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**BIOLOGY CONTRIBUTION**

# DEVELOPMENT OF A PORCINE DELAYED WOUND-HEALING MODEL AND ITS USE IN TESTING A NOVEL CELL-BASED THERAPY

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**Purpose: A delayed full-thickness wound-healing model was developed and used for examining the capacity of adipose-derived stem cells (ASCs), either alone or in platelet-rich fibrin gels, to promote healing.**

**Methods and Materials: Four pigs received electron beam radiation to the dorsal skin surface. Five weeks after radiation, subcutaneous fat was harvested from nonirradiated areas and processed to yield ASCs. Two weeks later, 28 to 30 full-thickness 1.5-cm$^2$ wounds were made in irradiated and nonirradiated skin. Wounds were treated with either saline solution, ASCs in saline solution, platelet-rich plasma (PRP) fibrin gel, ASCs in PRP, or non-autologous green fluorescence protein–labeled ASCs.**

**Results: The single radiation dose produced a significant loss of dermal microvasculature density (75%) by 7 weeks. There was a significant difference in the rate of healing between irradiated and nonirradiated skin treated with saline solution. The ASCs in PRP-treated wounds exhibited a significant 11.2% improvement in wound healing compared with saline solution. Enhancement was dependent on the combination of ASCs and PRP, because neither ASCs nor PRP alone had an effect.**

**Conclusions: We have created a model that simulates the clinically relevant late radiation effects of delayed wound healing. Using this model, we showed that a combination of ASCs and PRP improves the healing rates of perfusion-depleted tissues, possibly through enhancing local levels of growth factors.** 

**Adult stem cells, Wound healing, Disease models, Radiation injuries.**

## INTRODUCTION

The most challenging wounds to manage clinically are chronic wounds and wounds with poor healing potential because of pre-existing pathologies (*e.g.*, diabetes) or radiation-induced alterations of skin and subcutaneous tissues. In particular, postmastectomy breast reconstruction, neck dissections, and extremity sarcoma resections are fraught with complications such as slow healing, dehiscence, infections, excessive scarring, and poor cosmesis in up to 67% of procedures (1, 2). These problems likely result from late effects of radiation, such as fibrosis and loss of the skin microvascular network (3).

An improved understanding of normal and pathologic healing processes has resulted in implementation of a myriad of therapeutic modalities for improving wound healing. However, the beneficial effects of most of these agents have not been validated in late-stage clinical trials of efficacy. Development programs for novel therapies to treat complicated wounds are hindered by a paucity of preclinical animal models that closely mimic human pathophysiology, use current clinical practices, and most importantly, are predictive of the therapeutic potential of agents or devices. The anatomy and physiology of swine, particularly with respect to cutaneous blood supply and wound-healing characteristics, have made this species the

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standard model for plastic surgical and wound-healing studies (4, 5). Porcine skin, like that of humans, heals primarily through epidermal cell migration (4). Furthermore, it has also been shown that porcine skin responds to radiation in a similar time- and dose-dependent manner to human skin (6, 7).

The use of cell- and protein-based therapies, in particular, to enhance the healing process is rapidly expanding (4). Platelet-derived growth factor (PDGF), a well-known factor involved in normal wound healing, is approved in the recombinant form as a treatment for wounds (5). Vascular endothelial growth factor (VEGF-A) and stromal cell–derived growth factor 1$\alpha$ (SDF-1$\alpha$) have also been shown to accelerate wound healing in animal models (8, 9). A major shortcoming is that these treatments only supply individual factors in short boluses and do not take advantage of the complex interplay of the multitude of factors involved in wound repair. Theoretically, cell-based therapies are an advancement in that pluripotent stem or progenitor cells assist healing through both regenerating lost tissues at the wound site and providing a sustained source of many beneficial factors (10, 11).

Adipose-derived stem cells (ASCs) are abundant and easily isolated therapeutic cells present in the non-adipocyte (stromal) fraction of adipose tissues. In addition to their capacity to differentiate into cells of mesodermal, endodermal, and ectodermal origin (12, 13), ASCs secrete many potentially beneficial growth factors and cytokines (12, 14). Notably, though, ASCs do not produce PDGF, and therefore supplementation of this factor may enhance the beneficial effects of ASC, especially in wound healing.

Platelet-rich plasma (PRP) possesses multiple characteristics that make it an ideal adjuvant to ASCs for promoting repair of damaged tissues. In particular, PRP is a rich source of growth factors and cytokines involved in wound repair, including PDGF. After clotting, platelet concentrates form insoluble fibrin matrices that may be useful for containing ASCs at the wound site. Interestingly, there is a dose response between platelet concentration and proliferation of human adult mesenchymal stem cells and fibroblasts, as well as production of Type I collagen by these cells (15).

In a previous study, we used healthy, normal juvenile swine to show the safety and neovascularization benefit of ASCs embedded in PRP-derived fibrin matrix in a full-thickness wound model (16). Here, we extend those studies by showing a significant improvement in healing rates and repaired tissue quality of irradiated healing skin by ASCs embedded in a PRP matrix. In addition, we show that the porcine model of delayed wound healing developed for this study could be useful for evaluating the therapeutic potential of new treatment modalities in a system that is physiologically similar to human skin and uses modern clinical procedures.

## METHODS AND MATERIALS

### *Phase I study: Dose determination and microvasculature effects study*

*Radiation injury.* Animal care was performed according to the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals, and the protocol was approved by the institutional animal care committee. In the first phase of the study, pigs were irradiated to determine dose tolerance and microvascular effects of a single dose of electron beam radiation on porcine skin. Three female Yorkshire pigs weighing 30 to 35 kg were used. Once anesthetized via inhaled isoflurane, the pigs were transported to the Department of Radiation Oncology, Indiana University Cancer Center. Each pig received a single fraction of 16, 18, or 20 Gy with 6-MeV electrons to an 18 × 40–cm field on each side of the paraspinal dorsal skin surface of the animal, generated by a Siemens Mevatron linear accelerator (Erlangen, Germany). The radiation level was calculated to ensure that greater than 90% of the prescribed dose would be limited to a maximum depth of 2 cm. Doses were verified by use of silicon diode dosimeters. The borders of the fields were marked out with pen and then subsequently tattooed to allow for precise delineation of the treated area as the pig grew in the weeks after treatment. Afterward, pigs were extubated and recovered before returning to their cages.

*Skin assessment.* The pigs were housed for 12 weeks to allow late effects of radiation to develop. Trained personnel examined the skin to determine the degree of erythema and the presence of moist or dry desquamation, as well as any untoward systemic effects. The pigs were fed a standard laboratory diet, cleaned, weighed, and monitored daily. Every 7 days, they were sedated with ketamine and xylazine, and two 8-mm punch biopsy specimens of skin in the irradiated fields were aseptically taken. Biopsy specimens were also taken from normal, nonirradiated skin.

*Immunohistochemical analysis of skin microvasculature.* All biopsy samples were fixed in 10% formalin, embedded in paraffin, sectioned, and then examined histologically. The 5-$\mu$m sections from each punch biopsy specimen were stained with anti–smooth muscle $\alpha$-actin (clone 1A4; Sigma-Aldrich, St. Louis, MO) to determine vessel density according to a standard three-step immunohistochemical procedure (17). After washing, sections were incubated with conjugated antimouse IgG antibodies (Vector Elite Kit PK-6102; Vector Laboratories, Burlingame, CA), followed by development with diaminobenzidine, and counterstained with hematoxylin A. Adjacent sections were stained with nonimmune IgG from the same species by use of the same dilution as the primary antibody as negative controls. Sections from normal porcine skin served as positive controls. Each section was examined microscopically for the number of positively stained lumen-containing vessels in ten fields per slide, which were averaged together for sections. Within-group and between-group means were compared with respect to time, treatment, and irradiation. Significance was detected with a two-tailed $t$ test.

### *Phase II study: Treatment of delayed-healing wounds with ASC and PRP*

*Delivery of 20 Gy radiation.* A similar protocol to that in Phase I was used. Female Yorkshire pigs ($n = 4$), weighing 30 to 35 kg, received a single 20-Gy dose. They were then maintained on a standard porcine laboratory diet without intervention for 7 weeks, allowing time for the late effects of radiation.

*Autologous adipose tissue harvest and processing.* Five weeks after the radiation treatment and 2 weeks before wound creation, the pigs were anesthetized for adipose harvest through a 10-cm-long × 3-cm-deep incision in the nonirradiated dorsal hump, followed by adipose tissue excision (approximately 30 g) with a No. 10 blade scalpel. Adipose tissue from transgenic Yorkshire pigs expressing green fluorescence protein (GFP) under control of the ROSA26 promoter (National Swine Resource and Research Center,