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INTRADERMAL INJECTION OF AUTOLOGOUS DERMAL FIBROBLASTS
IMPROVES WOUND HEALING IN IRRADIATED SKIN

by

Peter C. Ferguson

A thesis submitted in conformity with the requirements for the degree of Master of
Science
Graduate Department of Institute of Medical Science
University of Toronto

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This thesis is dedicated to my wife Melinda, 

my daughter Erica, and my son Kyle. 

You are my strength, and my motivation to strive for excellence.
INTRADERMAL INJECTION OF AUTOLOGOUS DERMAL FIBROBLASTS IMPROVES WOUND HEALING IN IRRADIATED SKIN

by Peter C. Ferguson, Master of Science Thesis, 1998

Graduate Department of Institute of Medical Science
University of Toronto

Abstract

The combination of radiotherapy and surgical resection is effective in the management of several solid tumours. Despite its well-recognized benefits, the use of radiotherapy prior to surgery is associated with a high incidence of significant surgical wound healing complications. Radiation-induced damage to dermal fibroblasts has been proposed as an important cause. In this study, we investigate the effect of intradermal injection of autologous dermal fibroblasts in a rat model of combined preoperative irradiation and surgical wounding. Our results demonstrate that injection of normal, unirradiated fibroblasts significantly improves healing of the irradiated surgical wound. These cells are likely better able to respond to the proliferative, migratory and synthetic demands of the wound healing environment, as injection of irradiated cells has an equivalent effect on healing as injection of medium alone. Implantation of autologous fibroblasts may be a viable clinical option used to reduce the incidence of wound healing complications in surgical oncology patients undergoing preoperative radiotherapy.
I owe an enormous debt of gratitude to one man - Dr. Robert Bell. He stands alone as a supervisor, mentor and role model. He has instilled in me the enthusiasm, dedication and work ethic required to become both a surgeon and a scientist.

I would like to thank many others for their intellectual, technical and emotional support in bringing this work to fruition. To Erin, Jas, Jay, Jennifer, Chris, Maria, Leslie, Aileen and Krista - I am truly appreciative of your guidance and assistance.
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### ABBREVIATIONS

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>α-Minimal Essential Medium</td>
</tr>
<tr>
<td>CIP-1</td>
<td>Cyclin dependent kinase Inhibitor Protein</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic Acid</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor - Alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor - Beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
</tr>
<tr>
<td>UTS</td>
<td>Ultimate Tensile Strength</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WAF-1</td>
<td>Wild type p53 Activated Fragment</td>
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</tbody>
</table>
INTRODUCTION

Combined cancer treatment using both irradiation and surgery has, over the past 25 years, changed the clinical approach to the local management of several different solid tumors. Combined therapy has resulted in improved functional and/or cosmetic outcomes in breast carcinoma, soft tissue sarcoma, rectal carcinoma and head and neck cancers. The benefits of combining radiation and surgery treatments to preserve function is perhaps best demonstrated by a comparison of historical and current protocols for extremity soft tissue sarcoma treatment. Prior to the use of combined therapy, radical resection of entire limb muscle compartments or proximal extremity amputation were the accepted standard treatments for soft tissue sarcoma - it was recognized that conservative local tumor excision was complicated by a very high risk of local tumor recurrence. Current protocols employing irradiation and conservative tumor resection permit the preservation of functional muscle, nerve and vascular tissue with local tumor control rates equal to or better than radical surgery alone (Suit et al., 1981; Tepper et al., 1985; Bell et al., 1989; Bell et al., 1991; Suit et al., 1994; Wilson et al., 1994; Spiro et al., 1995; Suit et al., 1996; Ngan, 1997).

An important question for combined management is whether to administer the radiation pre- or postoperatively. Although there is no difference in terms of tumor control between the two (Nielsen et al., 1991; Sadoski et al., 1993; Wilson et al., 1994; Spiro et al., 1995; Cheng et al., 1996), most protocols for combined management call for initial surgical resection of the tumor followed by irradiation of the tumor bed following completion of wound healing. There are two major potential advantages to using irradiation prior to, rather than after surgery. First, irradiating the lesion in situ prior to
surgery may permit the radiation oncologist to reduce the total radiation dose (Suit et al., 1981; Tepper et al., 1985; Suit et al., 1994; Wilson et al., 1994; Spiro et al., 1995; Suit et al., 1996) and thereby decrease the late effects of irradiation on normal tissues surrounding the tumour (Withers, 1992). Current protocols for combined management of soft tissue sarcoma, for example, call for a total dose of 50 Gy when radiation is administered prior to surgery and 66 Gy when postoperative treatment is used.

A second therapeutic advantage for preoperative radiation relates to the volume of tissue treated. When irradiating the tumour bed following surgery, it is necessary to treat a considerable volume of normal tissue that has been potentially contaminated by surgical dissection around the tumour. It has been shown that if radiation is administered prior to the surgical dissection of the tumour, substantially less normal tissue is treated (Nielsen et al., 1991). This reduction in the volume of tissue treated is also important in decreasing the late effects of radiation on normal tissues (Tokarek et al., 1994). The use of irradiation prior to surgery may therefore decrease the late complications of combined management both by decreasing the total radiation dose and decreasing the volume of tissue treated with radiation, which may contribute to improved patient function (Karasek et al., 1992).

The major detriment to using radiation prior to surgery is the risk of wound healing complications. This problem is particularly troublesome in the management of extremity soft tissue sarcoma. Surgery in a previously irradiated field has been reported to result in complications in up to one half of patients (Skibber et al., 1987; Quill et al., 1990; Bujko et al., 1993; Saddegh et al., 1993; Peat et al., 1994; Cheng et al., 1996). Approximately half of these complications are severe enough to warrant additional surgical procedures, and some may even result in extremity amputation (Bujko et al.,
1993; Saddegh et al., 1993; Peat et al., 1994). The occurrence of a wound complication after extremity sarcoma surgery is also associated with poor functional outcome (Bell et al., 1991). In addition to the pain and inconvenience suffered by patients, wound healing complications following cancer surgery are remarkably expensive for the health care system.

Impaired wound healing following preoperative irradiation is also a clinical concern for treatment of malignancies other than soft tissue sarcoma. Patients with rectal (Whiting et al., 1993), breast (Alund et al., 1977; Danoff et al., 1985; Badr el Din et al., 1989; Guenier et al., 1990; Sauter et al., 1993) and head and neck (Donald, 1978; Griffin et al., 1979; Mantravadi et al., 1981; Girod et al., 1995; Hom et al., 1995) cancers appear to be at an increased risk for wound healing complications following preoperative irradiation. Any measures likely to improve wound healing in previously irradiated tissue could potentially be applied to a wide range of cancer patients. This study aims to achieve this by addressing the radiation-induced impairment of parenchymal cell function in dermal tissue.
LITERATURE REVIEW

Wound Healing

The process of dermal wound healing is a complex and dynamic interaction between soluble growth factors, extracellular matrix molecules and several cellular components. The ultimate result of this process in adult mammals is the formation of a collagenous scar, the function of which is to maintain dermal integrity and to withstand forces applied to the skin. The sequence of cellular and molecular events can be arbitrarily divided into three overlapping phases: inflammation, tissue repair and tissue remodeling (Clark, 1996).

Inflammation

The inflammatory phase begins with tissue injury, in this case, surgical wounding. The immediate processes of this phase involve interaction between damaged endothelium, platelets and the coagulation cascade. Exposure of subendothelial collagen in damaged vessels allows for platelet adhesion and aggregation, which contribute directly to hemostasis and also initiate the coagulation cascade. Aggregated platelets become activated, resulting in discharge of α-granules, which contain the proteins fibrinogen and fibronectin. Further platelets directly adhere to these proteins via the αIIbβ3 integrin receptor (Yamada et al., 1996). Circulating and platelet-derived fibrinogen is converted to fibrin via the coagulation pathway. This fibrin clot establishes hemostasis, and also plays a crucial function as the provisional matrix of wound healing, facilitating influx of later cellular components (Yamada et al., 1996), specifically monocytes (Ciano et al., 1986) and fibroblasts (Grinnell et al., 1980; Brown et al., 1993). A second major function of activated platelets is the liberation of stored growth factors essential for wound healing,
most notably transforming growth factor - beta (TGF-\(\beta\)) (Sporn et al., 1992) and platelet derived growth factor (PDGF) (Ross et al., 1990).

Concomitant with extravasation of blood products, neutrophils are the first leukocytes to arrive at the site of wounding, increasing to a peak at 24-48 hours (Riches, 1996). These cells respond to numerous chemoattractants released by platelets, the coagulation pathway and damaged tissue. Activated neutrophils degrade tissue and destroy invading bacteria via phagocytosis and liberation of various proteolytic enzymes. In the absence of a continuous source of activation, such as persistent bacterial infection, neutrophils become inactivated and sloughed or consumed by tissue macrophages. Despite this seemingly critical role, animals rendered neutropenic have normal wound healing (Simpson et al., 1972), perhaps due to an overlap in their function with cells of mononuclear lineage.

In response to specific chemoattractants, circulating blood monocytes gain access to the wound through injured and intact vascular endothelium. After undergoing transformation to mature tissue macrophages, perhaps in response to contact with the provisional matrix (Riches, 1996), these cells carry out wound decontamination similar to neutrophils. However, by far their most important function is the continued secretion of growth factors that direct much of the remainder of the wound healing process (Riches, 1996). Animals rendered deficient in functioning macrophages demonstrate marked delay in their wound healing (Leibovich et al., 1975).

The end of the inflammatory phase, therefore, is marked by hemostasis, the presence of a provisional matrix of fibrin and fibronectin, sterilization of the tissue and the
presence of growth factor-secreting macrophages, marking the transition into the phase of tissue repair.

**Tissue Repair**

This phase begins at approximately the third day after wounding and involves the initiation of processes that restore the appearance and function of the injured skin. Re-epithelialization, angiogenesis and fibroplasia occur via the respective actions of keratinocytes, microvascular endothelial cells and fibroblasts.

Restoration of the epithelial cover is essential to prevent tissue dessication and subsequent patient morbidity. This is more crucial in excisional wounds or full thickness injuries such as burns, but the principles also apply to surgical incisions. In response to tissue injury, and perhaps to specific epidermal growth factors such as transforming growth factor-α (TGF-α) (Clark, 1996), basal keratinocytes at the edge of the wound detach from the underlying basement membrane and proliferate actively, usually commencing on the second post-wounding day. A monolayer of cells moves across the underlying dermis until epithelial integrity is re-established. These cells then re-attach to newly synthesized basement membrane and revert to their previous, more static phenotype. In incisional wounds, this process is rather short, and epithelial coverage of the wound is present by the fourth day post-wounding.

The processes of angiogenesis and fibroplasia combine to form the essential repair tissue of dermal wounding, granulation tissue. Angiogenesis is necessary to provide oxygen and nutrients to newly forming dermal tissue. Once again, this process may be more important in wounds with large dermal defects rather than incisional wounds. However, with the presence of large volumes of dead space that must also be filled by
granulation tissue, neovascularization is essential. At the site of tissue injury, macrophages present from the inflammatory phase release factors that stimulate microvascular endothelial cells to secrete proteases that digest the underlying basement membrane. This permits the migration of endothelial cells from the parent vasculature into the newly forming granulation tissue, in response to endothelial cell chemotaxins such as fibroblast growth factor (FGF) and the fibronectin present in the provisional matrix (Madri et al., 1996). The endothelial cells in this migrating capillary loop are replaced by proliferation of more proximal cells, stimulated by the important vascular endothelial growth factor (VEGF), among others (Clark, 1996). Downregulation of these various stimuli cause cessation of angiogenesis and involution of some blood vessels once the tissue is adequately vascularized.

Formation of the other component of granulation tissue, the fibrous stroma, is undertaken chiefly by dermal fibroblasts. A complex “dynamic reciprocity” exists (Clark, 1996) between these cells and their environment. They not only synthesize extracellular matrix in response to soluble factors secreted by macrophages and platelets, but also undergo phenotype modulation on contact with existing extracellular matrix, and release growth factors that act in paracrine and autocrine fashions. The actions of fibroblasts are ultimately responsible for restoring structure and function to the injured dermis.

Fibroblasts exhibit several population phenotypes throughout the course of dermal wound healing. Whether these represent changes in individual cells or temporal domination by cells of different phenotypes is not certain. Initially, quiescent fibroblasts at the wound margin respond to several products of the inflammatory phase which promote replication and migration. Cytokines mitogenic for fibroblasts include PDGF (Heldin et
fibroblast growth factor (FGF) (Abraham et al., 1996) and to a lesser extent TGF-β. Replicating fibroblasts are stimulated to migrate into the wound, generally commencing on the fourth day. These fibroblasts migrate from the deep subcutaneous tissue and the adjacent dermis (Dale et al., 1997). PDGF and especially TGF-β are potent chemotaxins for fibroblasts (Clark, 1996; Roberts et al., 1996). These factors, liberated by platelets and macrophages, are also trapped in the provisional extracellular matrix (Nathan et al., 1991; Yamada et al., 1996), further increasing their local concentration. They may be released to carry out their biological effects upon degradation of the matrix.

Migration is further facilitated by interactions between fibroblasts and the extracellular matrix. Both TGF-β and PDGF (Clark, 1996) up-regulate the expression of integrins, the dimeric surface receptors that directly link the extracellular matrix to the cell’s cytoskeleton. Some of these receptors bind to molecules expressing the RGD (Arginine-Glycine-Aspartic acid) tripeptide sequence. Fibronectin, present in the provisional matrix from the plasma infiltrate and also secreted by several wound cells, contains this sequence. Fibronectin, through direct binding to migrating fibroblasts, likely serves as a scaffold to promote cellular infiltration into the provisional matrix (Knox et al., 1986). Fibrin present in the provisional matrix also contains an RGD sequence and certain integrins are specific for this molecule. Fibrin also permits TGF-β to promote collagen synthesis in tissue fibroblasts (Clark et al., 1995), and facilitates upregulation of integrin expression. The provisional matrix of fibrin promotes fibroblast proliferation, migration and enhances synthesis of collagen, all features of fibroblasts found in granulation tissue (Brown et al., 1993; Clark et al., 1995). The glycosaminoglycan hyaluronic acid is also plentiful in provisional matrix, and increases in early granulation tissue. This molecule,
whenhydrated,maypermitcellrecruitment(Toole,1981)andmaydirectlypromotecell
motilitybyinteractionwithspecifichyaluronanreceptors. Theearlyprovisionalmatrixof
thehealingwound,therefore,exhibitsseveralfeaturesthatpromoteinfluxofadjacent
fibroblasts,whothemselvesdisplayamigratoryphenotypefacilitatedbynumerous
chemotacticelements.

Oncepresent,thepheno typeoffibroblastschanges tooneof synthesis of
extracellularmatrixproteins. Initiallyfibroblastsproduce largequantitiesoffibronectin,
presumablytoincrease theabilityofmorecells togainaccess tothewound. This is soon
supplanted,however,byaugmented synthesis ofcollagens, mostnotablytypesIandIII.
These cells have abundant rough endoplasmic reticulumwhich has been shown to be
manufacturingtheprocollagen precursorsofmature collagen(Welchetal.,1990). Themain
stimulus for collagen synthesis is the growth factor TGF-β(Ignotzetal.,1986;
Roberts et al., 1986; Clark et al., 1995). TGF-βleads toincreased promotoractivity in the
procollagen genes(Inagakietal., 1994; Chungetal., 1996), resulting in increased
procollagenmRNA(Kaharietal., 1990; Reed et al., 1994) andincreasedmaturecollagen.
Cellsinthedeeplayersofgranulationtissuearetthe firsttodemonstrateexpression of the
alpha 1(I)procollagen gene(Scharffetter et al., 1989). Furthermore, TGF-βinduces
 suppressionofcollagenasegeneexpression(Mauviel et al., 1996)and stimulation of
 productionof tissue inhibitors of metalloproteinases (TIMPs)(Bigget al., 1996). The
existingfibrin provisional matrix facilitates this upregulation of collagen secretion(Gillery
et al., 1992; Clark et al., 1995; Tuan et al., 1996). Initially, themain collagen synthesized
is type III, afibrillarmolecule normally expressedinembryonic skin and scant in normal
adult dermis. Aftera delay of 5 days, the synthesis oftype I collagen increases. Both the
collagen mRNA expression and secretion of procollagen molecules are maximal between days 7 and 14 post-wounding. These times are comparable between humans (Springfield, 1993) and rats (Levenson et al., 1965). The ordered synthesis of fibronectin, type III then type I collagen corresponds with an increase in wound strength continued during tissue remodeling.

The major product synthesized by granulation tissue fibroblasts is type I collagen. Type I collagen is a fibrillar heterotrimer, with the three chains arranged in helical fashion. A repeating amino acid sequence of Glycine-X-Y is prominent, with X often representing proline and Y hydroxyproline. Lysine and hydroxylysine are also frequently found in this amino acid sequence. Glycine is required for the tight triple-helical structure and mutations that change this order are often associated with a highly unstable molecule. The imino acid proline stabilizes the backbone of each of the chains. Hydroxyproline is responsible for establishing hydrogen bonds between chains, increasing the stability of this linear molecule. After cleavage of the carboxy- and amino-terminal non-helical domains in the extracellular space, assembly of the mature collagen molecules into fibrils takes place. Covalent cross-links between different collagen molecules at the hydroxylysine residues contribute to the overall strength of this construct.

Not only do the thickness and degree of cross-linkage in the collagen bundles contribute to the strength of the healing wound, but their orientation is also essential to resist applied forces (Doillon et al., 1985; Clark, 1993; Clark, 1996). Myofibroblasts, a specialized phenotype of fibroblast (Gabbiani et al., 1971; Gabbiani et al., 1976), are involved in wound contraction and alignment of synthesized collagen bundles. Whereas many tissue repair fibroblasts undergo apoptosis after collagen synthesis is down-regulated
(Clark, 1996), a certain subset persist. This final fibroblast phenotype has many features of smooth muscle cells, including expression of α-smooth muscle actin (Gabbiani et al., 1981; Desmouliere et al., 1992) in its cytoplasm. This abundant actin along the cytoplasmic face of the cell membrane links to the extracellular matrix via integrin receptors. Fibronectin, which has binding sites both for integrins and type I collagen, is a key link in establishing a complex cell-matrix-cell network. Contractile forces generated intracellularly are thus transmitted to the matrix and other cells, leading to contraction of the entire wound (Bell et al., 1979; Bell et al., 1983). These spindle-shaped myofibroblasts are in fact aligned along the lines of contraction, a feature not seen with other cells in the healing wound (Clark, 1993).

The tissue repair phase, therefore, encompasses integrated neovascularization, re-establishment of epithelial cover, and synthesis of abundant type I collagen that is contracted by myofibroblasts. This all takes place by 10-14 days after wounding. Thereafter, remodeling is responsible for the end product of healing - the mature scar.

**Tissue Remodeling**

The process of remodeling encompasses gradual alterations in the extracellular matrix designed to maximize the mechanical performance of the scar. In fact, remodeling is undertaken at the periphery of a large wound at the same time granulation tissue formation is taking place in the wound center. Alterations in the composition and arrangement of the extracellular matrix both take place. Accompanying these changes is a slow reduction in the cellularity, resulting in a relatively hypocellular dermis in the area of the wound.
Tissue remodeling takes place over several months to up to two years as the mass of newly synthesized collagen is converted to a mature scar. This process depends on continued degradation and synthesis of collagen by residual fibroblasts. The signal to undertake this continuous revision is not known. Non-specific proteinases and matrix metalloproteinases specific for different molecules in the extracellular matrix are secreted by fibroblasts leading to matrix breakdown. This process is retarded by the presence of TIMPs, and reversed by synthesis of new collagen and other molecules. Therefore, the remodeling process is a delicate balance, any alteration of which can lead to complications of wound healing.

The processes described above contribute to create a scar that is able to carry out functions of normal dermis. Like any collagen-rich tissue, a principal function of dermis is to resist forces applied to the tissue, either external or internal. The synthesis and remodeling of dermal collagen correlates with increasing strength of the healing wound. Specifically, the strength reflects the amount and thickness of collagen fibers, the degree of cross-linkage between fibers, and the orientation of these bundles.

In the immediate post-wounding period until approximately 5-7 days after wounding, the extracellular matrix is composed mainly of fibronectin and hyaluronic acid, neither of which impart significant strength to the healing wound. Between 7 and 14 days post-wounding, the synthesis of collagen is at its maximum. Therefore, there is a rapid increase in wound strength over this time as collagen is assembled. This rapid gain persists for up to four weeks after incision (Springfield, 1993). The final strength is not reached until several months after wounding, and subtle changes in wound matrix composition may take place up to two years later. Despite this formation of a scar rich in type I collagen
matrix, the final strength is still inferior to that of unwounded dermis, reaching a maximum of approximately 70-80% (Levenson et al., 1965; Springfield, 1993).

In summary, the process of wound healing has been shown to be composed of a multitude of complex interactions between various cell types, assorted extracellular matrix molecules and several soluble growth factors. Although the final product can adequately carry out the function of the original tissue, it is still mechanically inferior. Any alteration of these interactions may lead to a tissue that is significantly less able to maintain the integrity of the original dermis, potentially to the detriment of the wounded organism.

**Soft Tissue Mechanics**

The structure of the collagen molecule is designed to resist tensile forces. Cord-like tendons and ligaments have many parallel collagen fibers, and thus they are uniquely designed to withstand applied tensile forces in the direction of those fibers. Dermis has abundant collagen fibers as well, but they are less well organized, with the fibers in multiple directions. For this reason, dermis is able to resist tensile forces applied from many directions. Furthermore, the behaviour of these tissues under tensile loading depends on the rapidity that the force is applied. In other words, they demonstrate viscoelastic behaviour (Mow et al., 1994).

As a collagenous tissue is stretched in a single direction (uniaxial tension) at a constant rate, a typical force-displacement curve for that tissue can be obtained. With the initial displacement, there is minimal force applied. This "toe region" is where uncrimping of collagen molecules takes place and is the initial flat section of the force-displacement curve (Figure 1). When the collagen molecules are fully extended, a given displacement is associated with a greater increase in the applied force. This exponential appearance is an
idealized representation of the force-displacement curve for a collagenous tissue. Eventually the applied force exceeds that which the collagenous matrix can withstand, and the tissue fractures. The applied force at this point of failure is referred to as the breaking load. This is a structural property, depending on the geometry and cross-sectional area of the sample as well as the arrangement and structure of the collagen molecules in the tissue.

For each point on the force-displacement curve, corresponding values for the material properties of stress and strain can be determined. Strain is calculated by dividing the amount of elongation (displacement) by the initial length, and is dimensionless. Stress is the force normalized to the cross-sectional area of the sample, and is expressed in pascals (Pa). Stress refers to the internal force generated to resist the applied force, and depends on the fine architecture of the collagen matrix. This is a true material property, and its use eliminates bias when comparing multiple tissues or samples. From the force-displacement curve, therefore, a corresponding stress-strain curve can be determined (Figure 2).

Similar to breaking load, the stress at which the material fails is referred to as the ultimate tensile strength (UTS). It depends to a large extent on the type, thickness and degree of cross-linkage of the collagen fibers. In the final stages of tensile loading, prior to failure, the curve becomes linear. The slope of this linear region is referred to as the modulus of elasticity, and is a measure of the stiffness of the material, expressed in Pa. A stiffer material will undergo less deformation with a given stress than a more elastic one. The orientation of collagen fibers are an important determinant of the elastic modulus - tissues with fibers oriented parallel to the direction of loading tend to be stiffer than those with fibers less well organized. Finally, the area under the stress-strain curve up to the
point of failure is referred to as the toughness of the material, which is a measure of the amount of energy absorbed prior to failure. The toughness depends to some extent on all of the above features of the extracellular matrix.

The process of dermal wound healing described earlier re-establishes the integrity of the injured extracellular matrix both anatomically and functionally. This process is not perfect as the final scar fails to achieve the biomechanical parameters of unwounded skin. These parameters are generally adequate, however, to withstand normal physiologic stresses. Should physiologic loading surpass any of these values (ie. applied load or energy), then biomechanical failure of the tissue can result and manifest as wound dehiscence.
Figure 1 - Typical force-displacement curve obtained from stretching a soft collagenous tissue to failure in uniaxial tension. The maximum force applied prior to failure is the breaking load.
Figure 2 - Typical stress-strain curve obtained from stretching a soft collagenous tissue to failure in uniaxial tension. The maximal stress generated in the tissue prior to failure corresponds to the breaking load/the cross sectional area of the tissue at failure, and is called the ultimate tensile strength (UTS). The modulus of elasticity (elastic modulus, Young’s modulus) is the maximal slope of the linear portion of the stress strain curve prior to failure. The toughness, or energy absorbed prior to fracture, is obtained by calculating the area under the stress-strain curve prior to failure.
Radiation Effects on Cells and Tissues

Radiotherapy has proven to be a valuable form of adjuvant treatment in the surgical management of soft tissue sarcomas. Although ionizing radiation inflicts physical damage on malignant tissue, this is often at the expense of adjacent normal tissue. This may result in tissue dysfunction, which, in the case of the dermis, may be manifest as impaired wound healing. An optimal radiotherapy protocol imparts maximal damage to the tumour while affecting normal tissue to a minimal extent.

Ionizing radiation can either be in the form of particle beams (electrons, neutrons or protons) or high-energy electromagnetic radiation (x-rays, gamma-rays). In the case of electromagnetic radiation, energy is carried in bundles called photons. Gamma-ray photons are produced by the decay of radioactive nuclei. X-ray photons are produced by collisions between energetic electrons and atoms (Purdy et al., 1992). If a collision take place with a high-energy inner shell electron in the atom, that electron can be ionized and emitted to carry on the process. When a second electron from a lower-energy outer shell moves up to take its place, energy is released in the form of an x-ray photon. Alternatively, the incoming energetic electron may be deflected by the electric field close to the nucleus, and in the process, decelerate and lose energy, also emitted as an x-ray photon.

X-ray and gamma-ray photons produce their biologic effect by interacting with electrons in tissue that they encounter. This interaction is a two-step process (Withers, 1992). In the first step, the incoming photons interact with electrons in the atoms of the matter, providing sufficient energy for their escape from their orbit around the atomic nucleus. This loss of a negatively charged electron results in a positively charged atom, or ion, and the process is therefore termed ionization. In the electromagnetic spectrum, only
x-ray and gamma ray photons have sufficient energy to produce emission of an atomic electron, and they are therefore termed ionizing radiation (Tokarek et al., 1994). The second step occurs because the emitted electrons have kinetic energy. They can go on to interact with further electrons in the material, causing further ionization events and depositing energy. These electrons gradually slow down as their energy is dissipated. When one Joule (J) of energy is deposited in one kilogram of tissue, primarily in this second step, the tissue is said to have absorbed a dose of one Gray (Gy) of radiation.

The primary mechanism by which radiation causes cell death in tumours and normal tissue is injury to cellular DNA (Bristow et al., 1998). However, energy is deposited randomly throughout the cell and alternative mechanisms, such as cell membrane injury and microtubule damage, may also have roles (Withers, 1992). Although some direct DNA ionization may occur as a result of contact with high-energy electrons in the second stage noted above, the majority of these electrons react with cellular water to produce free radicals, such as the highly unstable hydroxyl radical. These short-lived species, when located within approximately 100 Å of DNA, interact with the molecule to produce various injuries. These may include formation of cross-links between DNA strands, alteration of the DNA base sequence, or formation of single- or double-strand DNA breaks (Bristow et al., 1998).

As a result of this damage to DNA, the progression of irradiated cells through the cell cycle is delayed. The G2 phase is the most common site of blockade (Bristow et al., 1998). More germane to a discussion of fibroblasts, however, is the blockade during the G1 phase. As cells move through the cell cycle, the tumour suppressor gene p53 surveys the DNA for damage at the G1/S cell cycle checkpoint (Tokarek et al., 1994). Should
damage be present, p53 binds to the promoter region of the p21^{WAF-1/CIP-1} gene resulting in the production of the p21^{WAF-1/CIP-1} protein (Sidransky et al., 1996). This protein inhibits cyclin dependent kinases that phosphorylate the pRb protein, a process which is necessary for cell cycle progression.

This delay allows cells to repair DNA damage from radiation. Should residual abnormalities be present when the cell attempts to undergo mitosis after re-entering the cycle, cell death can occur via the process of apoptosis (Sidransky et al., 1996). This mechanism is poorly understood, but may be related to p53 altering the balance between the apoptosis-promoting BAX protein and the counter-regulatory bcl-2 protein. Alternatively, the cell can be permanently blocked from progressing through the cell cycle, and in effect, terminally differentiated. The factors that govern exactly which process a given cell will undergo are not well defined, however the decision is likely cell-specific. In either event, the injured cell is unable to proliferate and contribute to the cell population, and is said to have sustained a lethal injury.

The response of normal tissues to ionizing radiation depends on many factors. Treatment variables include the volume of tissue irradiated, the total dose administered, and the fraction size (if the total dose is divided into several fractions). For any specified dose the incidence of radiation injury may increase as the volume of tissue exposed increases (Peters et al., 1981). Dividing the total dose into fractions allows for relative sparing of the effects on slowly proliferating tissue, while maintaining the desired effect on the tumour. For example, a conventional preoperative radiotherapy protocol for soft tissue sarcoma might consist of 50 Gy total in 2 Gy daily fractions. These smaller doses/fraction allow for repair of injury in slowly proliferating tissues.
There are several cellular determinants of a tissue’s response to radiation. Repair of sublethal DNA damage is a feature of many cells, and is the primary reason for increased tolerance of a dose if it is administered in fractions (Bristow et al., 1998). This, combined with the ability of tissue to repopulate from stem cells, lead to decreased tissue effects from the radiation. The redistribution of cells throughout the cell cycle and the reoxygenation of previously hypoxic tumour cells, both lead to increased effects of radiation on malignant tissue (Withers, 1992).

Cells possess several mechanisms to repair DNA injured by ionizing radiation (Squire et al., 1998). During the delay in progression through the cell cycle, cells invoke these in order to repair sublethal damage. In most human cells, damage can be repaired within 12 hours (Bristow et al., 1998). Once daily fractionation schedules, therefore, generally provide adequate time for cells in normal tissues to repair themselves. However, should a cell attempt to undergo mitosis before this repair takes place, or if the damage is non-repairable, then the cell may die attempting to divide. The repair of double-stranded DNA breaks is crucial, as unrepaired double-stranded breaks are likely responsible for many of the effects of ionization radiation (Squire et al., 1998).

In the course of an organ’s normal lifetime, parenchymal cells are continuously replaced by division of stem cells of that lineage present in that tissue. The expression of radiation injury depends, in some part, on the extent of injury to a tissue’s stem cell population. These stem cells tend to be more vulnerable to radiation injury as they proliferate more than the differentiated parenchymal cell population. As a result, they may die attempting mitosis following irradiation. Significant damage to this reserve population would eventually lead to hypocellularity of the tissue when the cells are stimulated to
proliferate to replace lost mature cells. There is a wide variation between tissues in terms of the number of stem cells, their mitotic activity and therefore their ability to withstand radiation injury themselves. The stem cells in slowly proliferating tissues tend to be less mitotically active themselves (Rubin et al., 1992). This contributes to the delayed expression of radiation injury in these slowly proliferating tissues. However, an earlier injury, such as surgical wounding after irradiation, can stimulate the stem cell population to attempt to proliferate sooner, thereby resulting in cell loss and perhaps accelerated tissue dysfunction.

The inherent radiosensitivity of a particular cell depends to some extent on its position in the cell cycle. Cells which are in the stages G2 and M (mitosis) are more susceptible to injury which would lead to cell death during replication. Those cells in the S and G1 phases are less vulnerable to lethal injuries (Withers, 1992). The distribution of cells throughout the cycle influences the effect radiation will have on a particular tissue, especially if the treatment is given in fractions. Tissues that have a large proportion of cells in the sensitive phases will obviously be more affected acutely by radiation than those that have a higher proportion of cells in the G1 or S phases. After cell death during mitosis, the remaining cells in these rapidly proliferating tissues are largely in the resistant phases. Progress through the cycle leads to a high proportion of cells being distributed once again into the more sensitive phases, which can then be affected by subsequent doses. In contrast, slowly proliferating tissues have a large number of cells in the resistant phases, and may tend to remain in these phases rather than redistribute throughout the cell cycle. This is yet another mechanism by which slowly proliferating tissues are spared the acute effects of radiation by fractionation.
Finally, the degree of oxygenation of the tissue influences the degree of radiation injury to tumours. Oxygen is the most potent chemical modifier of inherent tissue radiosensitivity (Withers, 1992). Radicals formed on the DNA by interaction with ionized cellular water can interact with molecular oxygen, resulting in the formation of peroxides and hydroxyperoxides on the DNA. These entities are more resistant to enzymatic degradation and are also more toxic than the free radicals. As a result, tissues that are better oxygenated may sustain a more substantial injury from radiation than hypoxic tissues.

The processes of DNA repair, repopulation by stem cells and redistribution in the cell cycle allow for differentiation of normal tissues into those that tend to demonstrate acute effects of radiotherapy and those whose effects are delayed. The collagenous dermis, which is composed largely of extracellular matrix and slowly proliferating fibroblasts with sparse stem cells, is an example of a late-responding tissue. The main manifestations of radiation injury, such as fibrosis and extremity edema, may not be expressed for months or even a year after treatment. As mentioned above, however, detrimental effects can be accelerated in this tissue if the stem cells and mature tissue cells are stimulated to proliferate, as would be found in the scenario of surgical wounding.

Irradiated skin demonstrates several histologic features, none of which are pathognomonic for radiation injury (Fajardo, 1992). The epithelium can be hypertrophied acutely. Chronically, cells in this layer become atrophic and may demonstrate marked cellular atypia or even dysplasia, suggesting a premalignant condition. There is usually loss of skin appendages including hair follicles and sweat glands. More relevant to this discussion are changes in the dermis, however. Fibrosis is a prominent delayed response
which can cause skin contractures and impaired patient function, especially if around joints. Fibrin, identified as fine lacy fibrils between collagen bundles and fibroblasts, may be present as a result of any radiation damage or any inflammatory stimulus. The abundance of both collagen and fibrin in the extracellular matrix suggests a stimulus for excessive formation, impaired metabolism or both. Dermal fibroblasts display atypical changes suggestive of radiation injury. These cells may be large and basophilic with elongated cytoplasm. Nuclear atypia, such as hyperchromasia and polyploidy, may also be prominent (Fajardo, 1992). Several changes in the vascularity of the dermis may also be noted in delayed radiation injury. There may be altered interaction between endothelial cells and inflammatory cells, such that further injury results in a reduced infiltrate of granulocytes, macrophages and lymphocytes. The microvascular endothelial cells lining capillaries are the most sensitive to radiation injury of any vascular cells, so these vessels demonstrate damage more readily than larger vessels. Dilatation, irregular vessels and sometimes thrombosis as a result of endothelial hyperplasia may occur in injured capillaries. These changes are typically delayed as microvascular endothelial cells are usually slow to proliferate. In the case of dermis, the delayed manifestations of radiation injury are likely due to combination of the above effects. However, it is likely that injury to dermal fibroblasts and their stem cells represent the most important contributor, and that injury to microvascular endothelial cells compound these effects (Withers, 1992; Springfield, 1993).

**Radiation Effects on Wound Healing**

Ionizing radiation administered prior to surgery has been shown to decrease the initial strength of the healing surgical wound to less than half that of unirradiated controls.
(Gorodetsky et al., 1988; Gorodetsky et al., 1991). Since the strength of the healing wound is a direct reflection of the thickness and orientation of its collagen fibres, as well as the degree of cross-linkage of these fibers (Diegelmann et al., 1975; Gabbiani et al., 1976; Doillon et al., 1985; Bernstein et al., 1993), it must be concluded that ionizing radiation somehow alters these components of the extracellular matrix. This decreased strength likely leads to the failure of early wound coaptation and the major wound problems observed in the clinic.

The mechanism by which ionizing radiation impairs wound healing is uncertain. Because the end result of the wound healing process - well-organized collagen molecules to resist tensile forces - is the product of many interrelated processes, there are many possible steps at which the effects of the ionizing radiation can occur.

Although it is possible for the collagenous extracellular matrix to be altered directly by radiation, there is no evidence that this occurs at the doses used in conventional radiotherapy. Ionizing radiation is used to sterilize other collagenous tissues, such as ligaments, for use as allografts. Examination of these unwounded tissues after exposure to a dose of 2 Mrad, or \(2 \times 10^4\) Gy, demonstrates a reduction anywhere from zero (Gibbons et al., 1991) to 15% (Fideler et al., 1995) in tensile strength. It is unlikely, therefore, that total doses of 50 Gy given in 2 Gy daily fractions would significantly impair performance of the existing extracellular matrix. There is also no evidence that radiation causes direct alterations in the structure of growth factors, which are present in picomolar or femtomolar concentrations, and would therefore experience ionization exceedingly rarely.

As has been discussed with respect to normal tissue effects, it is likely that radiation impairs wound healing through its alteration of the cellular population. Based on
the review of normal wound healing, there are a number of cell types that could potentially be affected.

The inflammatory phase features the appearance and contribution of platelets, neutrophils and macrophages. The function of platelets themselves is unaffected by ionizing radiation (Kaloidouris et al., 1981). Neutrophils exhibit decreased phagocytosis and superoxide production when exposed to irradiated dermis (Gabka et al., 1995). These cells, however, are not present to a great extent in dermal tissue and usually migrate into the extravascular space in response to injury (Clark, 1996). Therefore cells migrating into the surgical wound would not be affected directly by the previous dose of irradiation. Monocytes, similarly, migrate from the intravascular compartment in response to liberated growth factors (Riches, 1996), and once they contact the provisional matrix, differentiate into macrophages. Rodents that have received total body irradiation, impairing the function of their hematopoietic system, demonstrate decreased wound strength, presumably due to a decrease in the early precursors of macrophages (Cromack et al., 1993). The healing wound would therefore be deficient in mature macrophages. This defect in wound strength can be restored to normal levels by the exogenous application of TGF-β1, which indicates that the production of this factor is a key function of the macrophage in the wound healing process. In the same experiment, animals that had local radiation to their skin, which maintains systemic macrophage function, had decreased wound strength that could not be reversed by application of TGF-β1, which implies a different mechanism of action of this form of radiation.

The microvascular endothelial system is responsible for supplying nutrients and oxygen to support the newly-forming granulation tissue. The radiosensitivity of the
vascular system is heterogeneous, with capillaries and small arterioles demonstrating the greatest effect, in studies of rabbit ear skin (Dimitrievich et al., 1984). The earliest response to ionizing radiation is increased permeability of the microvasculature (Baker et al., 1989). This is likely due to a dissolution of the polysaccharides that bind adjacent endothelial cells together, along with a release of vasoactive substances. The extravasation of blood and plasma results in erythema and edema of the tissue. Within days, there is a preferential loss of small capillaries, resulting in decreased vascular surface area and volume as determined histomorphometrically (Dimitrievich et al., 1984). The distance between capillaries may increase from 150 μm to 300 μm (Dimitrievich et al., 1984), which may impair the supply of nutrients to the tissue. Within days to a few weeks, there is irregular proliferation of the remaining capillary endothelial cells, resulting in small vessels of abnormal size and shape. These irregularities can lead to altered flow and ultimately result in thrombosis. This has traditionally been proposed to result in tissue hypoxia, which may predispose to impaired healing (Robinson, 1974). However, more recent studies have demonstrated that the oxygen tension in unwounded irradiated tissues is normal (Springfield, 1993). It is possible that the microvasculature would be unable to keep up with the demands of the wound healing scenario, resulting in relative tissue hypoxia, but this has not been observed. It is not clear at this point what effect, if any, radiation damage to dermal vasculature has on subsequent dermal wound healing and gain in tensile strength.

Dermal fibroblasts are responsible for synthesizing and remodeling the extracellular collagenous matrix, and their function directly affects collagen fibre density, size, orientation and hence, wound strength. Fibroblasts proliferate actively in response to
wounding, likely as a result of the mitogenic effects of liberated PDGF and TGF-β. Irradiated fibroblasts, however, show marked impairment in their ability to proliferate in culture (Rudolph et al., 1988). This decreased proliferation is likely in response to DNA damage. The p53 tumour suppressor gene is expressed at high levels in irradiated fibroblasts, which in turn can activate transcription of the gene for p21\(^{\text{WAF-1/CIP-1}}\) (Di Leonardo et al., 1994). This protein inhibits cyclin dependent kinase activity necessary for cell cycle progression (Bernhard et al., 1995). The irradiated fibroblast is therefore arrested in the G1 phase, and is in effect terminally differentiated (Rodemann et al., 1991; Rodemann et al., 1996), and likely incapable of proliferating in response to surgical wounding. The alternative fate, as described earlier, would be associated with more severe damage, in which the cell may undergo apoptosis. Irradiated fibroblasts also demonstrate impaired mobility, as indicated by decreased outgrowth from skin explants (Lombard-Vignon et al., 1990). Irradiated wounds also demonstrate reduced amounts of fibrinogen, fibronectin and fibrin deposition early in the process (Wang et al., 1996). These materials normally form a scaffold that permits rapid access to the wound by migrating fibroblasts. The result is a decreased presence of fibroblasts in the early wounding scenario (Wang et al., 1994). In addition to radiation effects on fibroblast proliferation, the new collagen synthesized by irradiated fibroblasts is abnormal. Initially, there is a delay in the formation of collagen bundles (Stajic et al., 1969; Wang et al., 1994). Examination of the bundles that are formed reveals decreased proline hydroxylation (Archer et al., 1970; De Loecker et al., 1976), which leads to an unstable molecule less able to withstand tensile stresses. This mechanical effect may also be related to the fact that newly synthesized collagen fibres are thinner than normal (Wang et al., 1996).
In summary, radiation injury to dermal tissue potentially affects the biologic behaviour of several different types of cells. Of these, injury to dermal fibroblasts, which maintain the extracellular matrix, seems to be prominent in moderating the effects on wound healing. Through alteration of the performance of the various fibroblast phenotypes found in the coordinated repair of dermal wounds, exposure to ionizing radiation may lead to structural changes in the repaired dermis, rendering it unable to withstand the rigors of physiologic loading. The end result may potentially be clinical wound failure and substantial patient morbidity.
HYPOTHESIS AND RATIONALE

The assumption underlying this research is that preoperative irradiation would offer a significant long term advantage for combined local therapy of cancer if wound healing problems could be eliminated. The goals of local tumour cancer treatment are increasingly focused on decreasing long term morbidity and enhancing functional outcome. Solving the problem of wound healing complications after preoperative irradiation is therefore an important goal for improving local cancer management.

Ionizing radiation results in impairment of several functions of dermal parenchymal cells, most notably dermal fibroblasts. As these changes are largely permanent, it is no surprise that attempts to improve the function of cells present in post-irradiated tissue, using growth factors or other forms of chemical manipulation, have been inconsistent in their success. Furthermore, because the controlled secretion of growth factors in wound healing is so tightly regulated, it is difficult to predict what effect a given growth factor will have on a given day of administration.

It seems reasonable that the best chance to improve the outcome of radiation-injured dermal tissue is to introduce parenchymal cells that have not suffered radiation injury. Ionizing radiation induces an altered phenotype in dermal fibroblasts that many have postulated to be the main contributor to impaired wound healing. Normal unirradiated dermal fibroblasts, if present at the time of wounding, may be better able to respond to the demands of the wounding healing environment. Indeed experiments in rodents have demonstrated that addition of neonatal syngeneic fibroblasts to an irradiated wound site can improve the response of the healing scar to physiologic stress (Krueger et al., 1978; Krueger et al., 1978; Gorodetsky et al., 1991). For human use, however,
embryonic fibroblasts are not practical. In order to avoid immunogenicity against any injected cells, these fibroblasts would have to be autologous. It has been demonstrated, however, that dermal fibroblasts obtained from adults and elderly patients have impaired proliferation and migration in culture when compared with those from infants and children (Schneider et al., 1976; Schneider, 1979). Since the majority of patients with soft tissue sarcomas are adults or elderly, their fibroblasts would demonstrate this less active phenotype. It cannot be assumed, therefore, that the presence of these cells in an irradiated wound would be as effective in enhancing wound healing as highly proliferative embryonic fibroblasts. Although the applications of xenograft and allograft tissues in human organ dysfunction are becoming more common, the use of functional autograft from the same individual remains the most attractive option, providing adequate tissue can be obtained. In the case of skin/dermis, patients generally have a surplus that can be obtained with minimal morbidity or dysfunction.

It is therefore hypothesized that the introduction of normal, unirradiated autologous dermal fibroblasts to previously irradiated skin would improve healing of a surgical wound. We tested this hypothesis in a rat model in which animals underwent bilateral buttock irradiation concomitant with harvest of dermal fibroblasts. Autologous fibroblasts were subsequently reimplanted back into the individual animals prior to surgical wounding, and the wounds assessed biomechanically as a indicator of dermal healing.
METHODS

To detect a difference of 20% between groups with confidence of 95% and power of 80%, using a standard deviation of 70 g (estimate based on previous studies), a sample size of 21.3 is required. To allow for animal morbidity, it was decided that 26 animals should be used for this experiment.

Twenty-six female Wistar rats (Charles River Canada) weighing 225-250g underwent a three-stage procedure. In the initial stage, each animal was anaesthetized using intramuscular injection of ketamine (10 mg/kg) and xylazine (10 mg/kg), followed by inhalation of halothane. The dorsum of the animal was shaved and hair removed with depilatory cream. The dorsum was then cleaned using povidone and 70% ethanol washes. An oval-shaped full-thickness skin biopsy, 1 cm² in area, was obtained and placed in sterile medium on ice. The wound was closed with three staples. Following this, the animal was placed in a plexiglas box, which allowed a 2.5 cm diameter circular region of tissue over each buttock to be sequentially exposed to single fraction irradiation of 18 Gy from an x-ray source at a rate of 10.2 Gy/min. The skin biopsy was disaggregated physically by chopping it into 1 mm³ fragments and then digested sequentially in 10 ml of 0.2% type I collagenase (Sigma Chemicals) suspended in the α-modification of Minimal Essential Medium (α-MEM) for 90 minutes and 10 ml of 0.05% trypsin suspended in phosphate-buffered saline (PBS) for 60 minutes. The digested cells and skin explants were suspended in α-MEM without RNA/DNA + 2000 g/l glucose + 100 IU/ml penicillin + 100 μg/ml streptomycin + 20% fetal bovine serum and cultured at 37°C with 5% CO₂ for three weeks. Medium was changed thrice weekly and cells passaged once confluence was reached.
Three weeks later, the cells were harvested by 0.05% trypsin digestion, stained by trypan blue and viable cells were counted using a hemocytometer. A total of 10x10⁶ cells were isolated and suspended in 1 ml of α-MEM. The skin on the buttocks was shaved and washed using the same protocol as in stage one. The irradiated fields were identified by skin changes such as alopecia and erythema. In the center of a randomly assigned side, the autologous cells were injected intradermally into the donor using a 3 cc syringe with a 21-gauge needle to minimize cellular damage. The contralateral side received the same injection of α-MEM only. After 15 minutes, a full-thickness longitudinal incision 2 cm in length was made through the bleb in the center of the irradiated field bilaterally. The wounds were immediately closed with three staples. Postoperative analgesia was provided by a subcutaneous injection of 0.1 ml of Temgesic. Staples for all wounds were removed seven days postoperatively.

In the final stage three weeks later, the animals were sacrificed by CO₂ inhalation and a 3 cm x 3 cm full thickness square of skin containing each wound was harvested. The skin containing the wound was cut into parallel 6 mm wide strips perpendicular to and containing the wounds, using a custom made cutting jig. The center two strips from each wound were used for biomechanical analysis, and a third strip, containing the end of the wound, was fixed in 10% formalin for histologic evaluation.

The biomechanical parameters evaluated were the load to failure, ultimate tensile strength, modulus of elasticity and fracture toughness. The thickness of each strip in the area of the wound was measured using digital calipers. Each strip was then mounted in a 37° C PBS bath in an Instron tensilometer. A photograph was taken of each strip after pre-loading it with 2 g of weight. The strip was then stretched in uniaxial tension at a rate
of 5 mm/minute until it fractured. The load at failure was determined from the resulting force-displacement curve. This was defined as the load generated immediately prior to visible failure of the skin strip.

In order to obtain a stress-strain curve, the cross-sectional area of the skin strip must be calculated. From the photographs, the surface area of each skin strip was able to be determined. These dimensions were obtained using a digitizer with the width of the grips holding the skin strip in the photograph serving as a known reference measurement. The length of the strip was measured directly. Because the shape of the skin strip was not rectangular, the width was measured at six evenly placed sites along the length of the strip, and the mean width obtained. These two values, multiplied by the thickness obtained from the digital calipers, allowed estimation of the volume of the strip between the grips. With progressive stretching, this volume divided by the length of the strip gave the instantaneous cross-sectional area. The force-displacement curve could then be converted to a stress-strain curve, with stress representing the force normalized to the instantaneous cross-sectional area. The ultimate tensile strength (UTS) was the stress immediately prior to visible failure of the skin strip.

The final two values were determined from the stress-strain curve. The modulus of elasticity was defined as the maximal slope of the linear portion of the curve prior to failure. The fracture toughness was determined from the area under the stress-strain curve prior to failure, which represents the energy absorbed by the strip before fracture.

For each biomechanical parameter, the mean of the two strips for each wound was calculated. The cell-injected and contralateral medium-injected wounds from each animal were compared using a paired t-test, assuming normalcy of the underlying distributions.
Histologic sections for each wound were stained with hematoxylin & eosin and Masson's trichrome. The site of wounding was identified in each section by thickening and puckering of the epidermis, collagen fibers running in a linear longitudinal fashion from the epidermis to the underlying subcutaneous tissue, and disruption of the subcutaneous fat and panniculus carnosus. Quantitative histomorphometric analysis consisted of determination of the overall cell density within the wound. A grid was used on 400x power to count all cells with fibroblastic morphology in the wound and the adjacent 0.3 mm of dermis (0.15 mm on each side of the wound). Any cells with different morphology, such as microvascular endothelial cells and polymorphonuclear leukocytes, were not included. Counting began at the level of the papillary dermis immediately subjacent to the epidermis, and stopped with cells in the layer of reticular dermis immediately overlying the subcutaneous fat. All cell counting was performed in random order by a blinded observer, and results were compared using a paired t-test. Only samples with a clearly defined wound within the histological specimen were included in this analysis. For specimens in which a wound could not be definitively identified, that specimen and the contralateral specimen were excluded.

A second group of ten animals (control group I) underwent unilateral wounding of the buttocks only, without prior irradiation or injection. These animals were subsequently treated in identical fashion to the first group with respect to biomechanical analysis. The results from these wounds were compared to the irradiated wounds using unpaired t-tests.

A final group of seven animals (control group II) underwent identical irradiation, skin biopsy and fibroblast expansion ex vivo as the experimental group. At the time of the second stage of the experiment, a total of $10 \times 10^6$ fibroblasts were harvested and
suspended in 1 ml of α-MEM. Immediately prior to injection, the sample was irradiated with a dose of 30 Gy. This sample was then injected into a randomly assigned side as in the second stage for the first group. The remainder of the experiment and biomechanical analysis took place as noted above. The values for control group II were compared to those in the first two groups of animals using unpaired t-tests.
RESULTS

Of 26 animals initially entered in the experiment, nine were excluded, leaving 17 for analysis. The reasons for exclusion were in vitro cell contamination (two cases), animal death or morbidity (three cases) and an insufficient number of skin strips for analysis (four cases). In control group I, no animals were lost. In control group II, one animal was lost due to animal morbidity. For all skin samples tested biomechanically, the strip fractured along the wound.

For each of the 17 animals, the breaking load of the wounds injected with normal cells was greater than that of the wounds injected with medium only. This is depicted in figure 3, with the animal number indicated on the x-axis. Figure 4 illustrates the mean breaking loads of all groups of wounds. The wounds with cells injected had a significantly higher mean breaking load (491.8 g +/- 92.5 g) than that of the wounds with medium alone injected (298.3 g +/- 81.1 g, p<0.0001). Wounds from control group I had a significantly higher mean breaking load (658.8 g +/- 160.5 g) than the cell-injected wounds (p=0.0005). The mean breaking load of control group II (343.7 g +/- 135.2 g) was significantly lower than the cell-injected wounds (p=0.005) and not significantly different from the medium-injected wounds (p=0.34).

The ultimate tensile strength (UTS) was greater in the cell-injected wounds than the medium-injected wounds for all animals. The mean UTS of the cell-injected wounds (0.63 MPa +/- 0.15 MPa) was significantly greater than that of the medium-injected wounds (0.37 MPa +/- 0.09 MPa, p<0.0001). The mean UTS of control group I (0.79 MPa +/- 0.19 MPa) was greater than that of the cell-injected wounds (p=0.02). The mean UTS of the cell-injected wounds was higher than that of control group II (0.42 MPa +/-
0.23 MPa, p=0.01), which was not different from that of the medium-injected wounds (p=0.41). The mean UTS of all groups is depicted in figure 5.

The elastic modulus was greater in the cell-injected wounds than in the medium-injected wounds for all except two animals. The mean elastic modulus for the cell-injected wounds (4.31 MPa +/- 1.05 MPa) was significantly greater than that of the medium-injected wounds (2.92 MPa +/- 0.54 MPa, p=0.0001) and was similar to that for control group I (4.84 MPa +/- 1.56 MPa, p=0.30). The mean elastic modulus for control group II (3.40 MPa +/- 1.72 MPa) was not significantly different from that of the cell-injected wounds (p=0.12) or the medium-injected wounds (p=0.31). The data for elastic modulus is displayed in figure 6.

The mean toughness of the cell-injected wounds was greater than that of the medium-injected wounds in all animals. The mean toughness of the cell-injected wounds (74.1 kPa +/- 24.9 kPa) was significantly higher than that of the medium-injected wounds (40.6 kPa +/- 14.3 kPa, p<<0.0001), but significantly less than that of control group I (106.1 kPa +/- 20.1 kPa, p=0.002). The mean toughness for control group II (51.6 kPa +/- 32.8 kPa) was not significantly different from that of the cell-injected wounds (p=0.08) or the medium-injected wounds (p=0.26). The data for wound toughness is displayed in figure 7.

The wound could not be definitively identified in five histologic sections. These and the contralateral section for that animal were excluded from any further histomorphometric analysis. Of the remaining twelve paired samples, the cell density was greater in the cell-injected wound in eight. The mean of the cell-injected wounds (1212 +/-
396 cells/mm$^2$) was significantly greater than that of the medium-injected wounds (942 +/- 320 cells/mm$^2$, p=0.03). This data is depicted in figure 8.

Figures 9 and 10 demonstrate the typical histologic appearances of wounds in normal cell-injected and medium injected irradiated skin, respectively.
Figure 3 - Individual Breaking Load
Figure 4 - Mean Breaking Load

- Control group I
- Medium Injection
- Cell Injection
- Control group II
Figure 5 - Mean Ultimate Tensile Strength (UTS)
Figure 6 - Mean Elastic Modulus

- Control group I
- Medium Injection
- Cell Injection
- Control group II
Figure 7 - Mean Toughness

- Control group I
- Medium Injection
- Cell Injection
- Control group II
Figure 8 - Mean Cell Density
Figure 9 - Photograph of wound in medium-injected irradiated skin (H&E, 100x), demonstrating minimal cellular infiltrate in region of wound and adjacent dermis three weeks after wounding.
Figure 10 - Photograph of wound in cell-injected irradiated skin (H&E, 100x), demonstrating dense cellular infiltrate in region of wound and surrounding dermis. The lineage of these cells is not known, but most have a fibroblast-like appearance.
DISCUSSION

The ultimate goal of this investigation was to develop new techniques that can be used in the clinical setting. It was desirable therefore, to use a surgical model that closely resembled the clinical problem in testing potential new interventions. The model used in these experiments has features that both emulate and differ from the clinical situation.

The skin of a rat is different anatomically from that of a human. In human skin, the dermis is tethered to the underlying superficial fascia by thick collagenous bands (Cormack, 1984). Rats, like virtually all other mammals, lack this tethering and the skin is freely mobile over the fascia and underlying muscle. Despite this difference the process of wound healing is similar in virtually all mammalian species and the rat has commonly been used in studies of interventions to improve wound healing in irradiated skin. Prior to any human use, however, it might be justified to investigate procedures on a species whose skin more closely resembles humans, such as the pig (Quaglino Jr. et al., 1990; Lavker et al., 1991; Quaglino Jr. et al., 1991; Millar et al., 1996).

In conventional preoperative radiotherapy protocols, radiation is administered in divided doses (fractions) to alleviate the acute effects of radiation damage on normal skin. For impaired wound healing, an isoeffect curve has been defined in a rodent model. Gorodetsky (Gorodetsky et al., 1990) has determined that a single fraction of 18 Gy has an equivalent effect on wound healing as 40-50 Gy given in 2 Gy daily fractions. The effect of the single dose in this experiment is therefore similar to that of the protocol for preoperative sarcoma irradiation (Suit et al., 1993).

The time from radiation to surgery in this model was chosen to emulate the clinical situation. In clinical practice, it is generally accepted that a delay prior to surgery of
approximately 3 weeks after completion of irradiation is necessary to allow any acute effects on the skin to diminish. This is the same protocol used in this experiment. The three week interval between surgery and wound mechanical testing corresponds with the approximate time at which patients experience post-operative wound failure (Bell, personal communication). By three weeks following surgery, the wound is either solidly healed, or alternatively, remains weak and subject to dehiscence (wound failure) with minimal stresses. The strength of a healing wound increases minimally over the first week post-wounding, until collagen synthesis is established. Between two and four weeks postoperatively, a much more rapid gain in wound strength is noted as collagen fibers are actively synthesized, remodeled and contracted by fibroblasts and myofibroblasts. After the first four weeks, there is a more gradual gain in wound strength over the final several months to its final status, which is still somewhat inferior to that of normal, unwounded skin (Levenson et al., 1965). The effect of radiation causes a delay, rather than an absolute impairment of this process. In fact the final strength of a healed irradiated wound may be similar to that of a wound in normal skin (Gorodetsky et al., 1988; Springfield, 1993). It is at the time of most rapid gain in wound strength, when wound healing clinical complications are most apparent (at three weeks following surgery), that the effect of an intervention designed to improve wound healing would have the greatest clinical utility.

Several intraoperative factors that may contribute to impaired wound healing in the clinical situation are not addressed in this experiment. Ischemia of wound edges may occur as a result of excessive tension during retraction. This may lead to necrosis of the wound edges post-operatively and subsequent wound breakdown. Resection of large volumes of muscle results in a residual "dead space". This space is left to fill with hematoma and
seroma, which are prone to becoming infected because of stasis. Infected wounds with ongoing inflammation demonstrate impaired healing (Fu, 1979). Although irradiated skin does demonstrate an increased preponderance for infection (Ariyan et al., 1980), no obvious infections developed in these animals. A certain proportion of sarcoma patients develop infections in their wounds. This may or may not be associated with gross wound dehiscence.

All of these factors may be equally important as preoperative irradiation in sarcoma patients. Because this model is not entirely representative of a sarcoma wound, with no muscle being resected, the effect of these variables have been eliminated. Modification of the model to address the interaction between all of these negative modulators would be necessary to more closely mimic a true sarcoma wound.

The principal outcome measures in this study are biomechanical parameters evaluating the integrity of the collagenous dermal extracellular matrix and the appropriateness of these measures may be criticized. It is possible that fatigue testing should be carried out on these specimens as a more clinically relevant parameter of clinical wound healing. However, the parameters that were used in this investigation are used regularly in wound healing experiments.

Two other criticisms may be raised about our biomechanical testing protocol. First, it is questionable whether the mean breaking load of several skin strips should be used to assess a wound's entire biomechanical performance. One would expect the wound to fracture at its weakest point, thereby rendering the breaking load (and other parameters) of the stronger skin strip irrelevant. Ignoring these values, however, would lead one to ignore the effect of the injected cells across the entire wound. It is possible that the
concentration of injected cells differed along the length of the wound, which may explain variation in the biomechanical performances between skin strips within the same wound. Second, in reporting the biomechanical parameters as they apply to the wounds in the skin strips, these truly should apply to the wound and adjacent unwounded dermis contained between the testing grips. However, this entire construct, rather than just the wound, is also loaded under physiologic circumstances.

The results of this experiment demonstrate that a single-fraction dose of 18 Gy of ionizing radiation substantially reduces the breaking load of a healing surgical wound. This reduction is consistent with other studies (Gorodetsky et al., 1991; Cromack et al., 1993). Interpreting these findings using the isoeffect curve reported by Gorodetsky et al, the preoperative course of radiotherapy used to treat soft tissue sarcoma would probably cause a similar decrease in the clinical wound breaking load. A similar reduction is noted in the UTS, modulus of elasticity and toughness of wounds in irradiated skin, when compared with unirradiated controls (control group I). Intradermal injection of 10x10^6 normal, unirradiated autologous dermal fibroblasts three weeks after irradiation and just prior to wounding incompletely reverses the radiation-induced wound healing deficit, as evidenced by a significant increase in the breaking load of these wounds when compared with the medium-injected controls. The wounds that received injection of unirradiated cells also demonstrated significantly increased UTS, modulus of elasticity and toughness, when compared with the medium-injected wounds. These results indicate that the extracellular matrix in these cell-injected wounds is better able to resist tensile loading. Specifically, features of the extracellular matrix such as the amount of collagen, the thickness of collagen fibers, the degree of collagen cross linkage and the orientation of the
collagen fibers may all be augmented to provide this improved mechanical performance. Collagen synthesized by irradiated fibroblasts demonstrates impairment of all of these features.

Although there is a marked improvement in the healing of these cell-injected wounds compared to medium-injected wounds, their characteristics are still inferior to wounds in normal unirradiated skin (control group I). There are several potential reasons why this reversal was incomplete. There is likely a greater number or higher density of functional fibroblasts present in the unirradiated wound. Although 10x10^6 cells were injected, it is likely that only a portion survived and were active in the healing process. Further improvement in healing might be demonstrated with a higher dose of injected cells. A second important potential mechanism is injury to the dermal microvasculature. Although radiation injury does not result in hypoxic tissue in the resting state, there may be impaired neovascularization in the wounding environment. The inability of the microvasculature to supply nutrients to the advancing wave of fibroblasts would hamper their ability to form functional tissue. A final factor may be subtle alterations to the nature of the dermal matrix that might result from preoperative irradiation. This may include an altered cytokine profile or changes in the non-collagenous extracellular matrix molecules produced by native irradiated fibroblasts. Both of these could lead to moderate alterations in the response of the injected cells that would lead them to perform at an inferior level to normal fibroblasts in unirradiated wounds.

In the third group of seven animals (control group II), the effect of irradiating the cells with a single dose of 30 Gy immediately prior to injection was examined. Irradiating the cells resulted in a significant decrease in the breaking load of the healing wounds when
compared with wounds injected with normal unirradiated cells. Similar effects of irradiating cells prior to injection were observed in the measurements of UTS, elastic modulus and fracture toughness. The differences in elastic modulus and toughness approached but did not achieve statistical significance.

The assumption that the injected unirradiated cells are viable and active in the wound healing process would lead one to presume that the wounds injected with irradiated cells (control group II) would perform no better than those injected with medium only. This was not quite the case. The wounds that received injection of irradiated cells were slightly better biomechanically than those that received medium only. This was not a significant difference but the absolute values of all biomechanical parameters were consistently higher. It is possible that not all of these cells were killed by the radiation treatment given. A total of $10^6$ cells were suspended in only 1 ml of medium at $4^\circ$C for approximately 1 hour. This would probably lead to significant hypoxia in some of these cells which would confer a certain degree of radioprotection. Alternatively, or in addition, the irradiated cells may have been able to perform some of the differentiated functions necessary to improve wound strength without undergoing significant proliferation. This may have led to the marginal improvement in biomechanical performance of this group of wounds.

In general, the irradiated skin demonstrated loss of dermal appendages, epidermal hypertrophy, some swelling of the dermal collagen, and the occasional "radiation fibroblast" - large cells with prominent nucleoli. Usually, the wound was relatively easy to identify. However, in five sections (three in medium-injected wounds, two in cell-injected wounds), it was not possible to definitively identify the wound. This is likely because the
histologic sections were obtained from the strips at the ends of the wounds, with the center strips being used for biomechanical analysis. To prevent bias in interpretation of histological results all sections in which the wound could not be identified and the contralateral wounds from those animals were eliminated.

Of the twelve remaining pairs of wounds, the cell-injected wounds demonstrated a higher cell density in eight. The mean cell density of the cell-injected wounds was approximately 30% greater than that of the medium-injected wounds. There was, however, a large variability in these densities. This variability can potentially be explained by sampling. Although it was impossible to determine the lineage for each cell, the majority of the counted cells were fibroblastic in appearance - spindle-shaped and associated with the collagenous matrix. Microvascular endothelial cells and cells that were obviously of other lineage (e.g. polymorphonuclear cells) were not included in the cell count. It is possible for other cells in the wound healing process, such as macrophages, to have a fibroblast-like appearance, but it is probable that the majority of cells counted were dermal fibroblasts.

There are two potential sources of this increased cell density. It is possible that these cells are residual from those that were injected, as the majority did resemble fibroblasts. Alternatively, the process of injecting the normal cells may have increased the influx of local cells from the adjacent tissues. An increase in cytokines chemotactic for the cells involved in the wound healing process may have led to greater migration of these cells. Alternatively, the quality of the provisional extracellular matrix may have been different. Increased fibrin, fibronectin and hyaluronic acid may have led to an improved ability of these native cells to gain access to the wound in the cell-injected samples. Apart
from the difference in cell density, the two groups of wounds did not appear to be significantly different qualitatively. Other authors have noted difficulty in appreciating a difference histologically between irradiated and unirradiated wounds (Springfield, 1993). There were no changes to suggest a mechanism for impaired healing in the irradiated wounds. This implies that the difference may be in the physiologic function of the involved cells, and in the chemical nature of the extracellular matrix, rather than its gross organization.

These results suggest that the process of injecting unirradiated autologous dermal fibroblasts into the irradiated skin prior to surgical wounding ameliorated some of the harmful effects of preoperative radiation on wound healing. There are several mechanisms by which these cells might exert this positive effect on wound healing. These may be best grouped into "passive" and "active" mechanisms.

The "passive" mechanisms assume that the injected cells have no or a minimal role in the active wound healing process. However, it is possible that a component of this injection bolus upregulated the wound healing process in the native tissues beyond that found in the medium-injected group. It is plausible that the suspended cells secreted a soluble factor into the medium capable of augmenting the healing process upon injection. The wounds that received injection of irradiated cells were also suspended in a similar fashion. Irradiation does not result in instantaneous cell death, and it is likely that these cells were alive and metabolically active at the time of injection. However irradiation may have decreased production of growth factors, which may account for the impaired healing in this group of wounds. The suggestions that cell injection may have exerted an effect on wound healing through passive means requires that native cells in the wound are capable
of responding to growth factors or other cell signaling factors released by the injected cells. This ignores the evidence that fibroblast response to signals that normally result in proliferation, migration and collagen synthesis are inhibited by ionizing radiation. TGF-β improves healing of surgical wounds in systemically, but not locally irradiated animals, suggesting that this factor requires functional fibroblasts be present to exert its effect (Cromack et al., 1993). Conversely, addition of PDGF-BB has been demonstrated to improve healing of locally irradiated skin flaps (Mustoe et al., 1989). It is possible, therefore, that an increased level of PDGF-BB in the injected normal cell suspensions led to increased migration of cells from normal dermis at the periphery of the radiation field. Irradiation of the cells prior to injection may reduce this secretion of PDGF-BB. These explanations are less likely than the active mechanisms detailed below, because they ignore the multitude of evidence of radiation-induced impairment of fibroblast function.

Alternative mechanisms involve "active" participation of the injected cells in the subsequent wound healing process. This presumes that a portion of these cells survive the injection process and go on to act like normal fibroblasts in response to the wounding stimulus. The fact that irradiation of the cells largely abolishes the positive effect of cell injection on wound healing supports this mechanism of action. These irradiated cells would be expected to perform no better in response to the wounding stimulus than the native cells. Many functions of dermal fibroblasts are impaired by irradiation, and the injected competent cells would be expected to carry out some or all of these functions more efficiently. The most important factor is that injected normal cells would likely have a higher rate of proliferation than the native or injected irradiated cells. Lacking the DNA damage from irradiation, these cells would not be expected to become terminally
differentiated as a result of p53 upregulation (Sidransky et al., 1996). They would be able to respond to mitogenic growth factors liberated by platelets and macrophages, such as PDGF (Heldin et al., 1996) and FGF (Abraham et al., 1996). Indeed, the emission of these growth factors by the injected fibroblasts themselves may be increased. Irradiated fibroblasts have demonstrated abnormal migration both in vivo and in vitro (Lombard-Vignon et al., 1990; Wang et al., 1994). Fibrin serves as the provisional matrix in the early tissue repair phase, and facilitates influx of macrophages and fibroblasts. The fibrin content of irradiated excisional wounds has been noted to be decreased (Wang et al., 1996). The injection of normal fibroblasts may lead to increased fibrin in the early wound, thus improving the access of other fibroblasts and macrophages to the environment. Alternatively, expression of integrins may be altered. Both PDGF and TGF-β upregulate the expression of integrins for fibronectin (Clark, 1996), leading to better adhesion of migrating cells to these molecules in the provisional matrix. Injected normal cells may demonstrate increased expression of these integrins, resulting in improved mobility.

Ionizing radiation causes fibroblasts to synthesize collagen that is abnormal both in quantity (Stajic et al., 1969) and quality (De Loecker et al., 1976). These fibers are thinner, with decreased cross-linkage. It is probable that the normal cells present at the time of wounding are able to synthesize collagen that more closely resembles that in unirradiated skin. This might include a greater quantity of types III and/or I collagen, improved cross-linkage, thicker bundling, and orientation perpendicular to the wound, all of which are suggested by the biomechanical parameters. Irradiation of the cells prior to injection would result in synthesis of extracellular matrix of similar quality to that synthesized by the native irradiated cells.
In the clinical situation of resection of a solid tumour, several factors may lead to impaired healing of the surgical wound. Factors such as patient age, size and depth of the tumour, location of the tumour in the lower extremity and concomitant illnesses such as diabetes or peripheral vascular disease have been reported to increase the risk of wound breakdown, but this is not supported by all studies. Systemic corticosteroids decrease the inflammatory response and result in impaired healing of dermal wounds if administered pre-operatively (Shamberger, 1985). The presence of implanted sarcoma cells has been demonstrated to decrease the healing of deep wounds, but not superficial wounds in rats (Lawrence et al., 1987). These factors have not been investigated in sarcoma patients. Treatment with certain chemotherapeutic agents also results in impaired wound healing. Specifically, the agents cyclophosphamide, methotrexate and adriamycin are clearly detrimental to healing of the surgical incision (Shamberger, 1985; Drake et al., 1995). These agents are rarely used in conventional protocols for management of soft tissue sarcomas. Studies have most consistently demonstrated preoperative irradiation to be associated with an increased risk of wound complications (Skibber et al., 1987; Quill et al., 1990; Bujko et al., 1993; Saddegh et al., 1993; Peat et al., 1994; Cheng et al., 1996), and this is likely a major contributor in patients with soft tissue sarcoma.

The principle of harvesting autologous cells for re-implantation in patients has been demonstrated in other wound healing scenarios (Ronfard et al., 1991; Horch et al., 1994; Stark et al., 1994; Spilker et al., 1996). In burn patients, skin substitutes are created by harvest of keratinocytes, expansion in culture and growth on dermal equivalents. Fibroblasts are sometimes cultured concomitantly on synthetic membranes for use as a dermal equivalent on which to place these keratinocytes. Used in the absence of adequate
amounts of skin for grafting, these are then re-applied to cover full-thickness wounds. Techniques are therefore available for large-scale cell culturing and amplification for use in the clinical setting.

In summary, we have demonstrated that the intradermal injection of autologous dermal fibroblasts significantly improves the healing of incisional wounds that are impaired by ionizing radiation. This suggests that damage to native fibroblasts is likely an important factor leading to impaired healing in irradiated surgical wounds. Implanted normal fibroblasts are likely better able to meet the proliferative and migratory demands of the wound healing environment, and are able to synthesize and remodel collagen that is better able to withstand the mechanical requirements of the healing surgical incision. Further studies with composite cell implants that would be better suited to the clinical surgical wound than cell injection are warranted. Autologous dermal fibroblast implantation may be a viable clinical option and may be applicable to a wide range of cancer patients who experience high rates of wound failure as a result of combined radiotherapy and surgical management.
FUTURE STUDIES

Along with radiation effects on fibroblasts, the development of surgical "dead space" likely contributes to wound complications after cancer excision surgery. Excision of soft tissue sarcomas, for example, often leaves a large void within the wound under the skin closure. A similar situation is evident following lumpectomy for large breast cancers, radical neck dissection or abdomino-perineal resection. The space remaining after cancer resection extends the volume of the surgical wound that must be filled by granulation tissue and subsequent scar. Standard clinical techniques for dealing with this problem of "dead space" include multiply layered wound closure, prolonged wound drainage and compression of wound surfaces. More invasive techniques include transferring vascularized muscle tissue from another anatomical site to fill the space left by tumour excision (for example rectus abdominis muscle transfer to the thigh or pectoralis major muscle transfer to the head and neck).

These methods are far from universally successful and in the case of tissue transfer result in donor site morbidity and prolonged surgery. A tissue engineering substitute for vascularized tissue transfer would be a tremendous benefit for patients receiving preoperative radiation. This report demonstrates that transplantation of autologous fibroblasts enhances healing after irradiation. It can be hypothesized that wound healing would be further improved if fibroblasts could be distributed through the surgical site, especially if they were seeded on a bio-absorbable carrier that would initially fill the "dead space" in the wound.

Several biomaterials have been studied for their effect on fibroblast function. The ideal material to enhance wound healing would be one that would promote some or all of
fibroblast proliferation, migration and collagen synthesis. These are all important functions of the early wound healing fibroblast, and therefore would represent the ideal phenotype. The fibroblast phenotype is determined to a large extent by its extracellular matrix environment (Mauch et al., 1988; Grinnell, 1994; Clark et al., 1995) and fibrin has been shown to promote a fibroblast phenotype that, similar to the early wound fibroblast, is both proliferative and productive of collagen (Yamada et al., 1996).

Fibrin “glue” has been shown to decrease seroma formation in animal models (Eroglu et al., 1996; Wang et al., 1996; Kulber et al., 1997) and has been shown to increase the strength of irradiated bowel anastomoses (Saclarides et al., 1992). Fibrin matrix has served as a vehicle for attachment of autologous keratinocytes to burn wounds (Ronfard et al., 1991; Horch et al., 1994; Stark et al., 1994; Spilker et al., 1996). In vitro culture of dermal fibroblasts in non-retracting fibrin gels leads to prolonged steady proliferation (Tuan et al., 1996), or markedly increased proliferation (Gillery et al., 1989; Gillery et al., 1992; Gray et al., 1995) compared with collagen matrices. There is also vigorous collagen synthesis on fibrin matrices (Gillery et al., 1989; Gillery et al., 1992). Dermal fibroblasts are able to remodel a fibrin gel into a collagen-containing scar-like tissue in vitro (Tuan et al., 1996). In short, a fibrin matrix promotes a fibroblast phenotype similar to the early wound granulation tissue (Yamada et al., 1996). As fibrin provisional matrix is decreased early in irradiated tissue (Wang et al., 1996), implantation of fibroblasts in this material would most closely mimic the early wound healing scenario.

In an attempt to verify this impression that fibrin is the ideal substance, we have evaluated several potential bio-absorbable materials that might provide the “filling and seeding” effect desirable within a cancer excision wound. In testing these materials we
assumed that the best material would promote fibroblast proliferation in vitro, both to
demonstrate that proliferation of autologous cells would be possible in vitro prior to
implantation and as evidence that the material would promote fibroblast proliferation when
implanted in vivo. The materials assayed in semi-quantitative fashion for promotion of
dermal fibroblastic proliferation in vitro included: collagen sponges with and without
polyethylene glycol cross linkage, formic acid treated collagen sponges, agarose, alginate,
and fibrin. These materials have been tested by culturing $10^6$ rat fibroblasts in vitro on
the material for one week and then evaluating surface cell density using
histomorphometry.

Figures 11-14 are images representative of the typical fibroblast densities
throughout each of the materials. It is quite obvious that the fibrin gel contains the greatest
cell density of any of these materials. The materials agarose and alginate were fairly fluid
and likely inappropriate for use to implant in a wound. The other materials all were able
to maintain their shape. Further time course experiments using fibrin gels have shown that
surface fibroblast density is maximal three days after implantation and that fibroblasts
leave the surface of the fibrin to grow down into the substrate thereafter. Later cultures
also showed evidence of fibrin contracture as fibroblasts grew through the matrix. These
in vitro experiments suggested that proliferation of autologous fibroblasts in vitro for three
weeks followed by implantation in the wound three days after seeding on a fibrin implant
might be a realistic method for introducing a bioabsorbable carrier into the dead space that
would both fill the space and provide proliferating fibroblasts to the wound.
Figure 11 - Photograph of rat dermal fibroblast ingrowth into bovine fibrin clot 7 days after implantation (H&E, 100x). Cells were placed on top of a mature clot and cultured. Layering of cells on the surface and extensive growth into the stroma of the clot is noted.
Figure 12 - Photograph of rat dermal fibroblast ingrowth into plain bovine collagen sponge 7 days after implantation (H&E, 100x). Irregular layering of cells is noted with minimal ingrowth into the sponge.
Figure 13 - Photograph of rat dermal fibroblast ingrowth into polyethylene glycol-treated collagen sponge (H&E, 100x) 7 days after implantation. A single layer of cells is noted along the surface with minimal ingrowth into the sponge.
Figure 14 - Photograph of rat dermal fibroblast ingrowth into formic acid-treated collagen sponge (H&E, 100x) 7 days after implantation. Minimal growth of cells is noted along the surface of the sponge.
REFERENCES


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