and activation of wound macrophages (84). More recently, Connors et al. showed that the beads work by recruiting macrophages to the wound site, and by stimulating increased expression of TGF-β1 and its receptor (48). This technique, while interesting, is not currently used clinically, and the long-term effects of the injected foreign body beads were not addressed by the published studies. It was acknowledged, however, that some long-term foreign body effect could be a possibility with this form of treatment (77). More recent work used a bio-absorbable version of the charged bead, with similar albeit somewhat diminished effects on WBS

(46).

Thrombin: Thrombin is known to possess growth factor characteristics (57). A thrombin-derived oligopeptide, p^{517-30}, was shown to increase WBS in both surface and total body irradiated models of impaired wound healing in a rat model. It was thought that this oligopeptide may interact directly with fibroblasts, independent of macrophages (57). More recent work using the synthetic thrombin peptide TP508 showed similar results in a non-irradiated rat excisional wound model (210).

1.8 Potential Strategies for Prevention of Radiation-Induced Impaired Surgical Wound Healing

1.8.1 Fractionation of Radiation Dose: Actively dividing cells such as tumor cells, are most sensitive to ionizing radiation (27, 238). Skin cells generally divide slowly and as such are relatively resistant to radiation damage. Dividing the total radiation dose into fractions is done for two reasons. In theory, fractionation increases the likelihood of successfully catching rapidly cycling tumor cells in the radiosensitive stage of mitosis. The major reason, however, is that it allows the non-tumor cells that are sub-lethally injured to repair themselves (27,238). The result is increased tumor cell killing and less marked adverse skin effects. This principle was demonstrated in mice with WBS increasing as the number of fractions was increased from 4 to 24 (93). Dose fractionation is currently the clinical standard used in modern radiotherapy for cancer treatment (7). Engel et al. used pre-operative fractionated irradiation in a canine esophageal anastamosis model and showed no significant impairment of esophageal wound healing compared to non-irradiated controls (76). Goredetsky demonstrated a decreased effect of irradiation on rat skin WBS when the dose of radiation was fractionated (94).

1.8.2 High Energy Radiation Beam and Skin Sparing: Megavoltage sources of radiation generate beams capable of deeper penetration than previously employed orthovoltage sources (28). With higher beam energy the maximum dose of energy can technically be deposited deeper in the tissue, thus sparing the skin from the maximal dose of energy (28). Unfortunately, when dealing with more superficial tumors, such as those in the head and neck region, the maximal dose of radiation must be delivered to a relatively superficial area in order to adequately irradiate the tumor, and the benefits of megavoltage radiation with respect to skin sparing are diminished.

1.8.3 Radioprotectors: In an effort to limit the injury to normal tissues caused by radiation, and to increase the total radiation dose deliverable to tumor tissue, radioprotective substances have been investigated, (235). Of the various groups of substances tested, the aminothiol group, to which the drug Amifostine (Ethyol™, WR-2721, S-2[3-aminopropylamino-ethylphosphorothionate]) belongs, has been the most successful. In fact, Amifostine is the first broad-spectrum cytoprotective drug approved by international regulatory bodies for clinical use as a radioprotective agent (37).

Mechanism of Amifostine Action: As shown in Figure 2, Amifostine seems to work by scavenging free-radicals that would otherwise cross-link and damage DNA (37,70,235). Amifostine itself is a pro-drug, which requires alkaline phosphatase mediated dephosphorylation to be become the active metabolite WR-1065, a free sulphydryl compound (37). WR-1065 is further metabolized to WR-33278, which has the ability to bind to DNA to stabilize it and enhance normal cellular antimutagenic biochemical pathways (71). Tumor cells are relatively deficient in membrane bound alkaline phosphatase, which is a requirement for cellular uptake of Amifostine. Therefore, cellular uptake and protection of tumor cells by Amifostine is minimal (37).

Amifostine (WR-2721) NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-S-PO_3H_2

PH 6.6-8.2
Membrane Bound
Alkaline phosphatase

WR-1065 NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-SH

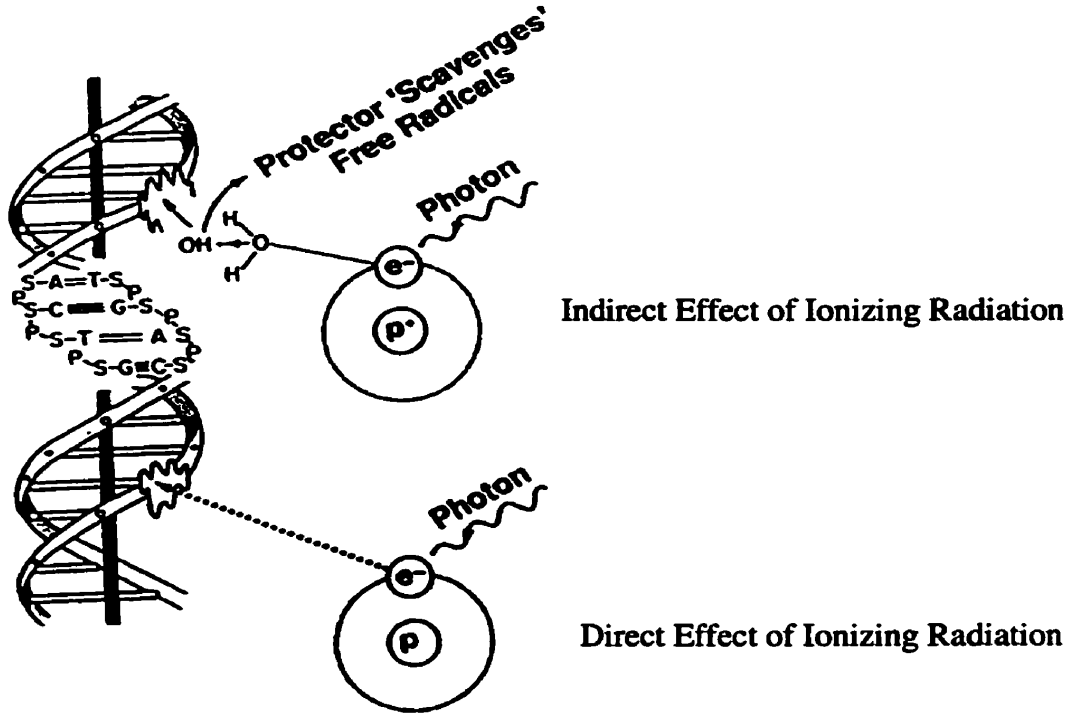

Figure 2. Mechanism of Amifostine Action. Amifostine is taken up into cells and converted to its active form only in the presence of cell membrane bound alkaline phosphatase, and a pH between 6.6 and 8.2. Cancer cells are relatively deficient in alkaline phosphatase activity, and the pH of tumor tissues is relatively acidic and does not favour Amifostine uptake and conversion to the active metabolite. Once taken up and converted to the active metabolite of Amifostine (WR 1065) acts to scavenge free radicals generated by ionizing radiation, thus protecting DNA from radiation damage. (100)

The low extracellular pH of tumors (pH <6.6) compared to normal tissues also diminishes the uptake of Amifostine into tumor cells. Amifostine requires a pH between 6.6 and 8.2 for cellular uptake (199).

Despite the consensus in the literature that Amifostine seems not to provide any clinically significant tumor protection to a broad range of human carcinomas, sarcomas, and leukemias (37, 38), there are several authors who maintain a more cautious position. These authors suggested that Amifostine may provide a small degree of tumor protection in certain cases (129). For example, in a single dose and fractionated irradiation study of mouse tumors, Stewart et al. showed that Amifostine had a protection factor of 1.2-2.5, with greater protection at lower radiation doses (Protection factor = radiation dose with protector/radiation dose without protector for same effect) (209). In another study, Milas et al. showed that Amifostine protected metastatic fibrosarcoma cells in mouse lung tissue by a factor of 1.28. In a follow up study, Milas suggested that larger tumors derive less protection from Amifostine, due to decreased blood flow and subsequent decreased drug delivery (148).

The pharmakokinetics of Amifostine make its use somewhat complicated. It has a short circulating half-life in plasma, with 90% of the drug being cleared within 6 min (37). Because of this short half-life, the optimal timing between drug administration and radiation remains unclear, with reports in the literature ranging from 15 to 60 min. The optimal one-time or repeated drug dose is also unclear, with reports in the literature ranging from 200-500 mg/kg, in mouse, rat and human experiments (37,50,70,208).

It has been demonstrated that there is some accumulated Amifostine toxicity with repeated doses, and therefore when using a fractionated dosing regimen, the dose must be

reduced (184). The principal side effects of Amifostine include nausea and vomiting, and hypotension, which seem to be dose dependant (37).

Protection of Tissues by Amifostine: In human studies, Amifostine has been reported to protect many tissue types from the effects of radiation. Bone marrow toxicity measured in terms of decreased WBC count was diminished in humans receiving palliative hemi-body radiation when Amifostine was given 15-30 min prior to radiation exposure (51). In a phase II trial involving head and neck cancer patients, salivary glands and oral mucosa were protected by Amifostine pre-treatment, as seen by lower incidences of xerostomia and mucositis (36). In a phase II trial, daily doses of Amifostine ($200mg/m^2$) were able to protect lung cancer patients from radiation-induced esophagitis (146).

In animal studies, protection of the gut and testis from gamma radiation has been shown in a mouse model (149). Intraluminal topical Amifostine at a concentration of 150 mg/ml protected the small intestine of rats from a single 11 Gy dose of radiation (61). Protection of the ciliated cells of the eustachian tube from a single radiation dose of 30 Gy has also been shown using a single intra-peritoneal Amifostine dose of 400 mg/kg in a chinchilla model (163). Immune system depression in mice following radiation of 5-9 Gy was alleviated by a 200 mg/kg intraperitoneal dose of Amifostine given 30 min prior to radiation exposure (200).

Skin Protection by Amifostine: There have been several studies examining Amifostine's ability to protect skin from single and fractionated doses of radiation. These studies, described below, have demonstrated that Amifostine can prevent some of the adverse skin reactions that typically follow irradiation (50,208,226).

Stewart and Rojas used single dose and fractionated radiation doses between 25 and 45 Gy to show that single Amifostine doses between 200 and 500 mg/kg injected 35-45 min prior to radiation exposure provided skin protection(208). Their findings were based on an arbitrary scale of observed skin reaction only (63), including erythema, desquamation, and ulceration.

In a rat non-wounded skin model, Constine et al. used an intraperitoneal Amifostine dose of 310 mg/kg given 15 min before radiation to demonstrate skin protection from single radiation doses up to 24 Gy (50). Timing the Amifostine dose 15 minutes prior to radiation is supported by Dorr (70). Again, this study examined grossly observable skin changes ranging from redness to skin breakdown, based on a scale by Moulder et al. (151) but did not examine WBS, histology or blood flow. Geng et al. showed that topical application of 0.3 mg of the active metabolite of Amifostine, WR-1065, could prevent alopecia in irradiated mice (87). A wide range of protection factor values have been reported in the literature (50).

It is important to note that these studies looked primarily at external skin reactions, and based their results on subjective rating scales. These studies did not examine the protective effect of Amifostine on wound breaking strength, skin histology, blood flow, growth factor profiles or the population and function of inflammatory cells, fibroblasts and capillaries in irradiated wound healing.

1.9 Rationale of this Research Project

The pathogenic mechanism of preoperative radiotherapy-induced impaired surgical wound healing is unclear and is the focus of this project. The literature reviewed thus far indicates that infiltration of functional inflammatory cells, collagen synthesis by fibroblasts and neovascularization play important roles in normal wound healing. Obviously, it is important to investigate the effect of preoperative radiotherapy on the wound densities of inflammatory cells, fibroblasts and capillaries, on wound collagen synthesis and on wound tissue content of the growth factors (TGF-β_1 and VEGF) that regulate the recruitment and function of these cells. To date, this area of research has not been undertaken. In addition, the specific use of a fractionated irradiation protocol use din this type of study has not been previously done.

Levels of other growth factors such as bFGF, TNF-α and EGF may also be affected by radiation, but, at this time, their roles in wound healing are less known than those of TGF-β_1 and VEGF and therefore they were not included in this study (54). In addition, research into radiation-induced impaired surgical wound healing thus far has focused on treating the problem after the damage has already been done. It is important to pioneer theinvestigation of the efficacy of prophylactic treatment with a radioprotective agent such as Amifostine for protecting normal skin tissue from radiation injury, and ultimately preventing post-operative wound breakdown. This approach will aim to reduce the length and cost of post-operative care, and reduce the need for secondary surgical revisions, which will mean less pain and suffering for patients.

A skin flap wound model was used in this project because skin flaps are routinely used for wound coverage after tumor excision. In addition, fractionated irradiation was used in this project because it is the clinical standard for radiotherapy.

1.10 Objectives

The objectives of this project were to investigate the pathophysiology and pharmacologic intervention for radiation-induced impaired surgical wound healing using a clinically relevant skin flap wound model in the rat. Specific emphasis was placed on the study of the deleterious effects of preoperative fractionated radiation on wound healing at the molecular, cellular and tissue levels. The efficacy of the radioprotector Amifostine in the mitigation of these deleterious effects of radiotherapy was also investigated.

1.11 Hypothesis

The hypotheses of this project were threefold:

(i) Preoperative fractionated irradiation causes impaired surgical skin wound healing in a manner similar to single dose irradiation;

(ii) Preoperative fractionated irradiation down-regulates the population and/or function of inflammatory cells, fibroblasts and capillaries, and the tissue content of growth factors essential to the wound healing process in surgical skin wounds;

(iii) Prophylactic treatment with the radioprotector Amifostine attenuates the deleterious effect of pre-operative irradiation on surgical skin wound healing.

1.12 Specific Aims

Aim 1: To investigate the deleterious effect of preoperative single dose and fractionated irradiation on wound breaking strength and wound tissue content of hydroxyproline (a marker of collagen content) and the efficacy of prophylactic treatment with the radioprotector Amifostine in restoration of wound breaking strength and wound tissue content of hydroxyproline.

Aim 2: To investigate the deleterious effect of preoperative single dose and fractionated irradiation on wound tissue content of macrophages, fibroblasts, capillaries, collagen and growth factors in surgical skin wounds at 3, 8 and 21 days post-operatively and the efficacy of the radioprotector Amifostine in alleviation of the deleterious effects induced by preoperative irradiation.

2.0 Materials and Methods

2.1 Animal Care

Adult male Sprague-Dawley rats (545 ± 10) grams were used for this project. All rats were housed in a room in the Animal Research Facility of the Hospital for Sick Children, under conditions of controlled light (12-h light/dark cycle) and temperature (22°C). All of the animals were individually caged and provided with food and water ad libitum through all phases of the experimental protocol. Animal care in this study was in compliance with "The Care and Use of Laboratory Animals" (Canada Council on Animal Care, 1980), and all experimental protocols were approved by the Animal Care Committee of the Hospital for Sick Children.

2.2 Irradiation Procedure

Rats were lightly anesthetized with 40-50 mg/kg of intramuscular Ketamine. The dorsal skin was shaved ~ 60 minutes prior to irradiation. The rats were placed in a specially designed plexiglass apparatus to allow for the exposure of only the desired segment of dorsal skin to be irradiated, thus sparing the remainder of the body from irradiation. The rat irradiation apparatus is shown in Figure 3. The size of the irradiated area was 4 x 10 cm. The area to be irradiated was marked out with an indelible marker using the following anatomic landmarks: The caudal boundary of the irradiated region was at the level of the pelvis. The lateral margins of the area included 2 cm on either side of the midline, as defined by palpation of the spinal column. The cephalad limit was the inferior angle of the scapula.

The animals received a radiation dose of either 20 Gy in one dose, or six fractions of 6.2 Gy delivered on a Monday-Wednesday-Friday schedule for two weeks. The

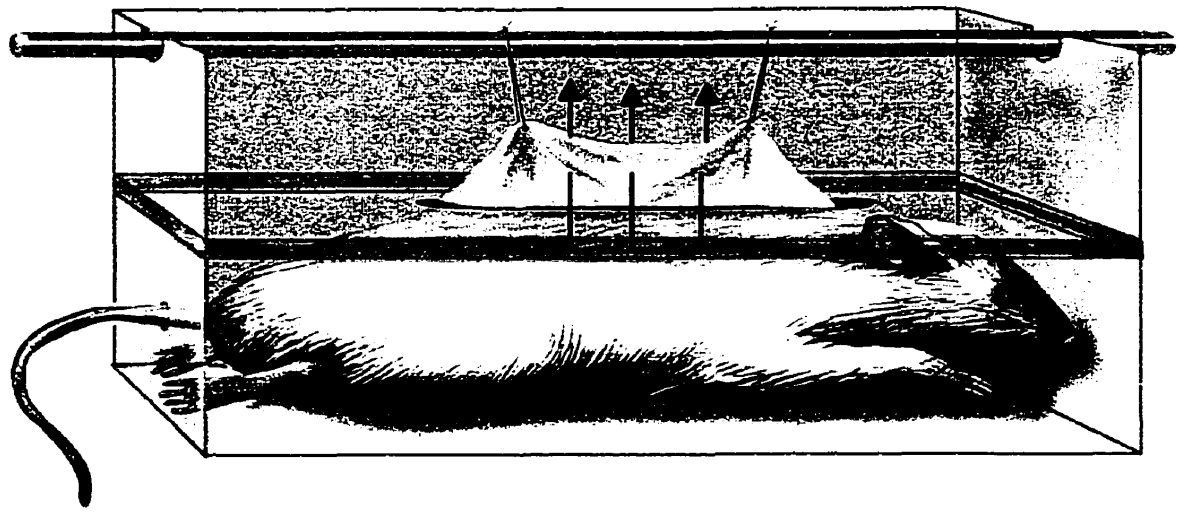

Figure 3. Rat Irradiation Procedure. Rats were anesthetized and placed in a specially designed plexiglass box. A 4 x 10 cm segment of dorsal skin was pulled through a slit in the box and held in place with sutures placed immediately prior to starting the irradiation procedure. This segment of skin was then irradiated using a 4 megavolt photon beam, directed perpendicular to the skin segment. The sutures were cut immediately after irradiation. The total time needed to deliver the radiation was 8.5 min for the single 20 Gy dose, and 2.8 min for each 6.2 Gy fractionated dose.

radiation source was a 4 megavolt linear accelerator (Varian, model 2100C 4/10, Palo Alto, California). All radiation was administered by registered radiation therapists at thePrincess Margaret Hospital, under the supervision of Dr Ivan Yeung, a radiation physicist.

A radiation dose of 20 Gy was chosen because it has been shown to produce significantly impaired wound healing (89), and it is within the 15-20 Gy range commonly used in other in vivo wound healing experiments using rats and mice, allowing our results to be compared with those done previously (23,24,28,66,69,92,123,155). The fractionated dose of 6.2 Gy was derived by calculating the iso-effective dose to 20 Gy, using the linear quadratic formula as suggested by Dr. Ivan Yeung (100). The α/β ratio used for the calculation was 10, which assumes an early radiation effect in normal skin (100). This protocol was meant to approach the clinical fractionated irradiation scenario, not to replicate it. In the clinical setting, fractionated irradiation involved daily doses of radiation for several weeks duration. Such a protocol was impossible to do with experimental animals because the Animal Care Committee did not allow the animals to be anesthetized so frequently, citing the potential ill-effects to the animals.

2.3 Administration of Amifostine

Amifostine was obtained from the Walter Reed Army Institute of Research (Rockville, Maryland, USA). Approximately 1 h prior to injection, it was dissolved in sterile isotonic saline to a concentration of 150 mg/ml. It was injected intraperitoneally using a 30 gauge needle 30 min prior to the delivery of radiation. The prophylactic doses of Amifostine given intraperitoneally prior to single and fractionated irradiation were 300 mg/kg and 150 mg/kg, respectively. The 300 mg/kg dose was estimated based on

effective doses used in rats in previous studies using Amifostine (50,216). The 150 mg/kg dose was based on our preliminary studies of the maximum dose of Amifostine tolerated by rats when given 6 doses of Amifostine over a 2-week period.

2.4 Operative Procedure

Justification for the use of the rat dorsal skin flap: Rats have been commonly used as models of impaired wound healing, with both the excisional and the linear incision wound models being used frequently. A skin flap is commonly used for wound coverage after soft tissue tumor resection, and thus the use of a random pattern skin flap was clinically relevant for the study of wound healing in irradiated skin. The skin flap model is substantially different from a simple incisional model, due to the nature of the blood supply to the wounded tissue. In the incisional model, normal blood supply exists on both sides of the incision. In the skin flap model, the blood flow is dependant on the pedicle only, since the flap is completely undermined and elevated off of the wound bed.

The dorsal skin was chosen because it is sufficiently loose to allow it to be pulled up so that the radiation dose could be delivered to the dorsal skin only, while the remainder of the rat was spared from irradiation (Figure 3). The blood flow in this skin segment during the irradiation procedure was assessed using a doppler flow meter, and found not to be compromised.

Skin Flap Surgery: Rats underwent surgery 5 to 6 weeks after irradiation. Anesthesia was achieved using Ketamine (40-50 mg/kg, i.m.) and sodium pentobarbitone (30-40 mg/Kg, i.p.). No intravenous access or airway instrumentation was required as the operative time was only 20-30 min. At surgery, a dorsally based, 3 x 8 cm random pattern

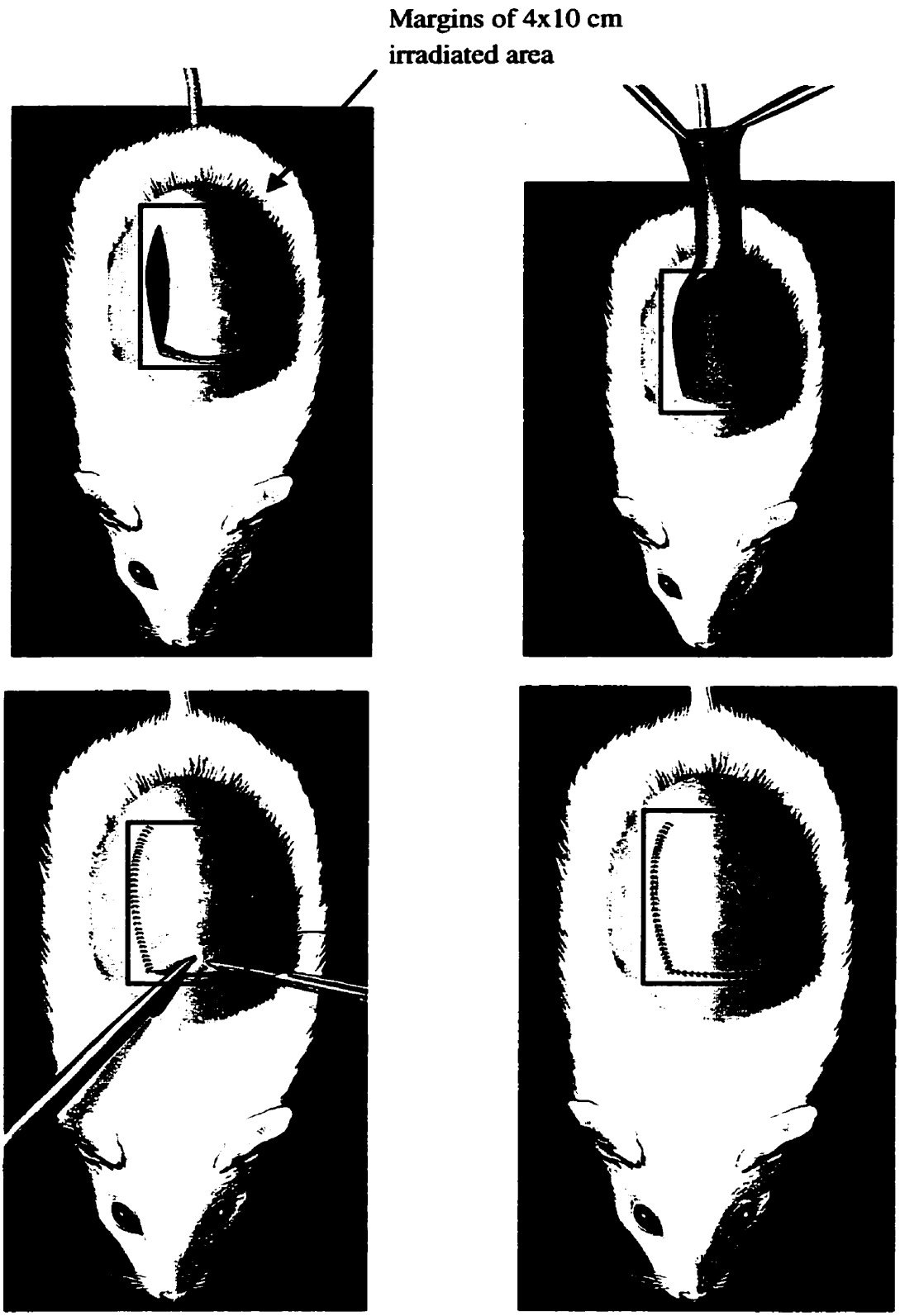

Figure 4. Diagram of Skin Flap Surgery. A 3x 8 cm random pattern, caudaly based skin flap was elevated within the region of skin previously irradiated. The only blood supply was through the pedicle of the skin flap. The flap was returned to the wound bed and sutured in place with 3-0 proline immediately after being raised.

skin flap (McFarlane Flap) (145) was raised on the dorsum of the rat, within the region of previously irradiated skin (Figure 4). The skin flap was carefully returned to the wound bed and the wound was closed immediately with interrupted 3-0 prolene sutures. Sterile technique was maintained during the surgical procedure. Post-operatively, the rats were kept in individual cages.

2.5 Tissue sampling

Biopsy Sites: All biopsies for the WBS test, and for histological and biochemical assessment were taken under pentobarbital anesthesia. All the sutures were removed from the incision prior to resecting the biopsy specimens. For the rats undergoing WBS testing, bilateral strips of skin centered over the suture line and 3 centimeters cephalad from the base of the flap were resected and used for the WBS test. These biopsies measured 3 x 1 cm (Figure 5). For the histological and immunohistochemical analyses, a 1 x 1 cm skin biopsy centered over the incision line taken from the same region as the WBS samples was used (Figure 5). The remainder of the suture line (approximately 100 mm long) in the viable portion of the flap was excised with margins less than 2 mm on either side of the incision wound, yielding approximately 400 mm^2 of tissue, weighing $\geq$ 2 grams. The wound tissue from both sides of the incision was cut into small pieces, snap-frozen in liquid nitrogen and pooled. A small portion (approximately 100 mg) of the pooled pieces from each rat was taken, weighed, and used for assay of hydroxyproline, a marker of wound tissue collagen content. The remainder of the pooled tissue from each rat was used for protein extraction for subsequent western blot analysis for TGF-β1 and VEGF.

The amount of skin needed to extract sufficient protein for a western blot analysis

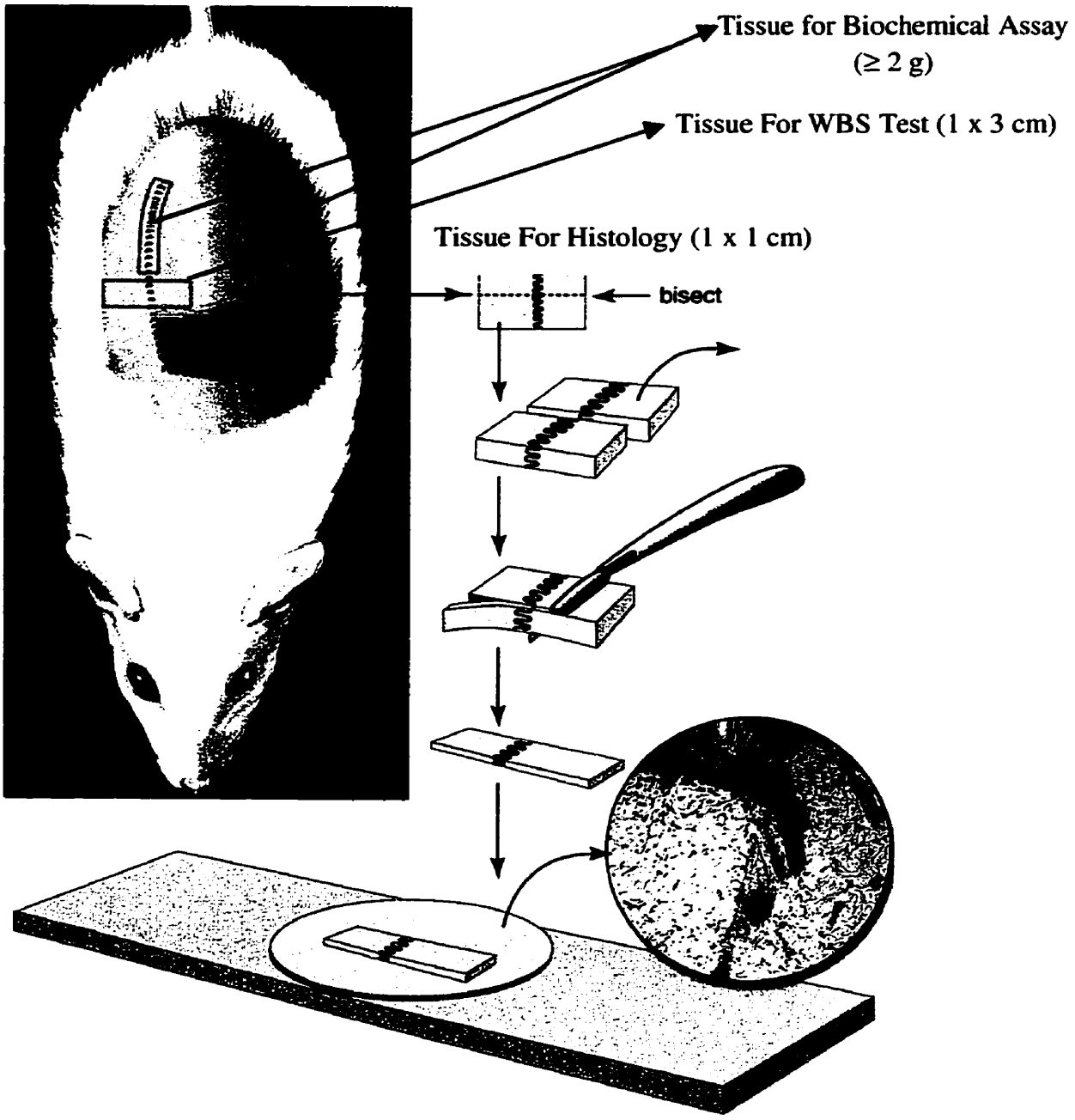

Figure 5. Diagram of Skin Flap Biopsies Locations. Locations for wound tissue samples taken for WBS, histology and for biochemical analysis are shown. For simplicity, the diagram shows only one biopsy site for WBS analysis, but in fact bilateral biopsies were taken. The orientation of the sample taken for histology is also demonstrated.

is about 1 gram (108), and was easily obtained by our biopsy technique. Immediately after the biopsies had been harvested, the rat was euthanized with an overdose of intracardiac pentobarbitone (100 mg/kg).

Justification of Wound Biopsy Technique for Biochemical Analysis: TGF-β_1 is known to be present in rat skin incisional wound tissues (58). In addition, it has been documented using pig excisional wound studies that TGF-β_1 is up-regulated in wound tissue compared to intact skin (128). In preliminary studies, using non-irradiated rats, we carried out western blot analysis for TGF-β_1 protein expression on biopsies taken from the wound sites, as described in the above section, and compared them to skin biopsies taken from non-wounded regions of dorsal skin from the same rat. The results showed a 43 $\pm$ 5% (n=3) increase in TGF-β_1 expression in the wound site tissue compared to the non-wounded skin tissue. These preliminary studies indicated that the difference in growth factor content between wounded and non-wounded tissue could be detected using western blot analysis despite the fact that the wound tissue biopsies contained a narrow margin of non-wounded tissue. In other words, the wound biopsies contained primarily wound tissue.

In the present studies, we chose to compare the pathophysiology of irradiated and non-irradiated wounds, and not intact irradiated skin. We did not examine intact irradiated skin because this has been done by other investigators (25,66,97). In addition, we were specifically interested in the clinical scenario of irradiated and wounded skin, not the simply the irradiated non-wounded situation.

Instron Tensionometer for WBS Test

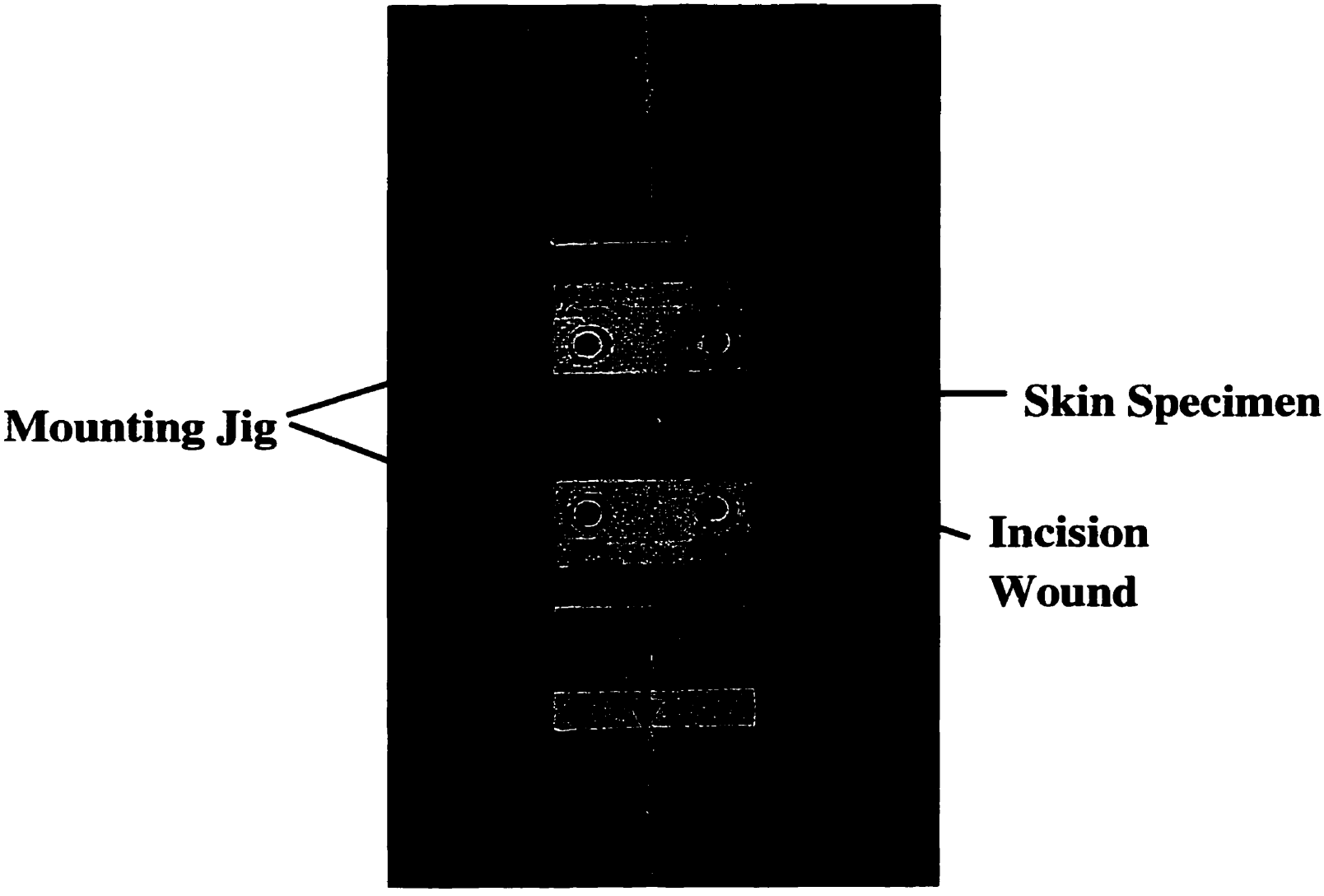

Figure 6. Wound Breaking Strength Procedure. An Instron Tensionometer apparatus was used for tensile strength assessment. Skin samples (1 x 3 cm) were carefully and securely mounted in a specially designed jig. They were then stretched uniaxially at 5 mm/min to the point of failure. The tensile load was sampled 10 times per second, and the data stored on a computer.

2.6 Skin Wound Tissue Studies

2.6.1 Wound Breaking Strength (WBS) Test

The Instron Tensionometer was used for the WBS test (Figure 6). All excised skin samples were kept moist in saline-soaked gauze in a closed plastic container until the time of testing, and all tests were completed within 4 h of biopsy. The test location was at the University of Toronto Department of Biomedical Engineering. Skin specimens were placed in an Instron tensionometer (Instron 8501 uniaxial testing machine, Instron Corporation, Canton, Massachusetts), using an MLP-25-CO load cell, and stretched uniaxially at a constant rate of 5 mm/min to the point of breaking to determine wound breaking strength. The peak load was taken as the maximum tensile load achieved during the course of the procedure, up to the point of wound failure. Two biopsies from each rat were used, and the results averaged. Results were expressed as peak tensile load in g/mm (width of skin wound).

2.6.2 Skin Flap Viability Assessment

Maximal surviving skin flap area was determined by first tracing the total and viable area of the skin flaps at the time of biopsy. The demarcation between viable and necrotic skin was clear to gross observation, when observed at least 7 days post-operatively. Areas of flap necrosis were identified by their black colour, rigid feel, and their lack of bleeding when cut. This technique of identifying viable and necrotic flap regions has been previously described in the same rat skin flap model (121). The surface areas of the surviving flaps were determined by taking a digital picture of the flap tracings, and analyzing them on a computer using the area measurement function of Fluorchem software, version 1.0 (Alpha Innotech Corporation, San Leandro, California).

2.6.3 Wound Histology and Immunohistochemistry

Protocol for Preparation of Histology Slides: The preparation of the slides was done at the pathology laboratories of the Princess Margaret Hospital, and of Women's College Hospital. Skin biopsies taken as described above (section 2.4) were fixed in 10% formalin for approximately 24 h, embedded in paraffin, and cut to a thickness of 3μm prior to undergoing specific staining for histologic evaluation and immunohistochemistry as described below.

Paraffin sections were de-waxed in toluene and hydrated through graded alcohol to distilled water. Prior to incubation with the primary antibodies, the sections were treated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and digested with 0.4% pepsin at 42°C for 5 min. The following procedures were performed at room temperature, inside a moist chamber, and with a phosphate buffered saline (PBS) wash after each step. Immunoreactivity was revealed using the Streptavidin-biotin system. After primary antibody incubation, the sections were incubated for 30 min with the linking antibody (Biotin-Goat anti-Rabbit IgG, 1:200 dilution, Vector Lab Inc. Burlingame, California), followed by incubation in Streptavidin-horseradish peroxidase (HRP), for 20 minutes (Streptavidin Kit, Signet Labs Inc, Dedham, Massachusetts). Slides were developed in AEC (0.0125% 3-amino-9-ethyl-carbazole, w/v, in 0.2M acetate buffer, pH 5.2) for 10 min. Slides were then counterstained in Mayer's Haematoxylin, and mounted with Crystal Mount (Biomeda Corporation, Foster City, California) (40).

Specific staining protocols for different cells and growth factors are briefly described here. For VEGF, the primary antibody was from Santa Cruz Biotechnology,

(Catalog # sc-152, Santa Cruz Biotechnology, Santa Cruz, California), used at a dilution of 1:800 and incubated overnight.

The protocol for the staining of macrophages and blood vessels was as above, with the following changes: Pepsin digestion was used for antigen retrieval, to enhance the antibody binding. The anti-macrophage antibody was a mouse anti-rat macrophage monoclonal antibody, (Catalog # MAB1435 Chemicon International Inc., Temecula, California), used at a dilution of 1:1000 for 1 h at room temperature.

The antibody used for blood vessels staining was a polyclonal rabbit anti-human factor VIII antibody, (Catalog # A 0082, Dako Diagnostics Canada Inc., Missisauga, Ontario) used at a dilution of 1:600 for 1 h. The secondary antibody was a biotin-conjugated anti mouse IgG, rat adsorbed, used at a dilution of 1:2000 (Vector Labs Inc., Burlingame, California). Similar protocols for macrophage and capillary staining have been published elsewhere (113). Hematoxylin and eosin slides were also made, and were used to assess wound fibroblast density, since no specific stains are currently available to specifically stain fibroblasts. Cells considered fibroblasts were those that were spindle shaped, with an elongated nucleus, and associated with adjacent collagen bundles. Any cell without these characteristics was not considered a fibroblast.

Cell and Vessel Counting: All of the cell counting was done in a blinded fashion. Wound densities of macrophages, capillaries and fibroblasts were assessed manually using eight high-power (250X) brightfield microscopic fields per slide, and using a Leitz Orthoplan microscope (Leitz Microscopes, Wetzlar, Germay) encompassing the areas immediately adjacent to the incision wound. The results are expressed as total number of positive cells summed over all eight fields examined.

2.6.4 Quantitation of Immunohistochemistry slides

After staining, slides were scanned in their entirety using a Polaroid Sprintscan 35 Plus Scanner (Polaroid Corporation, Cambridge, Massachusetts.), with the Pathscan Enabler (Meyer Instruments Inc., Houston, Texas.). The scanned slides were viewed using The MicroComputer Imaging Device (MCID) M2 (Imaging Research Inc., St. Catharines, Ontario). From the entire scanned slide, only a specified region of interest at the wound margin was analyzed for immunostaining. The dimensions of the region of interest assessed for positive staining were standardized. The region extended vertically from the dermal-epidermal junction, to the panniculus carnosis muscle, and laterally 500μm from the incision site. The total area assessed was approximately 2 mm^2. For each section, colour thresholding was inspected visually, and the software settings were adjusted if necessary. Results were obtained as the percentage of the total number of pixels in the field staining positive for VEGF. Similar protocols have been published (190).

2.6.5 Wound Tissue VEGF and TGF -β_1 Protein Expression

Protein Extraction from Biopsy Tissues: Total protein was extracted from frozen skin biopsies. Frozen samples were homogenized in a lysis buffer containing 1% NP40 detergent (non-idet p-40), 20 Mm Tris pH of 7.4, 150 nM NaCl, 5 mM EDTA, and 1 mM sodium orthovanadate, and one protease inhibitor tablet (cat # 1836153 Roche Biochemicals, Laval, Quebec) to protect the extracted proteins from serine, cysteine and metalloproteases. Homogenization was done on ice using a Polytron PT 10/35 homogenizer with a PTA 10 EC generator at a speed setting of 7 on the dial (18 900

rpm). The homogenization was done in 10 second bursts, followed by a 10 second rest, to keep the sample temperature from rising. After homogenization, samples were centrifuged (Hettich Micro 22R, Hettich, Tuttlingen, Germany) at 14 000 rpm for 30 min and at 4°C, and the supernatants containing the protein were collected. Protein concentration was determined using the Bradford assay (32).

Western Blot Analysis: In preliminary studies, the experimental conditions required for assessment of western blot band density were determined by several means. Different amounts of extracted total protein from control rats, ranging from 10-100 μg, were used to determine the appropriate amount of protein that should be used in the assay, so that a dose-response effect on band density could be measured. The amounts chosen for the assays (25 μg and 50 μg for TGF-β_1 and VEGF, respectively) were the ones resulting in intermediate band densities. Equal loading of protein into each lane of the gel was carried out using these amounts of total protein. In addition, the dilution of primary and secondary antibodies, as well as the incubation times were carefully titrated to result in band densities that were neither too light to be seen, or so dark that they would saturate the x-ray film. Primary antibody dilutions ranging from 1:100 to 1:1000 and secondary antibody dilutions ranging from 1:25 000 to 1:100 000 were tested until the optimum antibody concentrations were achieved, as described below. Finally, we tried x-ray film exposure times ranging from 5 seconds to 7 minutes until an appropriate band density was achieved (30 seconds for TGF-β_1 and 2 min for VEGF). Once these parameters were determined, they were used for all of the western blot assays, as described below.

Pre-cast tris-glycine polyacrylamide gels, 14% for VEGF blots, 16% for TGF-β_1

blots, (Catalog # EC6488 and EC6498, respectively, Novex Inc, San Diego, California) were loaded with equal amounts of protein from each sample (25 μg for TGF-β1, 50 μg for VEGF). Equal loading of protein into each lane of the gels based on a careful determination of protein concentration using the Bradford assay has been described by others (105,120,125). Recombinant purified protein (TGF-β1 cat # 4165, VEGF cat # 4045, Santa Cruz Biotechnology, Santa Cruz, California) was run as a positive control to ensure that we identified bands of the correct size. A molecular weight marker was also run for this reason (pre-stained broad-range protein marker, catalog # p7708S, New England Biolabs, Beverly, Massachusetts). Gels were run under denaturing conditions at 125 volts for 90 min in the Novex® Xcell II mini-gel system (Novex, San Diego, California). Proteins were electrophoretically transferred to PVDF (polyvinylidene fluoride) membrane (Catalog # IVPH00010, Millipore Corp., Bedford Massachusetts) using the Novex transfer module. The transfer was done at 25 volts for 2 h and 15 min. Nonspecific binding sites on the membrane were blocked for 1 h using a solution of 8% non-fat milk powder in PBST (phosphate buffered saline with tween-20). For the VEGF blots, 1% goat serum was added to the blocking solution to reduce the background staining.

The PVDF Membranes were then incubated in primary antibodies. For TGF-β_1, rabbit polyclonal antibody at a dilution of 1:400 was used for 2 h at room temperature (cat # SC-146 Santa Cruz Biotechnology, Santa Cruz, California). For VEGF, mouse anti-human monoclonal antibody at a dilution of 1:200 was used for 2 h at room temperature (cat # RDI-VEGF Nabmp, Research Diagnostics Inc, Flanders , New Jersy).

The PVDF membranes were washed and incubated with the appropriate

secondary antibody for 1 h at room temperature. For TGF-β_1 the antibody used was goat anti-rabbit IgG at a dilution of 1:50 000 (cat # 1706515 Bio-rad Laboratories, Hercules, California). For VEGF, the antibody used was goat anti-mouse IgG at a dilution of 1:50 000 (Catalog # 1706516, Bio-rad Laboratories, Hercules, California).

Chemi-luminescent detection was carried out using the enhanced chemi-luminescance protocol (ECL® Amersham Life Sciences, Buckinghamshire, UK), using Kodak X-OMAT AR® Scientific Imaging Film (Eastman Kodak, Rochester, New York). X-ray film exposure time was 30 seconds for TGF-β1 and 2 min for VEGF. Resulting bands were analyzed by densitometry, using Fluorchem 2.0 software (Alpha Innotech Corporation, San Leandro, California) and the Alpha-Innotech Fluorchem 8000 imaging system software (Alpha Innotech Corporation, San Leandro, California). Similar protocols for western blot assay of TGF-β1 (41,120,125) and VEGF (116,139) have been published elsewhere.

2.6.6 Hydroxyproline assay

Biopsy segments (approximately 100 mg) representing random portions of the wound site were frozen, lyophilized, weighed and delivered to the Protein Technology Center at the Hospital for Sick Children. The lyophilized skin biopsy was hydrolyzed by liquid phase reaction using hydrochloric acid of 6 molar concentration (6M HCl) with 1% phenol at 110°C for 24 h. The drying, evacuation of air, flushing with pre-purified nitrogen and hydrolysis were done on a Waters PICO-TAG amino acid analysis system (Waters Corporation, Milford Massachusetts). After hydrolysis, excess HCl was removed from the hydrolysis tube under vacuum and the hydrolysate was dissolved using 0.1 M

HCl. An aliquot of 5 µl was taken, dried and treated with a re-drying solution consisting of methanol:water:triethylamine (2:2:1) and dried again under vacuum. The sample was then derivitized for 20 min at room temperature using a derivitizing solution of methanol:water:triethylamine:phenylisothiocyanate (PITC) (7:1:1:1). The derivitizing solution was removed under vacuum and the sample was treated with the solution to remove any traces of PITC. The derivitized sample was dissolved in a given amount of sample diluent (pH 7.40) and an aliquot was injected into the amino acid analysis column. Column temperature was 38°C. The PTC-amino acids were detected at 254 nm. Results were calculated in pmol of hydroxyproline/mg dry tissue. This protocol has been published by others (30,42,47,102), and the measurement of hydroxyproline for indirect assessment of collagen content is well established in several animal models including the rat skin incision model (45,117,207).

2.7 Experimental Protocols

Aim 1: To investigate the deleterious effect of preoperative single dose and fractionated irradiation on wound breaking strength (WBS) and wound hydroxyproline content and the efficacy of prophylactic treatment with the radioprotector Amifostine in alleviating the adverse effects of irradiation.

Study 1: Single Dose Irradiation Protocol for the Study of WBS

Rats were randomized into one of three groups (n=6/group): (i) non-irradiated control (ii) single dose irradiation (iii) single dose irradiation with Amifostine pre-treatment. The irradiated groups were subjected to a single 20 Gy dose of radiation. The Amifostine treated group was given Amifostine intraperitoneally 30 min prior to irradiation at a dose of 300 mg/kg. The control group received sham radiation. Five to 6 weeks after the completion of irradiation, a 3 x 8 cm skin flap was raised on the dorsum of all rats. Biopsies for WBS were taken 14 days post-operatively, and the rats were sacrificed at that time with an overdose of sodium pentobarbitone.

Study 2: Fractionated Dose Irradiation Protocol for the Study of WBS and Wound Tissue Content of Hydroxyproline

Rats were randomized into one of three groups (n=8/group) (i) non-irradiated controls (ii) fractionated dose irradiation and (iii) fractionated dose irradiation with Amifostine pre-treatment. The irradiated groups were subjected to 6 fractionated doses of 6.2 Gy given over a 2-week period. The Amifostine treated group was given Amifostine intraperitoneally 30 min prior to each radiation fraction at a dose of 150 mg/kg. Five to 6 weeks after the completion of irradiation, a 3 x 8 cm skin flap was raised on the dorsum of all rats. Biopsies for WBS and wound hydroxyproline content were taken 14 days post-operatively, and the rats were sacrificed at that time with an overdose of sodium pentobarbitone.

Aim 2: To investigate the deleterious effect of preoperative single dose and fractionated irradiation on surgical skin wound tissue contents of macrophages, fibroblasts, capillaries, on growth factors, and on skin flap viability at 3, 8 and 21 days after surgery and to investigate the efficacy of the radioprotector Amifostine in alleviation of the deleterious effects induced by preoperative fractionated irradiation.

Rats were randomized into one of four groups (n=6/group): (i) non-irradiated control (ii) single dose irradiation (iii) fractionated dose irradiation and (iv) fractionated dose irradiation with Amifostine pre-treatment. The single dose irradiation group was subjected to a single 20 Gy dose of radiation. The fractionated irradiation groups were subjected to 6 fractionated doses of 6.2 Gy given over a 2-week period. The fractionation plus Amifostine treated group was given Amifostine intraperitoneally 30 min prior to each radiation fraction at a dose of 150 mg/kg. The non-irradiated control group received sham irradiation. Five to 6 weeks after the completion of irradiation, a 3 x 8cm skin flap was raised on the dorsum of all rats. In separate studies, rats were sacrificed and skin wound biopsies were taken on post-operative days 3, 8 and 21 for the following assessments: (i) histological assessment of wound density of capillaries, fibroblasts and macrophages and (ii) western blot assay of TGF-β1 and VEGF protein expression. Flap viability was also assessed in each study prior to taking skin biopsies. The times of wound biopsy (3, 8 and 21 days) were chosen to correspond with the three major phases of wound healing. Individual results for each biopsy time period were analyzed in isolation from the other biopsy time points, since the three wound healing phases represent distinct processes.

2.8 Statistical Analysis

All results are reported as mean ± standard error, unless otherwise stated. Statistical analysis was performed using the SPSS® statistical software version 10.0 for Macintosh. One way analysis of variance (ANOVA) followed by Tukey's least significant difference (LSD) post-hoc analysis were used for multiple comparisons of means. Statistical significance was set at $p<0.05$ for all of the ANOVA tests.

For the analysis of the western blot assays, the densities of the control bands were taken to represent 100%. Differences in protein expression in the experimental groups compared to the control were expressed as a percentage of the average control value for each blot. Statistical analysis was performed using a one sample Student t-test. (Figures 15, 18). A Bonferroni correction was used and statistical significance was set at $p<0.01$ for the western blot t-tests.

Mr. Derek Stephens, a biostatistician at the Hospital for Sick Children, was the consultant for all of the statistical analyses used in this thesis.

3.0 Results

3.1 Effect of Pre-Operative Single Dose Ionizing Radiation on Wound Breaking Strength with and without Amifostine Pre-treatment

Pre-operative single dose irradiation significantly ($p<0.05$) reduced WBS with or without Amifostine pre-treatment, compared to non-irradiated controls assessed 14 days post-operatively (Figure 7). Amifostine pre-treatment significantly ($p<0.05$) attenuated the effect of single dose irradiation on WBS, limiting the decrease in WBS to 20%, as opposed to a decrease of 45% of the control observed in the irradiated group without Amifostine pre-treatment.

3.2 Effect of Pre-Operative Fractionated Ionizing Radiation on Wound Breaking Strength with and without Amifostine Pre-treatment

Pre-operative fractionated irradiation significantly ($p<0.05$) reduced WBS compared to non-irradiated controls assessed 14 days post-operatively (Figure 8). Amifostine pre-treatment significantly ($p<0.05$) attenuated the effect of fractionated irradiation on WBS, limiting the reduction in WBS to 26%, as opposed to a decrease of 64% of the control observed in the irradiated group without Amifostine pre-treatment.

3.3 Effect of Pre-Operative Ionizing Radiation on Wound Tissue Hydroxyproline Content with and without Amifostine Pre-treatment

Pre-operative fractionated irradiation significantly ($p<0.05$) reduced the wound tissue hydroxyproline content compared to non-irradiated controls assessed 14 days post-operatively (Figure 9). Wound tissue hydroxyproline content in the Amifostine pre-treated group was not affected by pre-operative irradiation and no significant difference in wound tissue hydroxyproline content was found between the control and Amifostine pre-treated groups.

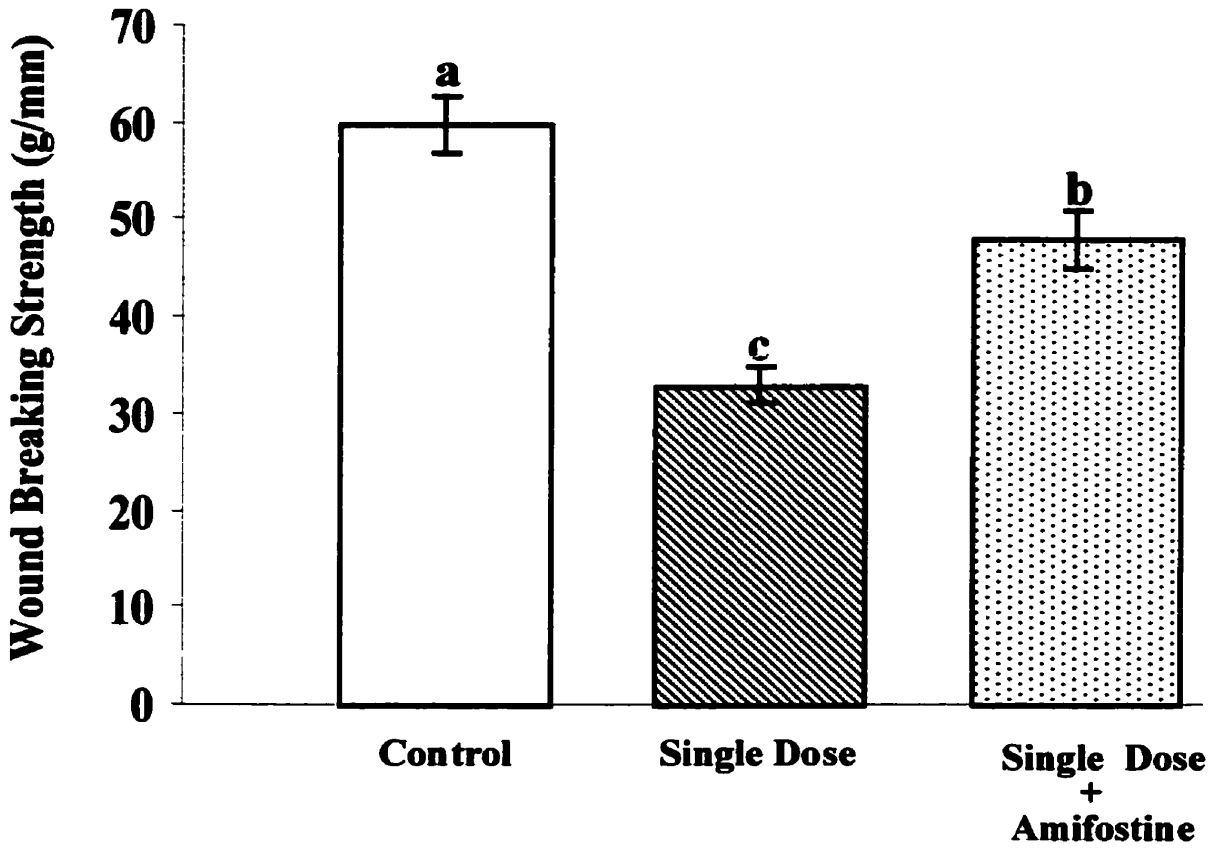

Figure 7. Effect of pre-operative single dose ionizing radiation on wound breaking strength 14 days post-operatively with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy radiation dose. Amifostine pre-treatment (300 mg/kg;i.p.) was given 30 min prior to irradiation. Skin flap surgery followed irradiation by 5-6 weeks. WBS test was done 14 days post-operatively. Bilateral skin biopsies (1 x 3 cm) were stretched uniaxially at 5 mm/min until the point of failure. Values are mean ± SEM, n=6 rats/group. Means without a common letter are significantly different. (a>b>c, $p<0.05$).

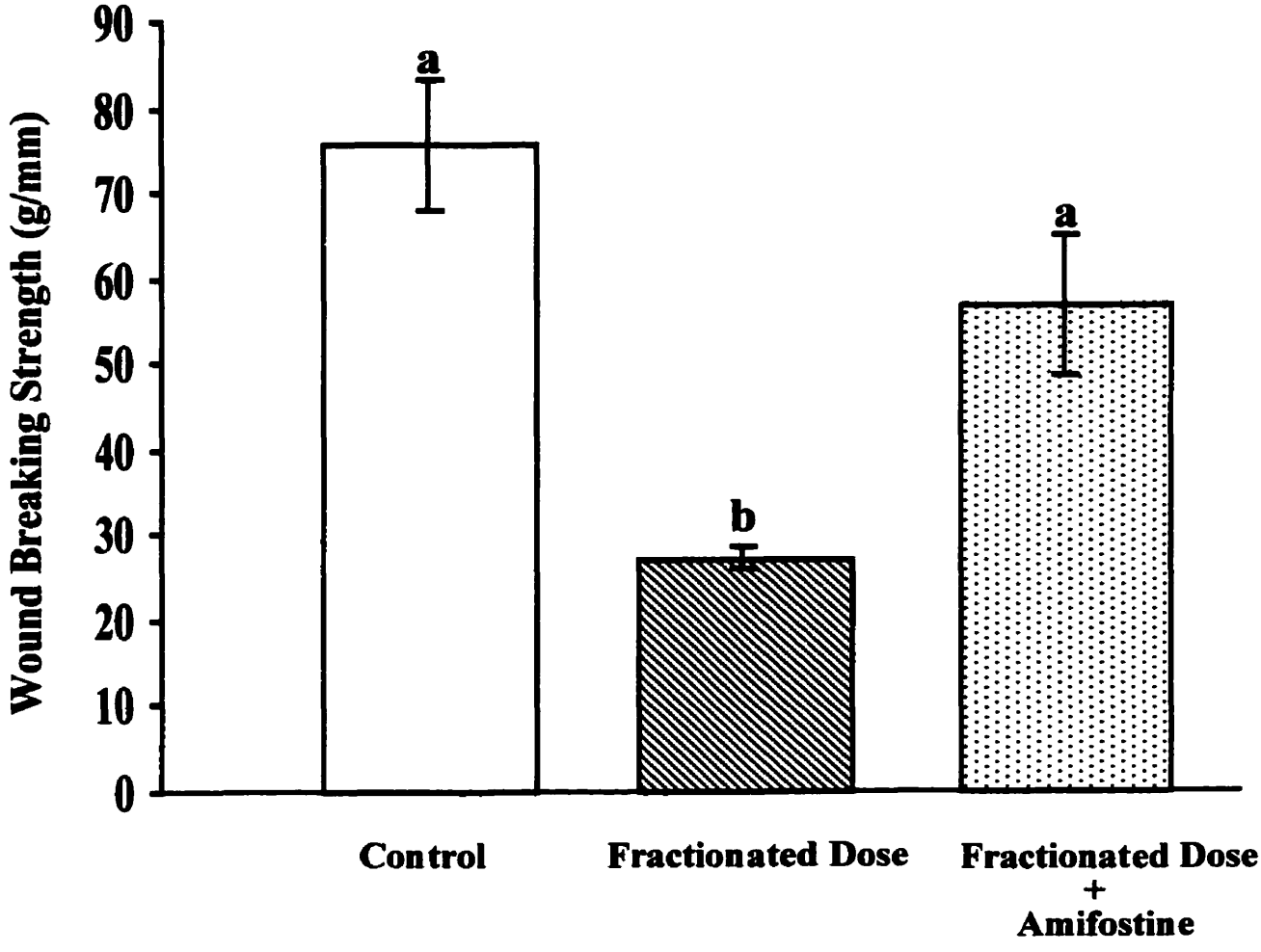

Figure 8. Effect of pre-operative fractionated ionizing radiation on wound breaking strength 14 days post-operatively with and without Amifostine pre-treatment. Rats were irradiated with 6 fractions of 6.2 Gy. Amifostine pre-treatment (150 mg/kg;i.p.) was given 30 min prior to each radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. WBS test was done 14 days post-operatively. Bilateral skin biopsies (1 x 3 cm) were stretched uniaxially at 5 mm/min until the point of failure. Values are mean ± SEM, n= 6 rats/group. Means without a common letter are significantly different. (a>b, $p<0.05$).

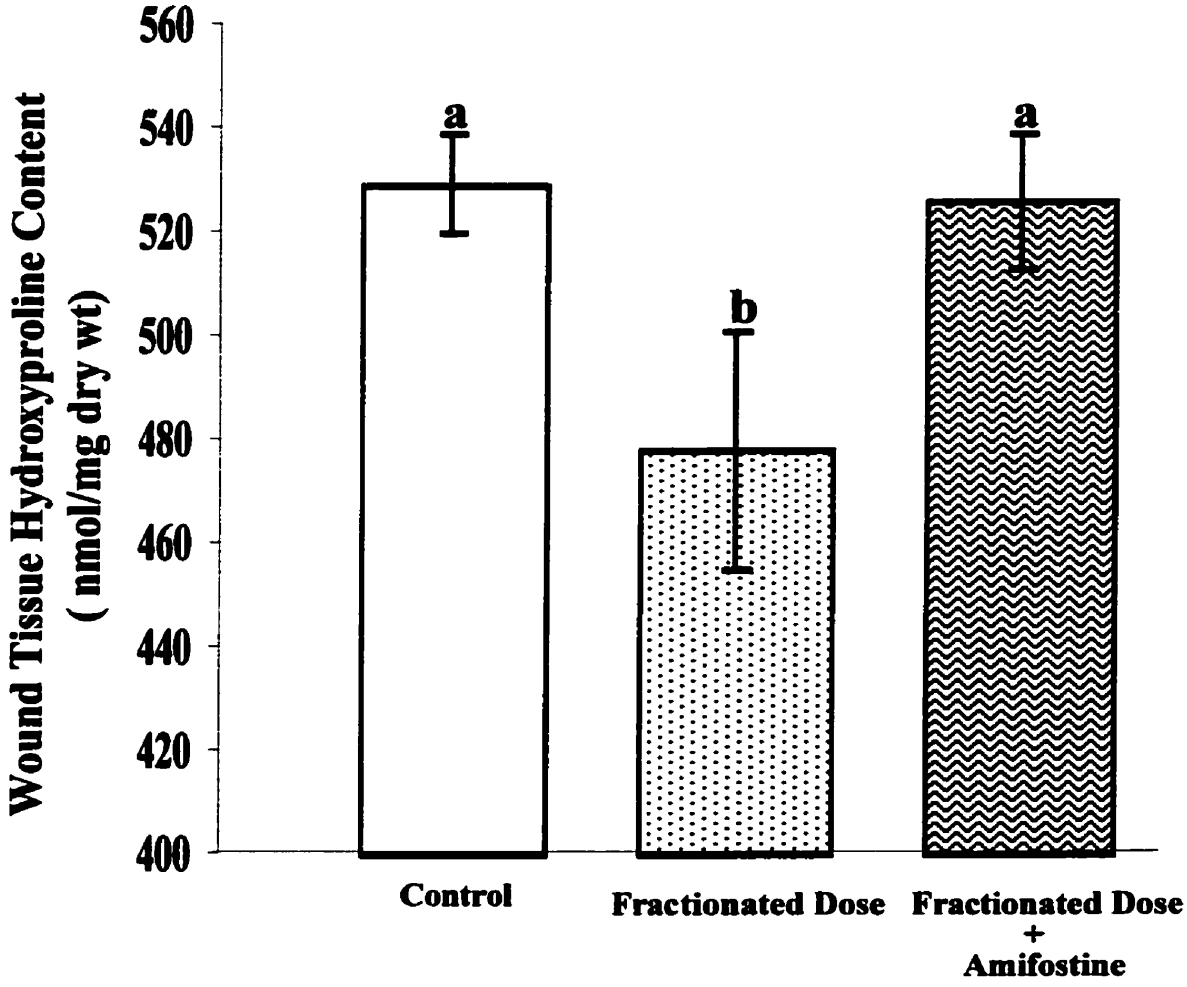

Figure 9. Effect of pre-operative fractionated ionizing radiation on wound tissue hydroxyproline content 14 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with 6 fractions of 6.2 Gy. Amifostine pre-treatment (150 mg/kg;i.p.) was given 30 min prior to each radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. Biopsies for hydroxyproline assay were taken 14 days post-operatively. Values are mean ± SEM, n=6 rats/group. Means without a common letter are significantly different. (a>b, $p<0.05$). Values are mean ± SEM, n=8 rats/group.

3.4 Effect of Pre-Operative Ionizing Radiation on Skin Flap Viability with and without Amifostine Pre-treatment

There were no significant differences in skin flap viability among control and treatment groups undergoing pre-operative single dose or fractionated irradiation, with or without Amifostine pre-treatment (Table 1). However, there were decreases in skin flap viability of 20%, 15% and 18% in rats receiving single dose irradiation, fractionated irradiation and fractionated irradiation with Amifostine pre-treatment, respectively, compared with the control on day 21 post-operatively, but these decreases were not statistically significant.

3.5 Effect of Pre-operative Ionizing Radiation on Wound Tissue Density of Macrophages, Fibroblasts, and Capillaries, with and without Amifostine Pre-treatment

Macrophages: Only data from the third and eighth post-operative days are discussed. Wounds biopsied 21 days post-operatively all had less than 15 macrophages/8 fields. The small number of macrophages observed in wound tissue on the 21st post-operative day is in keeping with similar descriptions of cellular infiltration into healing wounds reported by other investigators, because recruitment of wound macrophages occurs mainly within the first post-operative week (124, 239).

Representative histologic sections demonstrating the stained wound tissue macrophages on the third post-operaive day are shown in Figure 10. The brown stained macrophages are clearly visible in the dermis, and around the incision wound site. On the

Table 1: Effect of Ionizing Radiation on Skin Flap Viability at 3, 8 and 21 days post-operatively, with and without Amifostine Pre-treatment.

Groups	Area of Viable Skin Flap (cm^2)		
	3 Days Post-operatively	8 Days Post-operatively	21Days Post-operatively
Control	19.8 ± 1.5	16.6 ± 0.8	18.1 ± 1.1
Single Dose Irradiation	22.6 ± 0.8	17.7 ± 1.2	14.6 ± .7
Fractionated Dose Irradiation	21.3 ± 1.2	18.2 ± 1.5	15.5 ± 1.3
Fractionated irradiation + Amifostine	20.8 ± 0.5	16.8 ± 0.6	14.8 ± 1.0

Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. Skin flaps were traced, and the viable area was determined using digital imaging software. Values are mean ± SEM, n=6 rats/group.

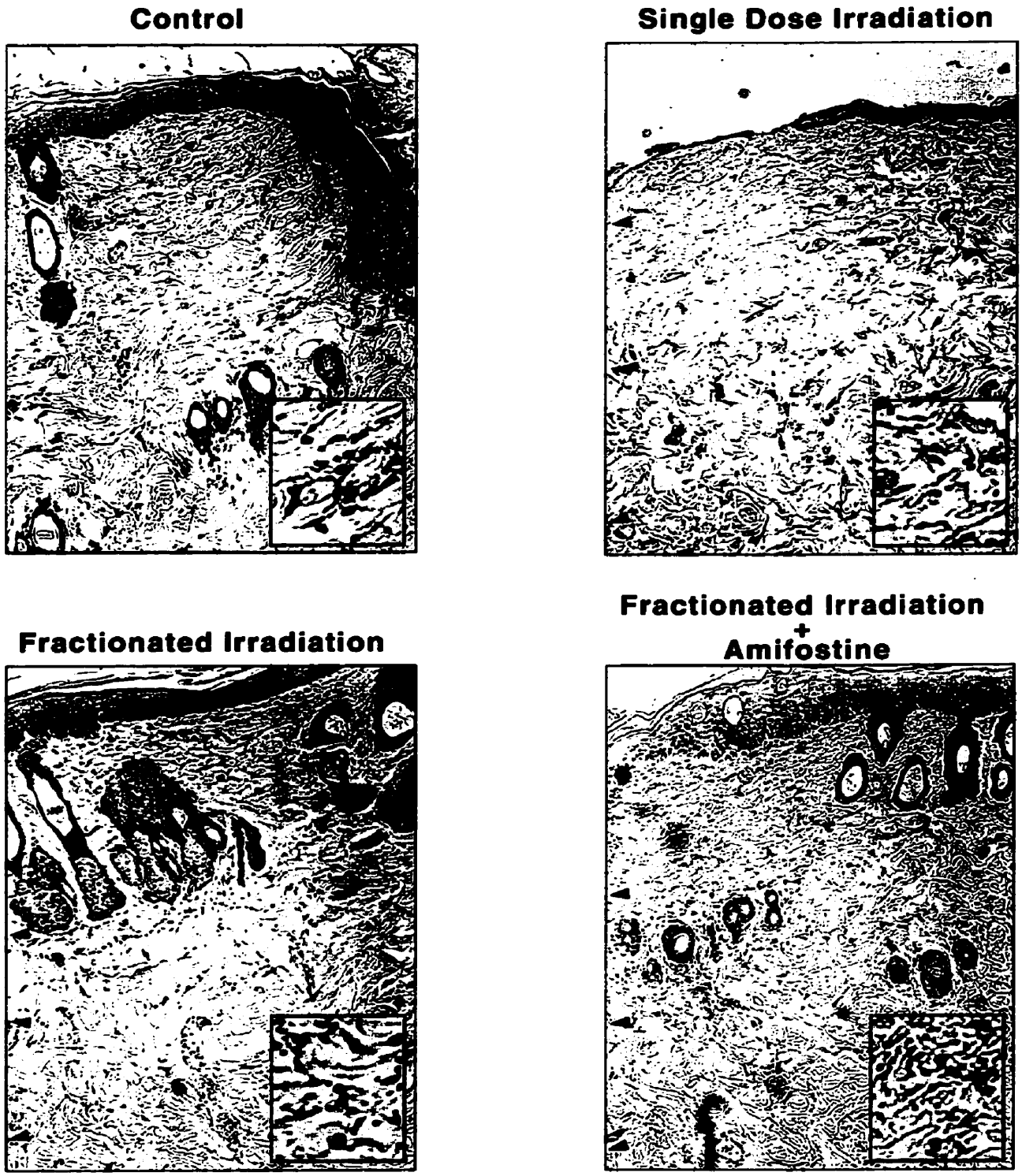

Figure 10. Representative histological sections of macrophage immunostaining on wound biopsies taken 3 days post-operatively. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fraction. Skin flap surgery followed irradiation by 5-6 weeks. Paraffin sections were stained with a macrophage specific antibody. Macrophages are seen in the figures (100X magnification) as brown coloured cells in the dermis. Arrowheads show the edge of the incisional wound. Figure inserts are higher magnification (250X magnification) views demonstrating the macrophage staining

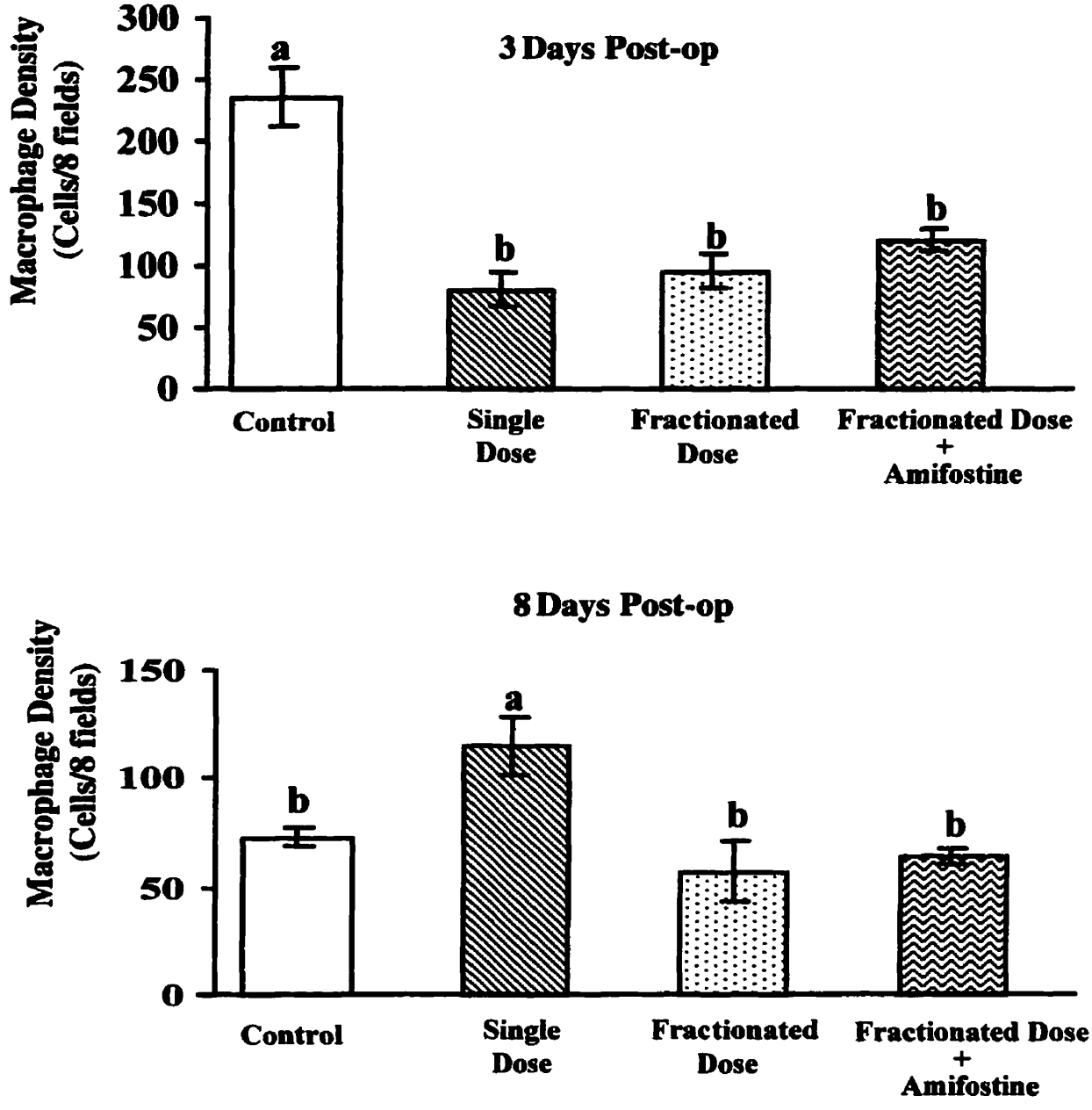

Figure 11. Effect of pre-operative single dose and fractionated ionizing radiation on wound tissue macrophage density 3 and 8 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. Macrophages were summed over 8 microscopic fields (250X). Values are mean ± SEM, n=6 rats/group. Means without a common letter are significantly different. (a>b, $p<0.05$).

third post-operative day, pre-operative single dose, fractionated dose, and fractionated dose radiation with Amifostine pre-treatment decreased the wound tissue density of macrophages by 66%, 59% and 48%, respectively, compared with the control (Figure 11), and the decreases were statistically significant ($p<0.05$). On the eighth post-operative day, the wound tissue density of macrophages was significantly ($p<0.05$) higher in the single dose irradiated group compared with the control and fractionated radiation groups with and without Amifostine pre-treatment. The wound tissue density of macrophages was similar among the control and fractionated radiation groups with or without Amifostine pre-treatment on day 8 post-operatively.

Fibroblast Density: Both pre-operative single dose and fractionated irradiation significantly ($p<0.05$) reduced the wound tissue density of fibroblasts on days 3, 8 and 21 post-operatively, compared to non-irradiated controls (figure 12). Amifostine pretreatment significantly ($p<0.05$) attenuated the pre-operative radiation effect on the decrease of wound tissue fibroblast density assessed on days 3 and 8 post-operatively, and restored wound tissue fibroblast density to a level similar to the control on day 21 post-operatively.

Capillary Density: Representative histologic sections demonstrating the immunostained wound tissue capillaries are shown in Figure 13. The red stained capillaries are clearly visible throughout the dermis. On the third post-operative day, pre-operative fractionated irradiation significantly ($p<0.05$) decreased wound tissue capillary density (Figure 14). The wound tissue capillary density of the single dose group was not significantly different from the control group. In the Amifostine pre-treatment group,

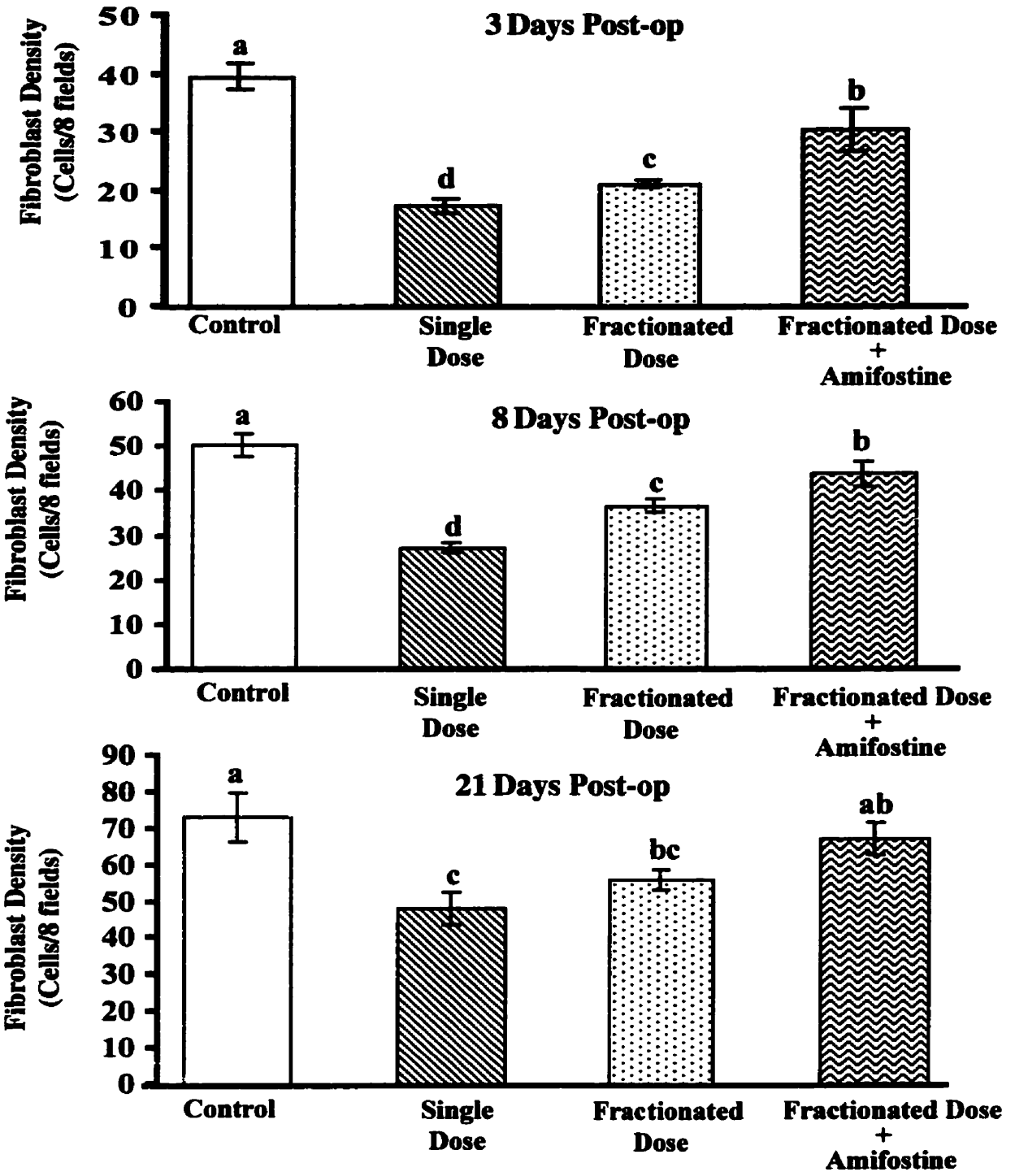

Figure 12. Effect of pre-operative single dose and fractionated ionizing radiation on wound tissue fibroblast density 3, 8 and 21 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation doses. Skin flap surgery followed irradiation by 5-6 weeks. Fibroblasts were summed over 8 microscopic fields (250X). Values are mean ± SEM, n=6 rats/group. Means without a common letter are significantly different. ($a>b>c$, $p<0.05$).

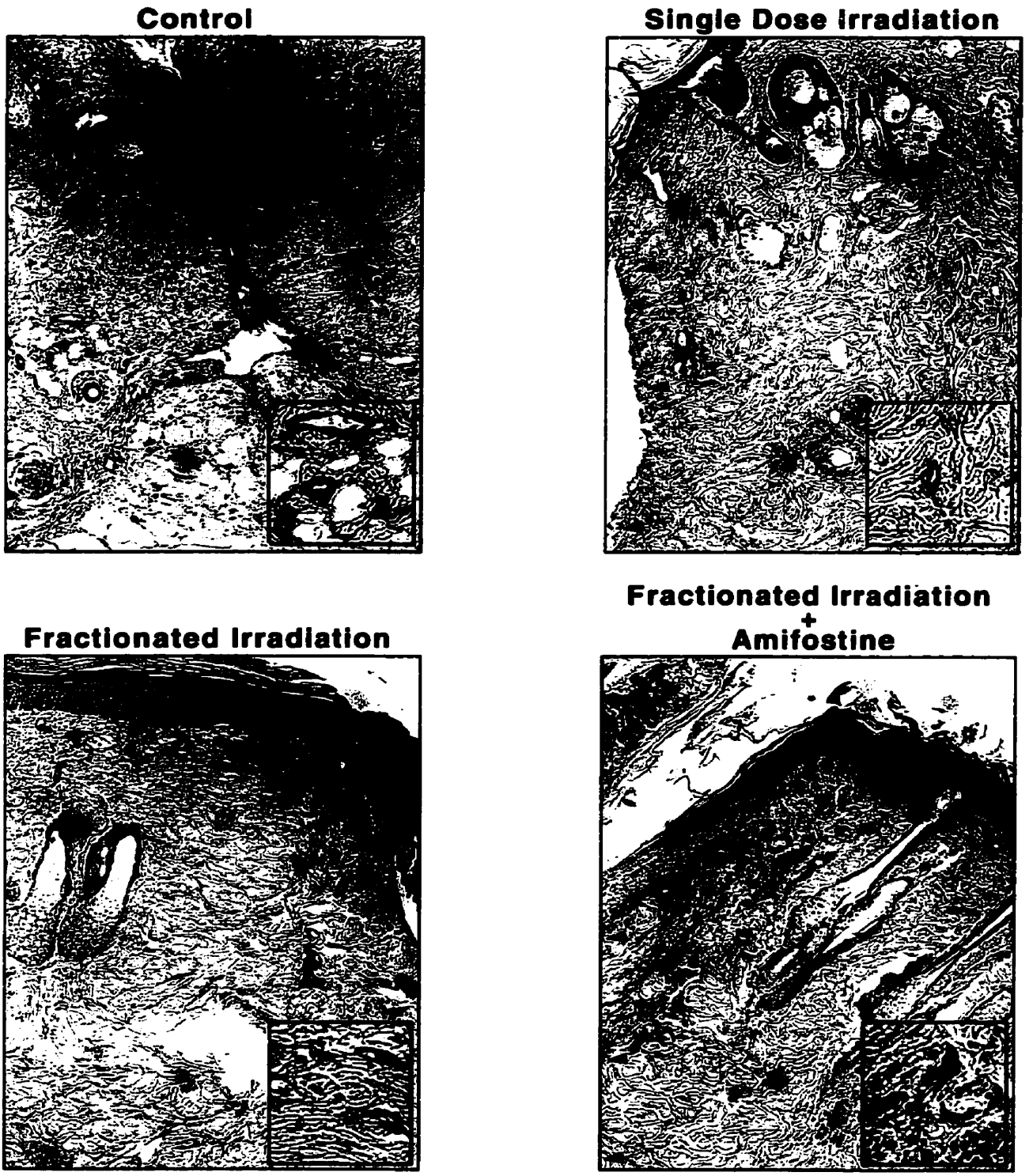

Figure 13. Representative histological sections of capillary immunostaining on wound biopsies taken 8 days post-operatively. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fraction. Skin flap surgery followed irradiation by 5-6 weeks. Paraffin sections were stained with an anti-factor VIII specific antibody. Capillaries are seen in the figures (100X magnification) as red coloured structures in the dermis. Arrowheads show the edge of the incisional wound. Figure inserts are higher magnification (250X magnification) views demonstrating the capillary staining

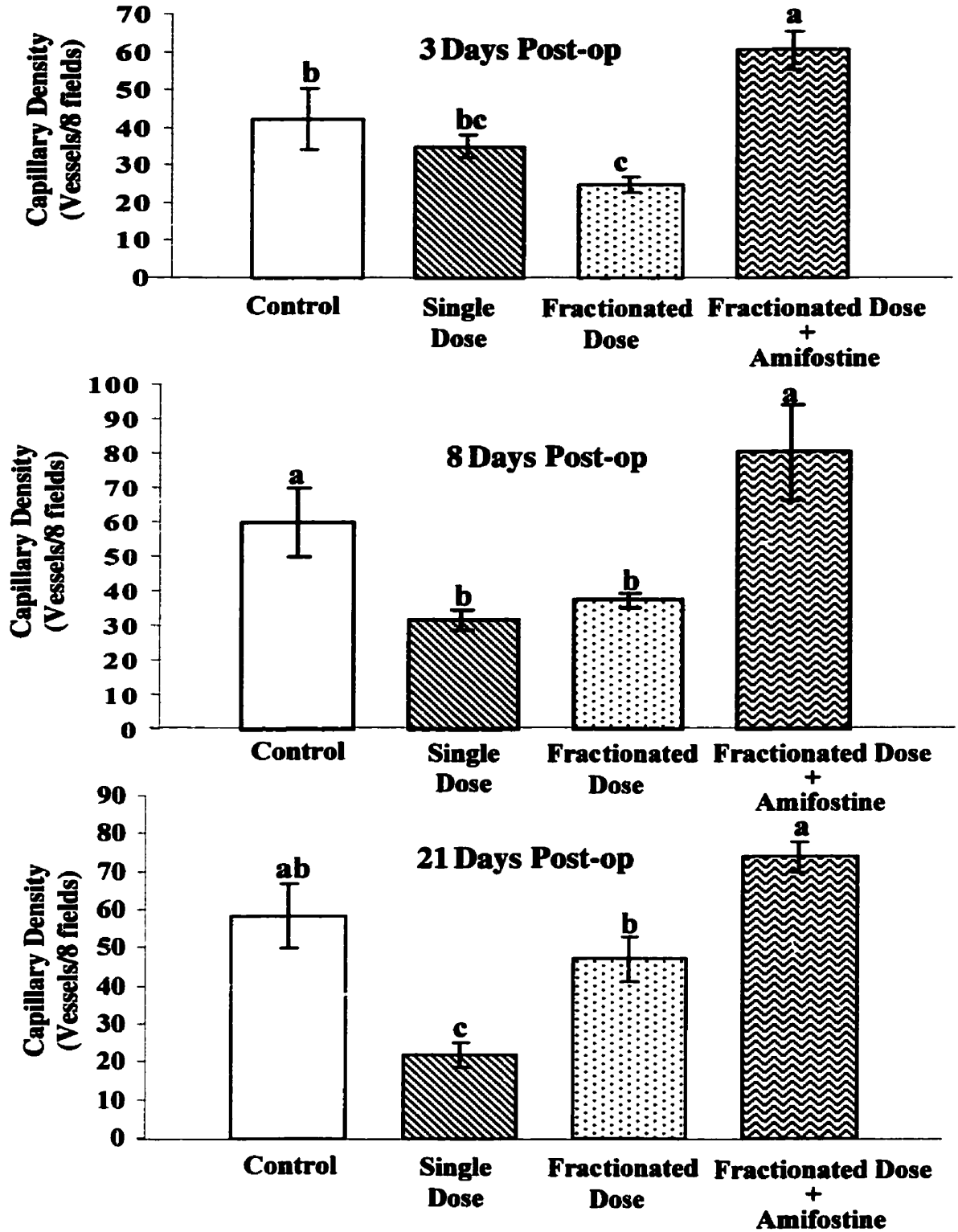

Figure 14. Effect of pre-operative single dose and fractionated ionizing radiation on wound tissue capillary density 3, 8 and 21 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg/i.p.) was given 30 min prior to each fractionated radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. Factor-VIII stained capillaries were summed over 8 microscopic fields (250X). Values are mean ± SEM, n=6 rats/group. Means without a common letter are significantly different. (a>b>c, $p<0.05$).

wound tissue capillary density was significantly ($p<0.05$) higher than the control, single dose and fractionated irradiation groups.

On the eighth post-operative day, wound tissue capillary density was significantly ($p<0.05$) lower than the control in both pre-operative irradiated groups (Figure 14). Wound tissue capillary density in the Amifostine pre-treated group was significantly ($p<0.05$) higher than both irradiated groups, but was similar to the control.

On the twenty-first postoperative day, wound tissue capillary density was significantly lower than the control in the single dose irradiated group (Figure 14). Wound tissue capillary density in the Amifostine pre-treated group was significantly ($p<0.05$) higher than in both the single dose and fractionated irradiated groups, and not significantly different from the control.

3.6 Effect of Pre-operative Single Dose and Fractionated Ionizing Radiation with and Without Amifostine Pre-treatment on Wound Tissue TGF-β1 and VEGF Protein Expression.

TGF-β1 Protein Expression Studied by Western Blot Assay: On the third post-operative day, no significant change in wound tissue TGF-β1 protein expression was observed in any of the groups compared to the control (Figure 15). Among the treatment groups, wound tissue TGF-β1 protein expression was significantly ($p<0.05$) lower in the fractionated group with Amifostine pre-treatment compared to the single dose groups.

On the eighth post-operative day, wound tissue TGF-β1 protein expression was significantly ($p<0.01$) up-regulated in the single dose group compared to the control. Among the treatment groups, wound tissue TGF-β1 protein expression in both the fractionated irradiation group and the fractionated irradiation group with Amifostine pre-

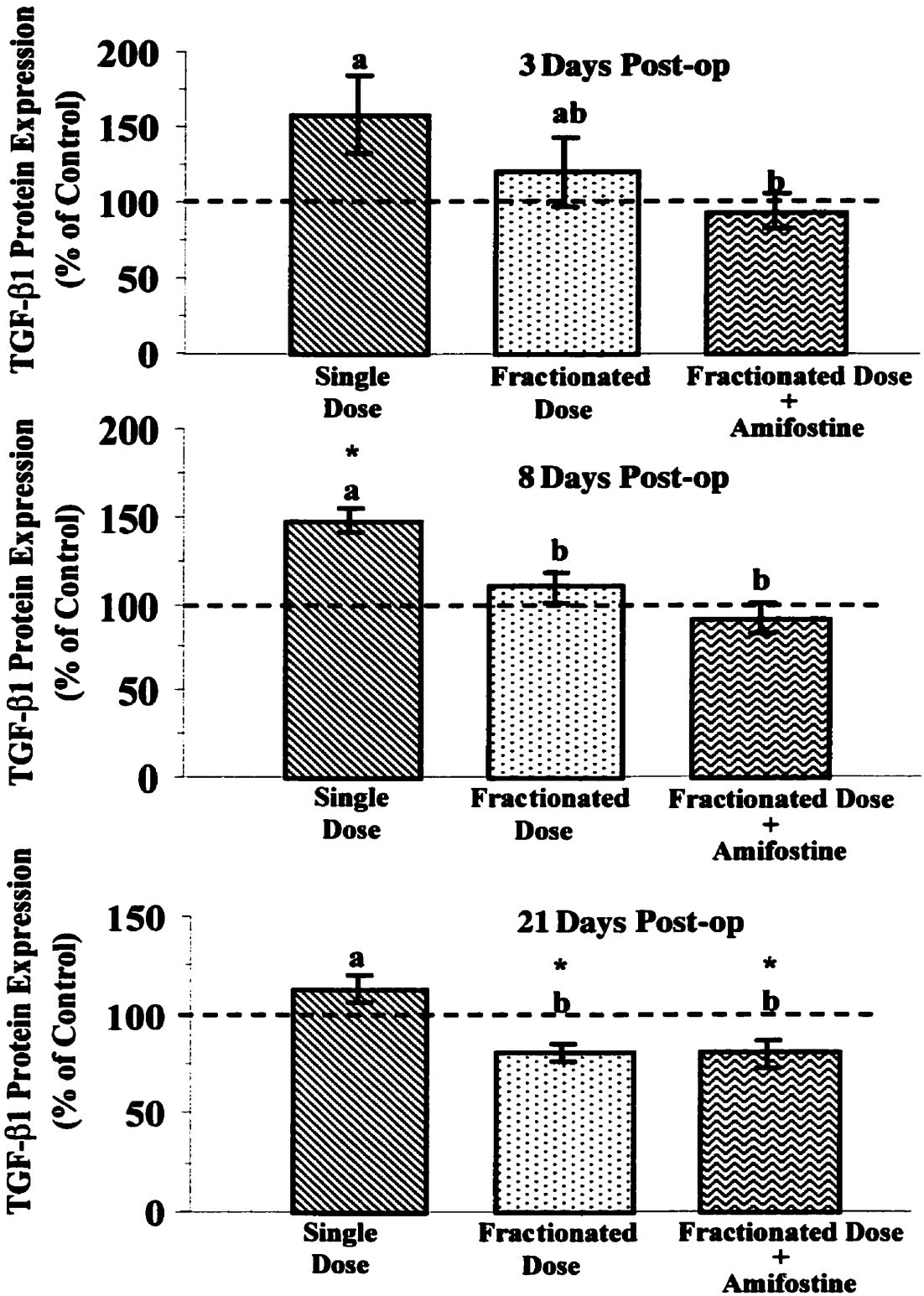

Figure 15. Effect of pre-operative single dose and fractionated ionizing radiation on wound expression of TGF-β1 3, 8 and 21 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p) was given 30 min prior to each fractionated radiation dose. Skin flap surgery followed irradiation by 5-6 weeks. The dashed line at 100% represents the level of TGF-β1 expression in the non-irradiated control wound tissue. Treatment groups that are significantly different from control are marked with an asterisk ($p<0.01$). Values are mean ± SEM, n=6 rats/group. Among treatment groups, means without a common letter are significantly different. (a>b, $p<0.05$).

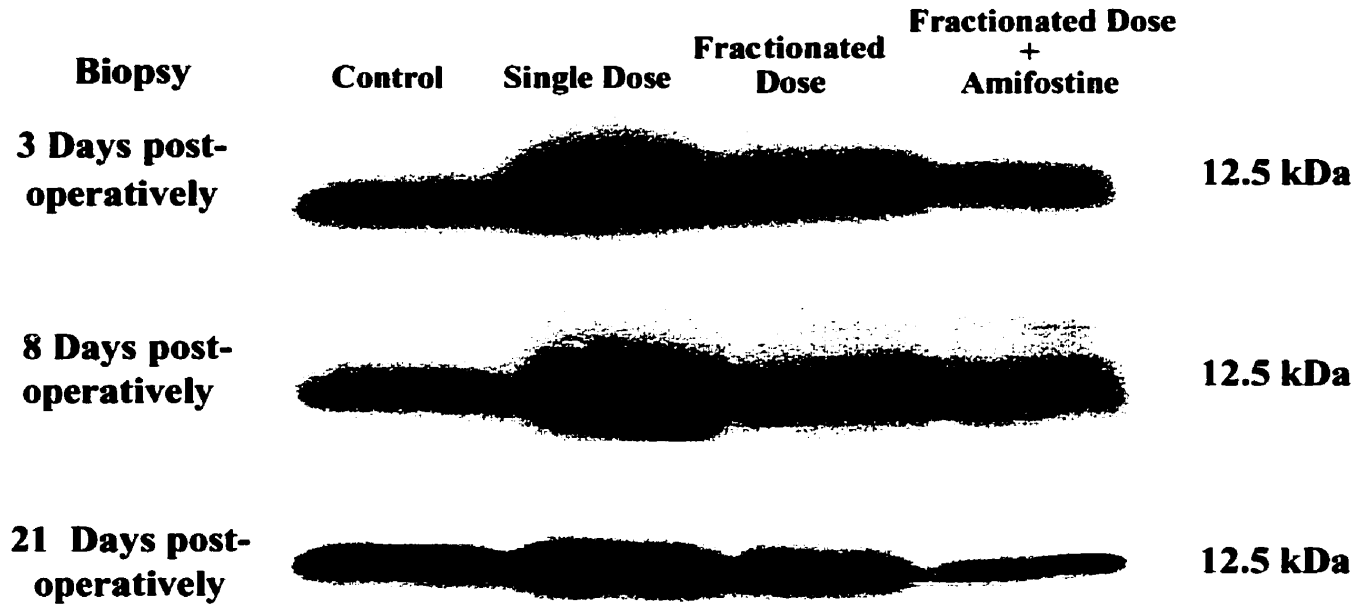

Figure 16. Representative western blot results for TGF-β1 protein expression. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Surgery followed irradiation by 5-6 weeks. Wound tissues were studied for TGF-β1 protein expression 3, 8 and 21 days post-operatively. The western blot results demonstrate upregulation of TGF- β1 expression by ionizing radiation compared with the control, and attenuation of this effect by Amifostine pre-treatment. Each band represents an individual rat.

treatment was significantly ($p<0.05$) lower than in the single dose group (Figure 15).

On the twenty-first postoperative day, TGF-β1 protein expression was significantly ($p<0.01$) down-regulated in both the fractionated and fractionated with Amifostine pre-treatment groups, compared to control. Among the treatment groups, TGF-β1 protein expression was significantly higher in the single dose irradiation group compared to the fractionated irradiation group, and the fractionated irradiation group with Amifostine pre-treatment (Figure 15). Representative western blot results for each biopsy time point are shown in Figure 16.

VEGF Expression Studied by Immunohistochemistry: Representative results of VEGF immunohistochemical staining are shown in Figure 17. Immunohistochemical staining for VEGF was significantly ($p<0.05$) reduced in the single dose, fractionated dose and fractionated dose with amifostine pre-treatment groups compared to control (Figure 18).

Among the treatment groups, immunohistochemical staining for VEGF was significantly ($p<0.05$) different in each group, with the single dose having the least positive staining, the fractionated group with Amifostine pre-treatment having the most, and the fractionated group without Amifostine pre-treatment falling in the middle.

VEGF Expression Studied by Western Blot Assay: On the third postoperative day, wound tissue VEGF protein expression was significantly ($p<0.01$) down-regulated in the fractionated group compared to the control (Figure 19). There were no significant differences among the treatment groups.

On the eighth post-operative day, wound tissue VEGF protein expression was

significantly ($p<0.01$) down-regulated in the single dose irradiation group compared to the control (Figure 19). Among the treatment groups, wound tissue VEGF expression was significantly ($p<0.05$) lower in the single dose group compared to the fractionated irradiation group and the Amifostine pre-treated group.

On the twenty-first postoperative day, wound tissue VEGF protein expression was significantly ($p<0.01$) down-regulated in both the single dose and fractionated groups compared to the control (Figure 19). Among the treatment groups, VEGF protein expression in the fractionated irradiation group and the fractionated irradiation group with Amifostine pre-treatment groups were significantly ($p<0.05$) lower than in the single dose irradiation group. Representative western blot results for each biopsy time-point are shown in Figure 20.

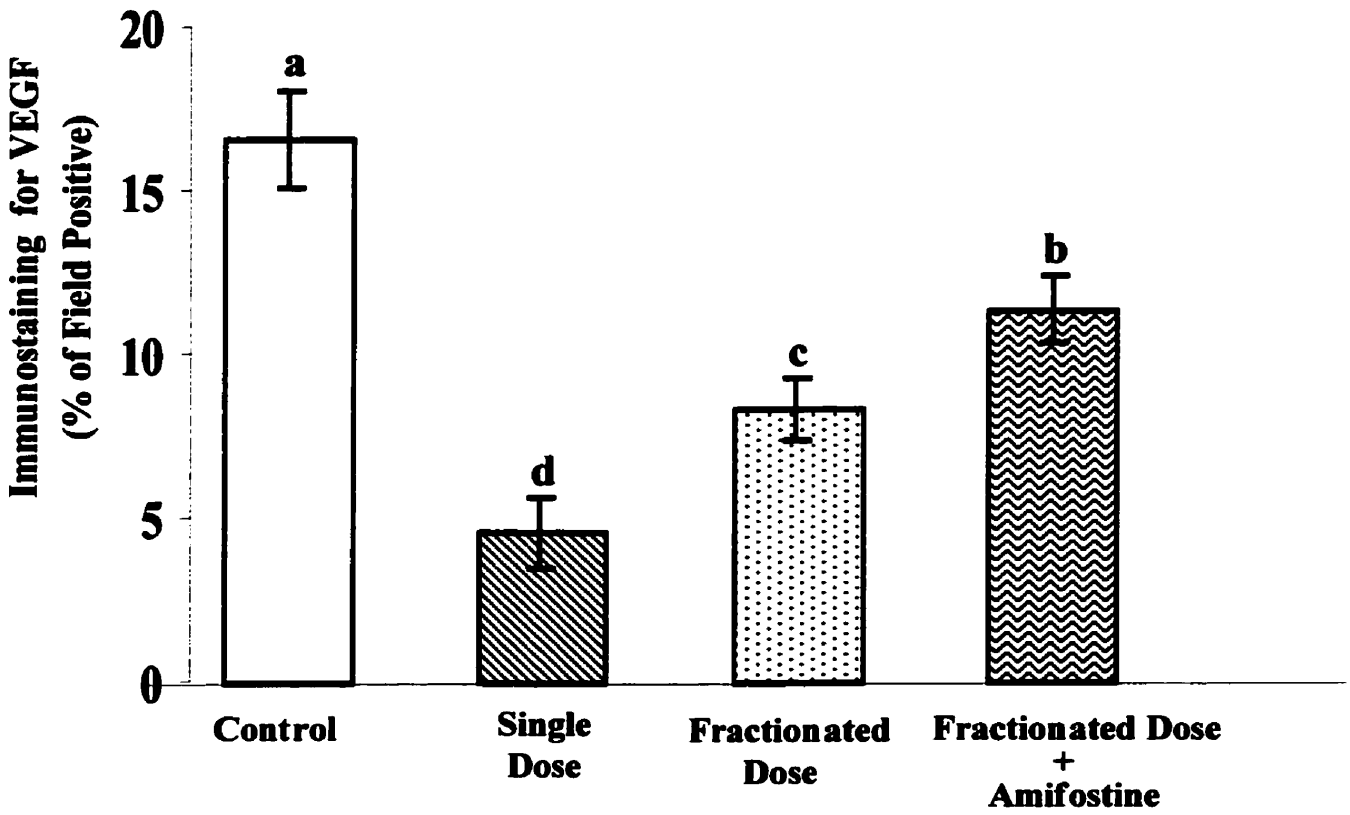

Figure 18. Effect of pre-operative single dose and fractionated ionizing radiation on immunohistochemical staining of wound tissue VEGF 21 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Surgery followed irradiation by 5-6 weeks. Biopsies were taken 21 days post-operatively. Paraffin sections were stained with anti-VEGF antibody. Slides were assessed by digital image analysis. Values are mean ± SEM, n=4 rats/group. Means without a common letter are significantly different. (a>b>c, $p<0.05$).

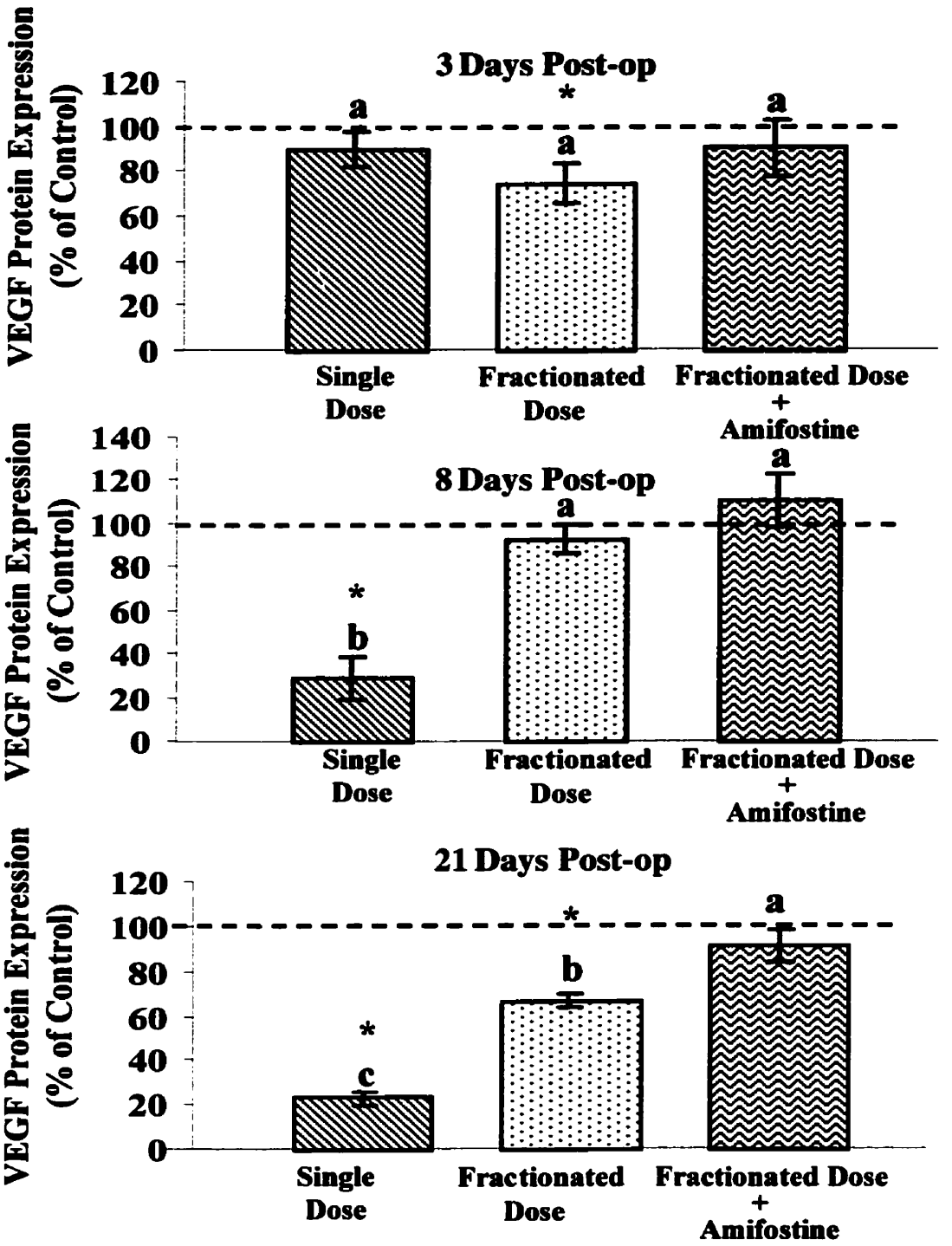

Figure 19. Effect of pre-operative single dose and fractionated ionizing radiation on wound tissue expression of VEGF 3, 8 and 21 days post-operatively, with and without Amifostine pre-treatment. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Surgery followed irradiation by 5-6 weeks. The dashed line at 100% represents the level of VEGF expression in the non-irradiated control wounds. Treatment groups that are significantly different from control are marked with an asterisk ($p<0.01$). Values are mean ± SEM, n=6 rats/group. Among treatment groups, means without a common letter are significantly different. ($a>b>c$, $p<0.05$).

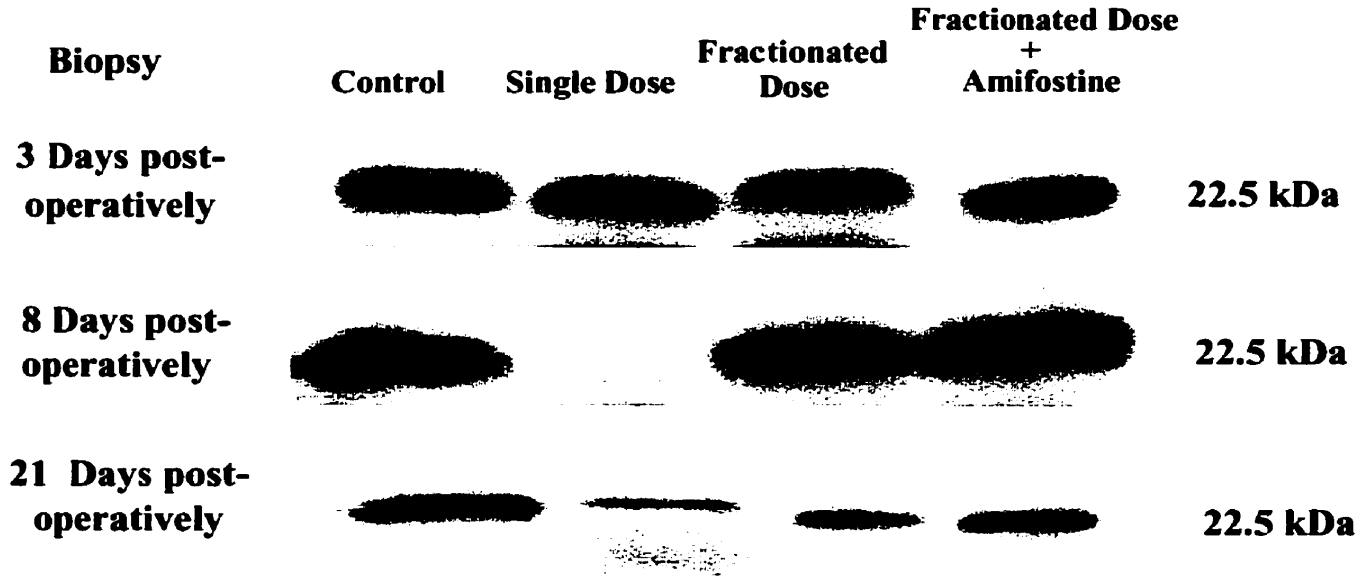

Figure 20. Representative western blot results for VEGF protein expression. Rats were irradiated with a single 20 Gy dose or 6 fractions of 6.2 Gy. Amifostine treatment (150 mg/kg;i.p.) was given 30 min prior to each fractionated radiation dose. Surgery followed irradiation by 5-6 weeks. Wound tissues were assayed for VEGF protein expression 3, 8 and 21 days post-operatively. The western blot results demonstrate downregulation of VEGF expression by ionizing radiation, and attenuation of this effect by Amifostine pre-treatment. Each band represents an individual rat.

4.0 Discussion

4.1 Important New Techniques and Findings in this Project

A combination of radiotherapy and surgery is the current standard of treatment for many forms of cancer. There is clinical evidence to indicate that pre-operative fractionated radiotherapy increases the incidence of impaired surgical wound healing (12, 35, 98, 110). Using incisional and excisional wound models and single doses of radiation in rat and mouse models, other investigators demonstrated that pre-operative radiation significantly reduced WBS, a marker of impaired surgical wound healing in the skin (26, 69, 79). However, little is known about the pathogenic mechanism of radiation-induced impaired surgical wound healing. Here, we used a clinically relevant skin flap surgical wound model and fractionated dose irradiation, as well as the radioprotector Amifostine as a pharmacological probe to investigate the pathophysiology of radiation-induced impaired surgical wound healing at the molecular, cellular and tissue levels. Several important new observations were made.

It was observed that pre-operative single dose or fractionated irradiation with a total dose calculated using the linear quadratic model (with an α/β ratio = 10) to be equivalent to a single dose of 20 Gy caused impaired surgical wound healing as indicated by a significant decrease in WBS compared to the control measured on day 14 postoperatively (Figures 7 and 8). The decrease in WBS caused by fractionated irradiation was also associated with a significant decrease in wound tissue content of hydroxyproline, a marker of tissue collagen content (Figure 9). Amifostine treatment given 30 minutes prior to single dose or fractionated irradiation significantly improved WBS (Figures 7 and 8) and wound tissue content of hydroxyproline (Figures 9)

Pre-operative single dose and fractionated irradiation significantly decreased wound

tissue macrophage density on day 3 post-operatively (Figure 11), and wound tissue fibroblast density on days 3, 8 and 21 post-operatively (Figure 12). Amifostine treatment given 30 minutes before each fractionated radiation dose significantly improved wound tissue fibroblast density at all time points studied (Figure 12) compared with time-matched controls.

Pre-operative single dose irradiation significantly reduced wound tissue capillary density on days 8 and 21 post-operatively compared with time-matched controls (Figure 14). Pre-operative fractionated radiation significantly reduced wound tissue capillary density on days 3 and 8 post-operatively compared with time-matched controls. Amifostine treatment given 30 minutes prior to each fractionated radiation dose restored the wound tissue capillary density to the control level at all time points studied (Figure 14).

In general, pre-operative single dose or fractionated dose irradiation did not affect wound tissue contents of TGF-β_1, except on post-operative day 8, when the wound tissue content of TGF-β_1 was significantly higher in the single dose irradiation group and on post-operative day 21, when the wound tissue content of TGF-β_1 was significantly lower in the fractionated irradiation group compared to their respective controls (Figure 15).

Pre-operative single dose irradiation significantly reduced wound tissue content of VEGF protein on days 8 and 21 post-operatively compared with time-matched controls (Figure 19). Pre-operative fractionated irradiation significantly reduced the wound tissue content of VEGF on days 3 and 21 post-operatively compared with time-matched controls. Amifostine treatment given 30 minutes prior to each fractionated radiation dose restored the wound tissue content of VEGF to the control level at all time-points

studied (Figure 19). Results from immunohistochemical studies also indicated that pre-operative single and fractionated irradiation significantly reduced wound tissue contents of VEGF on day 21 post-operatively compared to the control and Amifostine treatment restored wound tissue content of VEGF to the control level (Figure 17 and 18).

4.2 Effect of Pre-operative Single Dose and Fractionated Irradiation on Surgical Wound Healing and the Prophylactic Effect of the Radioprotector Amifostine.

Both pre-operative single dose and fractionated irradiation significantly impaired wound healing in our skin flap surgical wound model. This interpretation was based on significant reductions in WBS observed in the irradiated groups compared to the time-matched control. Compared to the control, the group that received a single dose had a WBS decrease of 60%, which is similar in magnitude to the decreases in WBS in skin incisional wounds observedby other investigators in rats using comparable single doses of radiation (56,79,153,155). Nearly all previous studies examining WBS in incisional wounds after irradiation used only single dose irradiation. Only one study examined WBS following fractionated irradiation to which we can compare our results. That study demonstrated a similar reduction in WBS following fractionated irradiation, but a linear incision wound was used, not a flap wound (94).

Archer et al. found a significant decrease in hydroxyproline in rat skin incisional wounds that were irradiated with a single 15 Gy dose immediately prior to wounding (9). Bernstein et al. measured wound collagen content in single dose irradiated guinea pig skin incision wounds and found a significant radiation induced decrease in type I collagen expression (25). Here, we extended this observation to pre-operative fractionated irradiation. In the present study, the significant decrease in WBS following

fractionated irradiation was also corroborated by a significant decrease in wound tissue hydroxyproline content, which is a marker for wound tissue collagen content. A decrease in wound collagen may, in part, contribute to impaired surgical wound healing as measured by a decrease in WBS (26). Also of note is a study by Devereux et al., that showed a significant decrease in new wound hydroxyproline content (using ^{3}H administered 20 h before sacrifice), but not in total wound hydroxyproline content in rat incisional skin wounds subjected to a single 20 Gy radiation dose (66). Thus, it is possible that in the current study, an even greater effect of irradiation could have been observed had we examined new wound hydroxyproline per se, instead of total wound hydroxyproline.

Prophylactic Amifostine treatment significantly attenuated the effect of pre-operative single dose and fractionated irradiation on WBS by 25% and 32%, respectively, compared with the corresponding control (Figures 7 and 8). This is the first demonstration that prophylactic Amifostine treatment is effective in prevention of radiation induced impaired surgical wound healing in skin.

4.3 Effect of Pre-operative Ionizing Radiation on Skin Flap Viability.

Skin flap viability in the control group measured 8 and 21 days post-operatively was 69% and 75% respectively (Table 1). Within the controls, skin flap viability measured 3 days post-operatively was slightly higher than when measured 8 and 21 days post-operatively, with a viability of 83%. The viability measurements taken on the third post-operative day most likely under-estimated the extent of skin necrosis, as it is generally accepted that an accurate measure of skin flap viability cannot be obtained until at least 7 days postoperatively.

Within each time period, single dose and fractionated irradiation with or without Amifostine pre-treatment did not result in any significant change in skin flap viability compared to the control. However, on day 21 post-operatively, the decrease in flap viability compared to control was 20%, 15% and 18% in the single dose, fractionated dose, and fractionated dose with Amifostine pre-treatment groups, respectively. Taking into account the variability of flap viability and the number of rats per group, it is possible that with a larger sample size, these differences could have reached statistical significance. At least one other study of irradiated rat skin flaps also found no significant reduction in skin flap viability after irradiation. In a study using a single 15 Gy radiation dose, Nall showed a non-significant 7% decrease in rat skin flap viability measured 7 days post-operatively (155). In contrast, Patterson demonstrated a reduction in viable flap length using a pig model subjected to single dose and fractionated irradiation (165).

In the present study, it is unclear why a greater trend towards significance in skin flap viability was observed on post-operative day 21 but not on post-operative day 8. It may have been due to the effects of the more slowly appearing effects of apoptotic cell death superimposed on the effects of necrosis, which were fixed by approximately 7 days post-operatively.

4.4 Mechanism of Pre-operative Radiation-Induced Impaired Surgical Wound Healing.

Previous studies of the effects of ionizing radiation on wound healing predominantly used single dose irradiation in linear incision or excisional wound models. Observations from these studies provided some insight into the mechanisms underlying radiation-induced impaired surgical wound healing. These observations included a

decrease in wound tissue inflammatory cell and fibroblast populations and a decreased amount of collagen production, or the production of poor quality collagen in irradiated wounds. In addition, some studies implicated a decrease in wound neovascularization, but others refuted this theory. Our findings discussed below support some of the theories and expand the current state of understanding of the pathophysiology of radiation-induced impaired surgical wound healing.

Preoperative single dose or fractionated radiation decreases wound tissue density and function of macrophages and fibroblasts and collagen synthesis, contributing to the pathogenesis of impaired wound healing.

It is a general consensus that during the inflammatory and proliferative stages of wound healing, the influx of macrophages and fibroblasts is crucial for normal wound healing (127). Macrophages clear the wound debris and secret growth factors such as monocyte chemoattractant protein-1 (MCP-1) that recruit more macrophages (88), and stimulate extracellular matrix remodelling, fibroblast proliferation, collagen synthesis by fibroblasts (TGF-β_1), and angiogenesis (VEGF)(29, 124, 239). Collagen synthesis by fibroblasts and neovascularization by angiogenesis play a central role is normal wound healing. Collagen produced by fibroblasts is the main determinant of tensile strength in a healing wound (131). Neovascularization provides blood supply which carries nutrients and oxygen to the healing wound (189, 224). Recently, the importance of fibroblasts for healing of radiated wounds was also demonstrated. Specifically, Ferguson et al. increased WBS in irradiated rat incisional wounds by injecting the wounds with autogenous fibroblasts (79).

Macrophages are also important for healing of irradiated wounds. This was

demonstrated by Galiano et al. who used charged beads to increase macrophage influx into irradiated rat incisional wounds, resulting in enhanced wound healing as measured by increase in WBS (83). However, the effect of preoperative irradiation on wound tissue content of macrophages required for wound healing has not been investigated. In the present study, we demonstrated that pre-operative single dose or fractionated irradiation reduced wound tissue density of macrophages in the rat skin. Specifically, it was observed that the wound tissue density of macrophages was significantly decreased in preoperative single dose and fractionated irradiation groups compared with the control on day 3 postoperatively, during the inflammatory phase of wound healing, when macrophages activity is normally at its peak (124, 203, 239). It is possible that the processes initiated by macrophages such as recruitment of more macrophages, secretion of growth factors, and stimulation of fibroblast proliferation and collagen production were adversely affected during and immediately after the inflammatory phase, contributing to the pathogenesis of impaired wound healing. Indeed, we observed that pre-operative single dose or fractionated irradiation caused a significant decrease in wound tissue fibroblast density assessed on days 3, 8 and 21 post-operatively (Figure 12), and wound tissue content of hydroxyproline and WBS assessed on day 14 post-operatively (Figure 9) compared with time-matched controls.

We observed an increase in wound tissue macrophage density in the single dose irradiation group on the eighth post-operative day. This increase may represent a radiation-induced delay of macrophage entry into the wound. Wang et al. noted a delayed infiltration of inflammatory cells in rat incisional wounds subjected to a single pre-operative radiation dose of 10 Gy (234) assessed 1, 3, 7 and 14 days post-operatively.

Stajic et al. described a similar delayed influx of inflammtory cells in excisional wounds in rats subjected to 7 Gy of total body irradiation 2 days prior to wounding (204). However, neither of the above studies quantified their findings, or made any specific reference to wound tissue macrophage density.

It is not clear how pre-operative irradiation caused a significant decrease in wound tissue macrophage and fibroblast density. The wound densities of macrophages and fibroblasts may be decreased due to the partial destruction of the local population of macrophages and fibroblasts via irradiation-induced necrotic cell death. With regard to macrophages, a proportion of the cells in the wound site will have been spared the effects of irradiation as they were recruited from the circulation at the time of wounding and were not present in the wound site at the time of irradiation. However, a significant proportion of macrophages involved in wound healing initially reside in the tissue adjacent to the wound site. Furthermore, in vitro, irradiated human fibroblasts were arrested in the G1 phase of the cell cycle, and were therefore unable to replicate in response to TGF-β1 signaling (183). Thus, it is possible that the decrease in wound tissue fibroblast density seen in the present study was due to inadequate fibroblast replication. In addition, it is possible that these cells were impeded from entering the wound site. While no mechanism of impaired cellular influx into irradiated wounds has been described, one can speculate that such a mechanism may involve changes in regional blood flow or vascular permeability, which could affect cell delivery to the wound site. Another possibility could involve changes in the ECM that can possibly impede the entry of macrophages or fibroblasts into an irradiated wound, or changes in the expression of the enzymes that facilitate cellular migration through the ECM to the

wound site. ECM changes following irradiation have been documented. These changes include the loss of hyaluronic acid, and the induction of tenascin, two ECM components considered important for cell migration (16). Taken together, these factors may account for the decrease in wound macrophage and fibroblast density.

The pathophysiologic importance of a decrease in wound tissue fibroblast density lies in the fact that fibroblasts produce collagen, the main determinant of tensile strength in a healing wound (131). As such, a decrease in fibroblast population or function, would likely be associated with decreased collagen production. The observed decrease in fibroblast density, and in wound collagen (as measured by wound tissue hydroxyproline content) were associated with wounds that were structurally weaker, and therefore would be more likely have wound healing complications. Wang et al. noted an association between deceased wound fibroblasts in dorsal incisions in rats following a single 10 Gy irradiation dose, and a decrease in the rate of wound healing. Unfortunately, Wang et al. did not quantify their findings with respect to wound fibroblast density (233).

In the present study, we documented quantitatively that pre-operative single dose or fractionated irradiation significantly reduced wound tissue fibroblast density on days 3, 8 and 21 post-operatively, compared with time-matched controls (Figure 12). In addition, since wound tissue fibroblast density was diminished at 3, 8 and 21 days post-operatively, we suggest that this decrease in wound fibroblast density was likely associated with decreased wound tissue content of hydroxyproline (Figure 9) and WBS (Figure 8) measured 14 days post-operatively.

In the present study, the decrease in wound tissue fibroblast density was not the only factor responsible for low collagen production as indicated by low wound tissue

content of hydroxyproline (Figure 9). The decreased WBS observed, despite seemingly plentiful wound tissue TGF-β_1 expression, may also be related radiation-induced cellular dysfunction. It is possible that the cellular processes of TGF-β_1 synthesis may be damaged by irradiation, resulting in structurally altered TGF-β_1 molecules that may be functionally inactive and unable to activate the TGF-β_1 receptors. Alternatively, the process of TGF-β_1 activation from its latent form may somehow be disrupted by irradiation, resulting in TGF-β_1 molecules being present in the wound, but not functioning as active growth factors. The limited improvements in WBS in irradiated animals models treated with exogenous TGF-β_1 may be related to the above hypotheses. A third explanation for impaired wound healing despite upregulation of TGF-β_1 protein expression is a possible radiation-induced decrease in activation of the TGF-β_1 receptor system, making it unable to respond to TGF-β_1. To date, these explanations are hypothetical, as no studies on these issues have been published.

Previous animal studies observed that single dose irradiation can up-regulate the expression of TGF-β_1 in non-wounded skin (137, 138, 176, 177). Fractionated irradiation has also been shown to up-regulate TGF-β_1 expression in the intact rat intestine (122). These observations are important in light of recent interest in TGF-β_1 as a therapy for radiation-induced impaired wound healing (24,56,155). Given that there is no downregulation of TGF-β_1 in irradiated wound tissue, the justification for adding exogenous TGF-β_1 to irradiated wounds for elimination of radiation-induced impairment of wound healing is that the endogenous TGF-β_1 is not effective or the fibroblast density in irradiated wounds is too diminished, or the fibroblasts are less sensitive to TGF-β_1.

These circumstances would require a higher level of TGF-β_1 for stimulation of collagen production. In the present study, pre-operative single dose or fractionated irradiation, in general, did not reduce wound tissue contents of TGF-β_1 on days 3, 8 and 21 post-operatively compared to time matched controls. However, the wound tissue TGF-β_1 content in the pre-operative fractionated irradiation group and the pre-operative fractionated irradiation group with Amifostine pre-treatment was lower than the control on day 21 post-operatively.

Pre-operative Irradiation Decreases Wound Tissue Density of Capillaries:

In the present study, significant reductions in capillary density were observed at all biopsy times following both single dose and fractionated irradiation (Figures 13 and 14). A substantial decrease in wound capillary density may cause a decrease in wound blood flow, a notion currently under investigation in our laboratory using the radioactive microsphere technique. Given the importance of re-establishing wound blood flow by neovascularization in normal wound healing (224), a substantial decrease in capillary density may have a significant negative impact on wound healing. In addition, the timing of the observed decreases in capillary density was important. On the eighth post-operative day, when wound angiogenesis was expected to be at its peak, significant decreases in capillary density were noted in both the single and fractionated irradiated groups. Other studies also reported changes in neovascularization and vascular density after irradiation in rabbit and rat models (68, 72).

There are two possibilities which may explain the observation that pre-operative irradiation caused a decrease in wound tissue capillary density in the present experiment. Pre-operative irradiation may have cause a reduction in macrophage density and

endothelial function in wound tissue, resulting in a decrease in VEGF synthesis or release which was essential for stimulation of angiogenesis (neovascularization) in the wound tissue. In addition, impaired endothelial function induced by pre-operative irradiation may also reduce the ability of these cells to respond (i.e proliferate) to the angiogenic stimulus of any VEGF present in the wound.

In contrast to our findings of decreased wound tissue VEGF expression caused by pre-operative irradiation, there is some evidence in the literature suggesting that ionizing radiation may up-regulate VEGF expression (6,85,95,227). However, there are substantial methodological differences in these studies. These studies were done using in-vitro tumor cell culture, normal quiescent tissues or ultraviolet irradiation. As well, one study examined VEGF production by only one cell type, fibroblasts, out of the many cell types that can produce it. Our study is different due to our use of an in-vivo wound healing model. In addition, there is some evidence to support our finding of radiation-induced VEGF down-regulation. One recent study using cultured osteoblasts exposed to single radiation doses ranging from 4 - 8 Gy found that irradiation decreased the amount of VEGF present in the in-vitro sample measured 9 days after irradiation (73). An extensive literature search failed to identify a single study using an in vivo wound healing model to investigate the effects of ionizing radiation on VEGF expression in wounded or unwounded skin. Our study is the first to report these effects.

4.5 Effect and Mechanism of Amifostine in Prevention of Radiation-Induced Impaired Surgical Wound Healing

Animal studies performed by other investigators demonstrated the ability of Amifostine to protect tissues such as gut, testis, and lung in mice (149), long bones in rats

(60), and mouse buccal mucosa (62) and hair follicles (87). In clinical studies, Amifostine was effective at protecting bone marrow (51), pelvic organs, (130), salivary glands (144) and esophageal tissue (217). The current study is the first to show that Amifostine, when given 30 minutes prior to single dose or fractionated doses of radiation, can significantly improve the tensile strength of healing skin wounds measures 14 days post-operatively.

Amifostine, a free-radical scavenging agent, is known to protect normal tissues from the damaging effects of ionizing radiation (39). Yuhas et al., using single dose x-ray irradiation of mice carrying implanted mammary tumors, demonstrated that the radioprotective effect of Amifostine is limited to normal tissue, and tumor cells are not protected (243). The mechanism of Amifostine action is via the uptake and conversion of the pro-drug WR-2721 to the active metabolite WR-1065. The uptake and conversion are dependant upon the presence of alkaline phosphatase and a local pH > 6.6. These conditions are present in normal cells. In general, tumor tissues are relatively deficient in alkaline phosphatase activity, and the extracellular pH of tumor tissues is generally less than 6.6. These properties of tumor tissues limit the uptake and radioprotective effects of Amifostine on tumor tissues. In normal cells, the active form of Amifostine (WR 1065) then acts as a free-radical scavenger and as a hydrogen donor (see Figure 2, page 34) and protects cellular DNA from the adverse effects of direct macromolecule injury (hydrogen donation) and from the indirect effects of radiation-induced free radicals (free radical scavenging). These adverse effects include cell death and apoptosis.

In the current study, the radioprotective effects of Amifostine were evident as Amifostine appeared to limit the decrease in wound tissue densities of capillaries (Figure

14) and fibroblasts (Figure 12) compared to the irradiated group without Amifostine pre-treatment. In addition to cell population, cell function may also have been protected. Preservation of fibroblast function was reflected in increased wound tissue hydroxyproline content, and subsequently in higher WBS in the Amifostine pre-treated group compared to the irradiated group without Amifostine pre-treatment (Figures 7,8 and 9). Protection of capillary function resulted in the ability of endothelial cells to proliferate in response to VEGF, resulting in a greater wound tissue capillary density than that observed in the irradiated group without Amifostine pre-treatment (Figure 14). It is currently not clear why Amifostine pre-treatment did not provide a protective effect on wound tissue macrophage density. It is possible that Amifostine pre-treatment provided some degree of preservation of macrophage function, allowing macrophages to produce some TGF-$\beta_1$and VEGF, and contribute to the wound healing process.

It is of interest to point out that the capillary density was significantly higher in the irradiated group with prophylactic Amifostine pre-treatment than it was in the non-irradiated control wound tissue (Figure 14). This observation led us to speculate that Amifostine pre-treatment protected endothelial cells from radiation injury and at the same time may also have had some angiogenic effects. Other investigators have demonstrated the angiogenic effect of Amifostine using a chick embryo model (104, 174).

5.0 Conclusions and Future Studies

5.1 Conclusions:

The following conclusions can be made based on the observations obtained from the present studies.

1. Pre-operative single dose and fractionated irradiation impaired surgical skin wound healing in rats as indicated by a significant decrease in WBS and wound tissue hydroxyproline content assessed 14 days post-operatively compared to non-irradiated, time matched controls.
2. Pre-operative single dose and fractionated irradiation caused significant reductions in wound tissue densities of macrophages (3 days post-operatively), fibroblasts and capillaries (3, 8, and 21 days post-operatively) compared to non-irradiated, time matched controls.
3. Pre-operative single dose and fractionated irradiation significantly down-regulated wound tissue protein expression of VEGF assessed 3, 8 and 21 days post-operatively compared to non-irradiated, time matched controls.
4. The pathophysiology of pre-operative radiation-induced impaired surgical skin wound healing may be in part attributed to the significant decreases in wound tissue densities of macrophages, fibroblasts and capillaries, and to a significant down-regulation of VEGF in irradiated wounds.
5. The radioprotective agent Amifostine administered by intraperitoneal injection 30 min prior to single dose and fractionated irradiation significantly attenuated the adverse effects of pre-operative irradiation on WBS, wound tissue densities of fibroblasts and capillaries, and wound tissue content of hydroxyproline and VEGF compared to irradiated groups without Amifostine pre-treatment.

5.2 Future Studies:

Previous efforts to treat impaired wound healing in irradiated skin have focused on the use of several modalities including the application of exogenous growth factors, the use of implanted fibroblasts, or exposure to hyperbaric oxygen. Our data suggest that prevention of radiation-induced impaired surgical wound healing is a viable alternative to treating the wound complications after they are established. The potential advantages of preventing these wound complications range from saving a significant amount of money in terms of health care costs, to sparing cancer patients the pain and suffering of prolonged hospitalization, long term wound care, and secondary operative procedures.

Our future studies in the field of radiation-induced impaired surgical wound healing will continue to focus on the prevention rather than the treatment of this frustrating clinical problem. It is hoped that several potential improvements can be made to Amifostine to reduce the undesirable side effects and increase its clinical utility. It may be possible to make Amifostine more potent, thus requiring a smaller dose. In addition, it may be possible to reduce the renal toxicity and hypotensive side effects, making the use of Amifostine less dangerous. Finally, it may be possible to produce a formulation of Amifostine that can be applied topically. A topical formulation would deliver the drug directly to the dermis, where it would have its maximal benefit in terms of preventing the surgical wound healing complication, and would avoid the frequently cited drawback of Amifostine, which is the need for invasive and painful intravenous access.

With respect to the timing of Amifostine administration, it may be useful to investigate shorter time intervals between injection and irradiation. We found no

difference in Amifostine protection when given 20 minutes or 30 minutes prior to irradiation. However, the literature is unclear on the best time to administer the drug, and testing a shorter interval such as 5-10 minutes may be informative.

With regard to the profile of wound growth factors, several future studies may be important. Given the alterations in wound tissue protein expression observed in the current study, assessment of both TGF-β_1 and VEGF gene transcription should be done using northern blot or RT-PCR studies. This would provide insight into the molecular level at which irradiation exerts its effect on the wound growth factor profile. Preliminary efforts to carry out these studies are underway in our lab.

The current study was limited in the scope of growth factors that were examined. The individual and combined roles of the myriad of known growth factors is only slowly emerging. It is therefore reasonable to examine other growth factors in the manner in which we examined those in the current study. In addition to TGF-β_1 and VEGF, there are several other growth factors that could be studied in the irradiated wound. These growth factors include TGF-β_2 and TGF-β_3, which are also known to be important in cutaneous wound healing (136). In addition, the effect of pre-operative irradiation on the wound tissue profile of PDGF-BB, a growth factor known to be important in the recruitment of macrophages and fibroblasts, can be studied. Furthermore, the effect of irradiation on growth factors influencing angiogenesis, such as bFGF can be investigated.

With respect to wound angiogenesis and blood flow, we will extend our current finding of decreased wound tissue capillary density by doing more functionally oriented studies of wound tissue blood flow. In fact, we have begun doing these blood flow studies using the radioactive micro sphere technique.

Further study of fibroblast function and response to Amifostine prophylaxis and growth factor stimulation/deprivation will also be a large part of our future work, since the fibroblast plays such a central role in the orchestration of wound healing.

6.0 References

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