



# Model of radiation-impaired healing of a deep excisional wound

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## ABSTRACT

Despite many well-recognized benefits, administration of ionizing radiation before surgical resection of malignancies is associated with a high risk of wound-healing complications. Most animal models investigating techniques to improve wound healing use a superficial wound. The goal of this study was to develop a novel model of radiation-impaired healing using a deep excisional wound, which is closer to the clinical situation. In the first part of this study, female Lewis rats were exposed to 0, 12, 15, or 18 Gy single-fraction radiation to the buttocks. Three weeks later, deep wounds were created by excision of the gluteus maximus muscle. Irradiated wounds had a lower rate of healing of the surgically created defect than unirradiated wounds ( $p < 0.001$ ), but there was no significant difference between the different doses of radiation. Impaired healing was still evident at 12 weeks. The second part of this study investigated the ability of porcine small-intestinal submucosa (SIS) to improve healing in this animal model. At 6 weeks, wounds implanted with SIS showed improved healing at all doses of radiation compared with unimplanted irradiated wounds. However, higher doses of radiation were still associated with a lower rate of healing. SIS induced a cellular response that was not evident in defects that did not receive SIS, suggesting that SIS has the potential to stimulate repair. This reproducible model of radiation-impaired wound healing closely resembles the clinical setting. The results indicate that this model can be used to investigate new biomaterials as possible therapeutic agents to enhance wound healing.

Surgical treatment of extremity soft tissue sarcoma has historically involved amputation or radical resection of entire muscle compartments. Current treatment protocols of extremity soft tissue sarcoma and other solid tumors have evolved to include combined management using both adjuvant radiation and surgery. This approach has allowed for preservation of functional tissue while achieving local tumor control rates equal to or better than radical surgery alone.<sup>1-11</sup>

Protocols for combined management include administration of radiation either before or after surgery. Both pre- and postoperative radiation protocols provide similar local control of sarcoma,<sup>3,12-15</sup> but there are two advantages to using preoperative irradiation. The usual radiation dose used for treatment before surgery, e.g., 50 Gy in 25 fractions, is lower than the standard postoperative dose (60-66 Gy in 30-33 fractions).<sup>2,3,6-8,15</sup> Furthermore, a smaller volume of normal tissue is exposed to radiation injury when adjuvant treatment is administered before surgery.<sup>12</sup> These factors may lead to a decrease in late radiation fibrosis and edema<sup>16</sup> and may improve functional outcome.<sup>17</sup>

The major disadvantage of preoperative irradiation is an increased incidence of subsequent wound-healing com-

plications. Excision of extremity sarcomas after irradiation is associated with a substantial risk of wound complications, many of which require second surgical procedures.<sup>13,18-22</sup> A recent prospective-randomized study comparing pre- and postoperative radiation therapy in extremity soft tissue sarcoma showed a twofold increase in wound complications in the preoperatively irradiated group (35% incidence of wound complications in patients randomized to receive preoperative treatment vs. 17% in patients receiving radiation following surgery).<sup>23</sup> Wound-healing complications are a major source of morbidity for patients receiving combined treatment and have detrimental effects on functional outcomes.<sup>10,24</sup>

Several factors contribute to impaired wound healing after irradiation. Radiation administered preoperatively has been shown to decrease the mechanical strength of the surgical wound in superficial incisional wound models.<sup>25</sup> Surgical resection of large soft-tissue sarcomas, as well as other solid tumors such as rectal, and head and neck

H&E Hematoxylin & eosin  
SIS Small-intestinal submucosa

carcinomas, often results in a large, deep surgical defect. Accumulation of hematoma and wound fluids in these defects can prevent the adherence of superficial to deep healing tissues. This large residual “dead space,” in combination with the decreased superficial wound strength, likely contributes to the high incidence of wound complications observed following preoperative radiation.

It is possible that filling this dead space may improve healing. One potential biomaterial that could be used is small-intestinal submucosa (SIS), a type I collagen-based xenogenic membrane produced from porcine small intestine. SIS has been shown to be effective in the reconstruction of bladder,<sup>26</sup> blood vessels,<sup>27</sup> small bowel,<sup>28</sup> abdominal wall,<sup>29</sup> tendon,<sup>30,31</sup> and other tissues. It supports blood vessel ingrowth.<sup>32,33</sup> Other investigators have shown that SIS does not induce an immunogenic reaction upon implantation and is remodeled to resemble normal connective tissue rather than scar tissue.<sup>29,31,34</sup> This effect may be due to the presence of growth factors that promote tissue repair, such as transforming growth factor- $\beta$ <sup>35</sup> and fibroblast growth factor-2.<sup>36,37</sup>

The majority of preclinical investigations designed to evaluate methods of improving impaired wound healing have used superficial excisional or incisional wounding models. The clinical scenario in clinical cancer surgery, however, is quite different in that tumors are removed with the creation of defects deep to fascia. The purpose of this study was to develop a model of radiation-impaired healing in a deep excisional wound. In addition, a potential therapeutic intervention, SIS, was placed in the defect and its effect on wound healing was evaluated.

## MATERIALS AND METHODS

Ethical approval for animal surgery was obtained from the Animal Care Committee of the Ontario Cancer Institute.

Female Lewis rats (Charles River, Saint Constant, QC, Canada) weighing 200–224 g were anesthetized with inhalational isoflurane. The animals were placed in a shielded Plexiglas box with positioning jigs that allowed a 2.5 cm diameter circular region of tissue over each buttock to be sequentially exposed to single fraction external beam radiation at a source rate of 10.2 Gy/minute. Both buttocks of female Lewis rats received a single fraction of 12, 15, or 18 Gy ionizing radiation ( $n=10$  animals/group).

Three weeks after irradiation, the animals underwent surgical wounding. Cefazolin (10 mg/kg) and 3 cm<sup>3</sup> normal saline fluid bolus were administered preoperatively by subcutaneous injection. The operative procedure was performed using a sterile technique under a laminar airflow hood. The animals were anesthetized, and a 2.5 cm full-thickness dorsal longitudinal skin incision was made over each buttock centered in the radiation field. The irradiated field was easily discernable as a result of hair loss. Medial and lateral flaps were created by elevating the skin and subcutaneous tissue off the underlying muscle. The lateral mass of the gluteal muscle was divided using cautery, and the sciatic nerve was identified and dissected off the underside of the gluteal muscle. The deep surgical defect was created by excising en bloc the entire gluteus maximus muscle from lateral to medial, while preserving the sciatic nerve. The resected muscle was weighed on an electronic scale to ensure uniformity in the size of the surgical defects.

One gram of muscle was resected in each case. Hemostasis was obtained, and the skin was closed with three staples. Perioperative analgesia was provided with a subcutaneous injection of 0.1 mL of Temgesic. Postoperatively, animals were housed separately. The animals were euthanized at either 6 or 12 weeks after surgery. Ten animals served as controls and although they underwent creation of surgical defects, they did not receive preoperative irradiation.

For the animals receiving implanted biomaterial, animals were treated as described above, except that sterile, lyophilized SIS (five sheets, 2×1.5 cm, obtained from Cook Biotech Inc., West Lafayette, IN), was placed, in a layered configuration mucosal side down, in the defect on one side before skin closure. The defects in the contralateral side did not receive SIS.

## Wound evaluation

The ventral skin from the thigh region was removed and the tissues were fixed in 10% formalin for 24 hours. Parallel longitudinal sections through the wound and surrounding tissues were obtained. The dorsal skin overlying the defect was retained to keep the tissues together. Animals were scored as visually showing residual surgical defects or showing no evidence of the surgical defect. Representative tissue sections were embedded in paraffin, and 5  $\mu$ m sections were cut and stained with hematoxylin and eosin (H&E).

The histological sections were examined and digital images were captured. The sciatic nerve was identified in each case and used to landmark and orient the specimen. A standardized Adobe PhotoShop (San Jose, CA) 2-cm grid template was centered over the sciatic nerve to ensure a minimum length of six grids overlying the defect. Wounds were classified as either healed or unhealed. An unhealed defect was defined as a persistent cavity with a thin fibrous lining. A healed defect had no cyst cavity.

For animals that received SIS bioimplants, incorporation of the implant into soft tissue, filling of the defect, and host response to the material were assessed histologically. Incorporation was defined as complete adherence of the SIS to the underlying muscle due to host tissue ingrowth and there was no delamination of the SIS. The implant was considered not incorporated if there was no adherence of the implant to the wound base or if the implant was extruded or delaminated during sectioning. Complete defect fill was defined as obliteration of the defect by the implant with complete adherence to both the underlying muscle and overlying panniculus carnosus and dermis. Partial defect fill was defined as adherence of the SIS implant to the underlying muscle but without complete adherence of the overlying dermis.

Cellular ingrowth into the implant was expressed as the percentage of cross-sectional area of implant containing granulation tissue (fibroblasts, inflammatory cells, or blood vessels) as measured using a standardized Adobe PhotoShop histomorphological grid.

## Statistical analysis

Analysis of significance was performed using SPSS (Chicago, IL) to perform a chi-square test of independence for

the four levels of radiation. Individual chi-square using Bonferroni's correction for multiple tests was also performed. ANOVA was used to evaluate significance for cellular ingrowth.

## RESULTS

Of the 80 animals in the study, five animals had to be euthanized before completion of the study and were excluded from subsequent analysis. These five animals, two of which received 18 Gy and three of which received 15 Gy, experienced early wound dehiscence. The remaining animals tolerated the procedure well and were fully mobile within their cages.

### Effect of radiation on wound healing

Radiation affected the extent of healing. Ninety percent of animals that received 18 Gy had unhealed defects at 6 weeks (Figure 1A). One specimen was damaged during sectioning and that animal was excluded from the analysis. Histologically, an unhealed defect consisted of a cavity lined by a thin layer of fibrous tissue (Figure 2A). There was no attempt at repair, no evidence of granulation tissue formation, and no vascular proliferation was seen. These histologic findings are typically seen as a result of ionizing radiation. At 15 and 12 Gy, 70 and 60% of the animals, respectively, had residual unhealed defects. Only one ani-

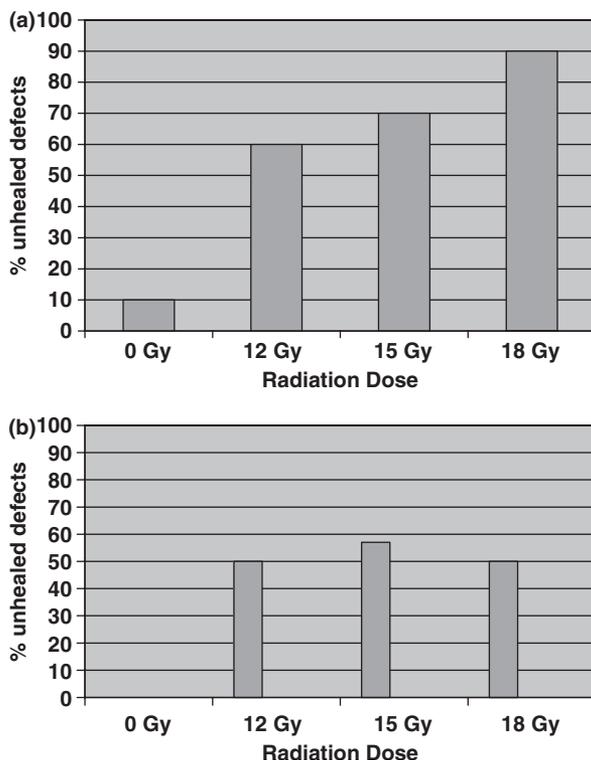
mal in the group of 10 that did not receive irradiation had a nonhealed defect and this was associated with the presence of postsurgical hematoma. When there was healing of the wound, this was characterized histologically by fibrosis and obliteration of the cystic cavity (Figure 2B). The proportion of unhealed defects in the irradiated and nonirradiated animals was statistically different (Pearson's  $\chi^2$  (3) = 18.750,  $p < 0.001$ ). The rate of nonhealing was lower in the 0 Gy group when compared with the 15 and 18 Gy group ( $p=0.03$  and  $0.005$ , respectively). At 12 weeks, the animals that received 12, 15, and 18 Gy had residual cavities in 50, 57, and 50% animals, respectively (Figures 1B, 2C, D). There was no significant difference between the groups.

### Effect of SIS on wound healing

Gross examination 6 weeks post-implantation revealed that all animals that received 0 and 12 Gy of radiation had some incorporation of the SIS, whereas 80% of animals that received 15 and 18 Gy showed some degree of incorporation (Figure 3). There was no difference between the groups in this regard. In the unirradiated animals, 20% had residual defects, while the animals that received 12 Gy had no residual defects. Forty percent of animals that had received 15 Gy and 60% of those that received 18 Gy had persistent cystic cavities (Figure 3). There was a significant difference between the 12 and 18 Gy groups in the number of residual defects. Comparison between the animals that received radiation and SIS implants and those that received no implant showed a significantly higher rate of unhealed or partially healed defects in the group without SIS at all doses of radiation.

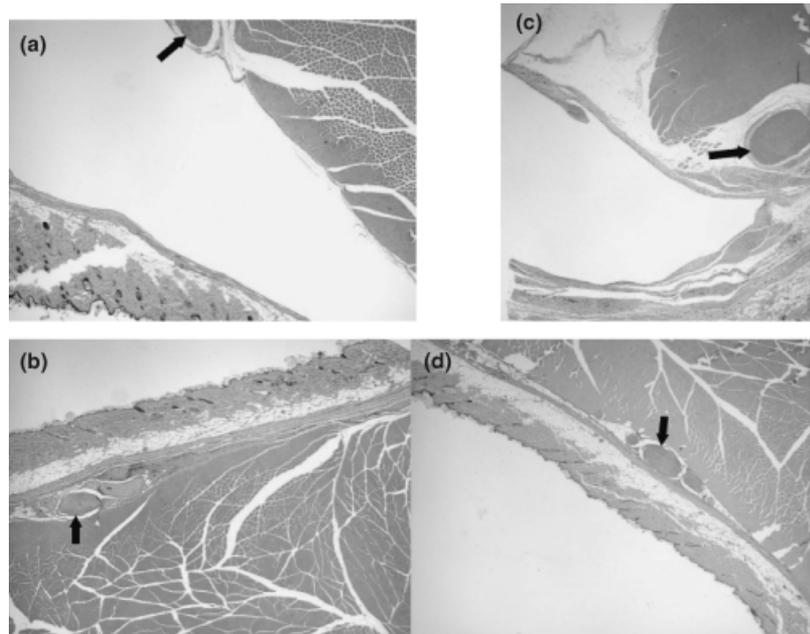
At 12 weeks, all defects in the unirradiated animals or those that received 18 Gy of radiation were filled. There was a 10% rate of unhealed defects in the animals that received 12 Gy, and in those that received 15 Gy, 29% of animals had unhealed defects. There was no significant difference between these groups.

Histological examination of the 6-week group revealed that the incorporated SIS implants displayed ingrowth by either fibroblasts or granulation-type tissue and this was associated with a mild to moderate chronic inflammatory infiltrate (Figure 4A, B). Only one implant in the 18 Gy group was infiltrated by acute inflammatory cells (polymorphonuclear cells), suggesting the presence of infection. There was more complete cellular ingrowth throughout the whole cross-sectional area of the implant (determined by digital histomorphometry) at lower radiation doses (ANOVA,  $F(3,40)=4.317$ ,  $p=0.01$ ). The 0 and 12 Gy groups (mean % area ingrowth was 100% and 97%, respectively) had significantly higher total area cellular ingrowth compared with both the 15 and 18 Gy groups (the mean % area ingrowth was 82% and 85%, respectively;  $p < 0.05$  post hoc multiple comparisons, Figure 5). A mononuclear cell infiltrate was evident in all incorporated SIS implants at 6 weeks. There was a tendency toward less complete cellular infiltration throughout the whole implant at the 18 and 15 Gy radiation level at 6 weeks. When the infiltrate was not uniform, it tended to be most abundant at the host-implant interface. At 12 weeks, the inflammatory response had subsided and breakdown of the SIS with ingrowth of fibroblasts and capillaries was



**Figure 1.** Effect of preoperative radiation dose on wound healing at 6 and 12 weeks. (A) 6-week observations; (B) 12-week analysis.

**Figure 2.** Photomicrographs of irradiated wound tissue at 6 and 12 weeks postoperative. (A) Photomicrograph of irradiated wound 6 weeks after muscle resection. The overlying skin is intact and the defect cavity is lined by a thin layer of fibrous tissue. (B) Photomicrograph of unirradiated wound at 6 weeks showing healing of the defect with fibrous tissue. (C) Photomicrograph of irradiated wound 12 weeks after resection showing a cystic cavity lined by fibrous tissue. Hemosiderin deposition is present. There is no evidence of healing. (D) Photomicrograph of an unirradiated wound at 12 weeks showing a healed defect with a very thin layer of fibrous tissue overlying the sciatic nerve. The arrow in each panel highlights the sciatic nerve. All sections were stained with H&E (original magnification  $\times 12.5$ ).



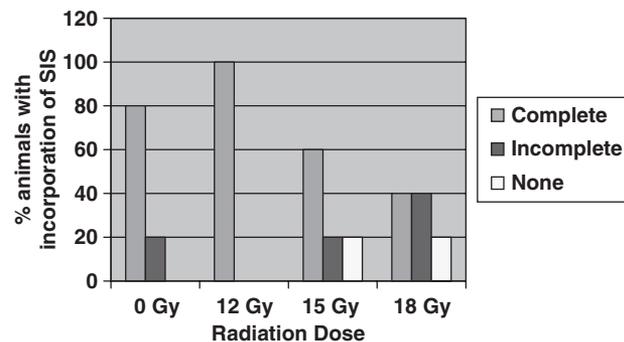
evident (Figure 4C, D). SIS was still present in all animals even at 12 weeks.

**DISCUSSION**

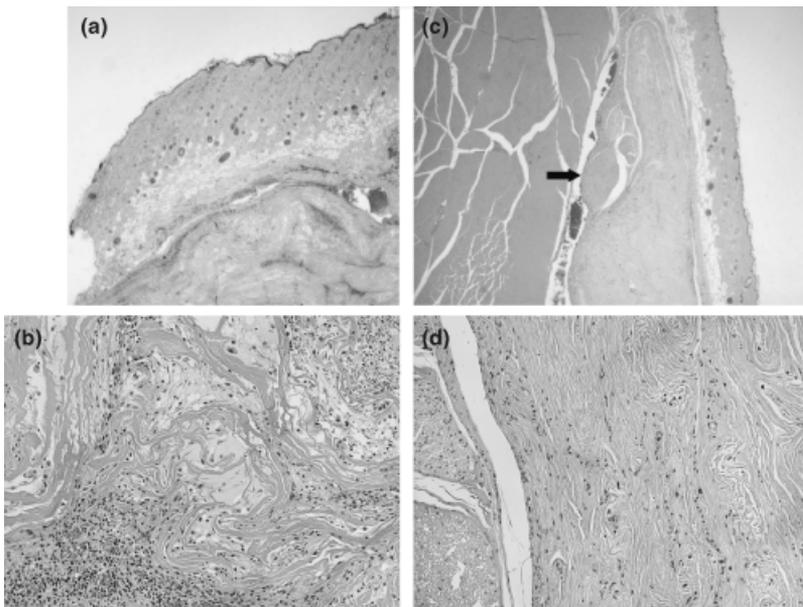
The primary goal of this study was to develop a model of deep wound healing after tissue resection and irradiation. In the clinical scenarios of soft tissue sarcoma, head and neck, rectal and breast carcinoma, altered deep wound healing in the presence of irradiation is a major cause of patient morbidity.<sup>23,38-41</sup> Impaired healing is often manifested as seroma or hematoma formation, chronic wound drainage with sinus formation, and persistence of surgically created “dead space.” Establishment of an animal model that resembles the clinical situation is important to be able to evaluate potential therapeutic modalities to overcome these complications.

The animals were handled consistently in this study to create a reproducible model. Each animal was positioned using the exact same technique and jig to ensure consistency. A standard dose of radiation was utilized and a constant volume of gluteus maximus was resected. The wounds were closed in exactly the same fashion and staples were removed on the same day. We rigorously examined various methods empirically for histological evaluation of a deep excisional wound. The technique that we utilized in this study was found to be effective for examination of an entire deep wound in situ without disturbing the tissue structure during processing. The fact that in the unirradiated animals the wounds were universally healed histologically, whereas in the animals that received 18 Gy the wounds were consistently not healed confirms the reproducibility and robustness of the model.

This study showed that reproducible deep wound defects are present 6 weeks postoperatively when 18 Gy of external beam radiation was administered preoperatively. In a previous study, we had evaluated increasing single-fraction doses of radiation between 10 and 20 Gy to determine the effect on biomechanical strength of wound healing. We determined that at 18 Gy, there was a marked reduction in the strength of the healing wound at 3 weeks.<sup>25</sup> We selected the 12 and 15 Gy doses in this experiment to investigate whether those with minimal biomechanical effect were still associated with histological evidence of impaired healing. There appeared to be improved healing with decreasing radiation doses, but a significant number of animals still had persistent defects. Only the unirradiated animals showed nearly universal healing of the deep wounds. The persistent defects at 6 weeks were demarcated by a thin fibrous lining with a paucity of granulation tissue or inflammatory infiltrate. The defects that healed did so primarily by collapse of the subcutaneous tissue onto the underlying muscle with



**Figure 3.** Effect of radiation doses on incorporation of SIS into healing wounds at postoperative week 6.



**Figure 4.** Photomicrographs showing incorporation of SIS into irradiated wounds at postoperative weeks 6 and 12. (A, B) Photomicrograph of irradiated wounds 6 weeks after implantation of SIS showing ingrowth with granulation tissue and a mild to moderate chronic inflammatory infiltrate. (C, D) Photomicrograph of irradiated wound 12 weeks after implantation of SIS. There has been resolution of the inflammatory response with breakdown of the SIS and ingrowth of fibroblasts and capillaries. The arrow indicates SIS (H&E; original magnification: A, C  $\times 12.5$ ; B, D  $\times 100$ ).

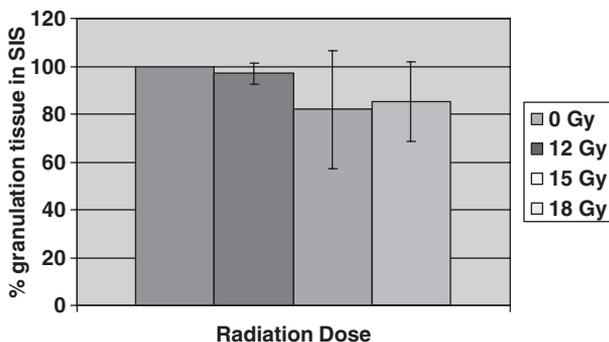
primarily fibrous healing histologically. This repair was similar for those animals that received radiation and those that did not.

Current preoperative radiotherapy protocols use dose fractionation to limit the effect on normal tissue while maximizing the effect on tumor cells. For impaired wound healing, an isoeffect curve has been defined in a rodent model. Gorodetsky et al.<sup>42</sup> have 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 on wound healing of typical preoperative sarcoma irradiation<sup>43</sup> is most closely mimicked by the single dose of 18 Gy in this experiment. The smaller doses of 12 and 15 Gy would be expected to have less detrimental effects on the wound-healing process and for this reason it was not surprising to observe repair in some animals at the lower radiation doses. The absence of wound healing may be due to several factors. Radiation may inhibit the animals' ability to resorb effectively the resultant seroma, preventing collapse of the subcutaneous layers onto the underly-

ing remaining muscle. The seroma may contain inhibitors of wound healing.<sup>44,45</sup> Ionizing radiation may inhibit the inherent ability of the panniculus carnosus to contract, which may be detrimental to dead space closure.<sup>45</sup> Ionizing radiation causes fibroblasts to exhibit reduced proliferation,<sup>46</sup> abnormal migration,<sup>47</sup> and both qualitative<sup>48</sup> and quantitative<sup>49</sup> abnormalities in collagen synthesis. It is clear that the combination of preoperative irradiation and deep muscle excision alters the normal woundhealing process.

The rat has some anatomic differences when compared with humans. For example, in human skin, the dermis is contiguous with the underlying superficial fascia whereas rats lack this tethering and the skin is freely mobile over the fascia. However, rats have been used by others to evaluate interventions to improve wound healing in irradiated skin, and their use is justifiable in this model.<sup>25,50</sup> Furthermore, the process of dermal wound healing is similar in all higher vertebrates.<sup>51</sup>

The 3-week interval between radiation and surgery simulates the clinical situation. It is generally accepted that a delay before surgery of approximately 3–6 weeks after completion of irradiation is necessary to allow any acute effects on the skin to diminish. The interval between surgery and 6-week histologic evaluation corresponds with the approximate time at which patients experience postoperative wound failure (R. S. Bell, personal communication). This also represents the time when there is most active collagen synthesis and contraction by myofibroblasts.<sup>52</sup> It is the time of most rapid gain in wound strength, when wound-healing complications are most apparent, that the effect of an intervention designed to improve wound healing would have the most apparent clinical effect. A delay in healing as a result of irradiation would likely be evident histologically at this time point. Although wounds do appear to heal clinically at time points later than 6 weeks, it is the early failures that cause problems and are of interest in this report.



**Figure 5.** Effect of radiation dose on development of granulation tissue in SIS implants.

At 12 weeks postoperatively, there were persistent defects in approximately 50% of the animals at all levels of irradiation, which was lower than at 6 weeks. This suggests that progressive healing and collapse of the dead space continues to occur in some animals after the initial 6-week interval. There does, however, continue to be factors that delay or impair the wound-healing sequence.

The lack of healing may be due to factors other than irradiation. For example, ischemia of wound edges may occur as a result of excessive retraction. This may result in necrosis of the wound edges, with resultant wound breakdown. No necrosis was seen at the wound edges histologically in these animals, suggesting that this was unlikely. Infected wounds with ongoing inflammation show impaired healing.<sup>53</sup> Although irradiated skin does show an increased preponderance for infection,<sup>54</sup> only one animal had evidence of an infection at the termination of the experiment.

There are inherent difficulties in quantifying the exact size of the wound defect in this animal model. A deep defect is a three-dimensional potential space and the dimensions of a residual defect may vary with animal movement. For this reason, we did not attempt to quantitate the size of the residual defect. In addition, care must be taken during the fixation, dissection, and sectioning of these soft tissue specimens so as to not disrupt the underlying architecture. In order to preserve the integrity of the defect, the specimen should be fixed before sectioning. This permits the dorsal skin and defect to remain intact. The critical size for impaired healing of a soft tissue defect is not known but clearly this study showed that it was attained with this extent of surgical resection.

Although surgical dead space can be managed by watertight surgical closure and prolonged wound drainage to remove accumulating seroma, these techniques are not always sufficient and may not enhance the healing potential of the underlying wound. More complex methods of deep wound management such as vascularized free tissue transfer are available<sup>55,56</sup> but carry significant risk of complications and morbidity.<sup>57</sup> The development of a bio-implant that fills dead space and enhances the healing process biologically would be a major contribution to surgical practice.

To confirm the utility of the animal model to evaluate implanted materials, wounded animals were treated with SIS. Histologic examination of SIS-implanted wounds at the 6-week point revealed fibrovascular granulation tissue at all doses of radiation, in contrast to the unimplanted irradiated wounds, which were devoid of such a response. This indicates that the wounds produced in this model do have the ability to mount a healing response under the right circumstances, and that implantation of the SIS provides an adequate stimulus to do so. There was a high rate of incorporation of the SIS implants into the defects (80% of specimens at 18 and 15 Gy). In the animals where the implant did not incorporate, there was no inflammatory response noted in either the graft or host. The unincorporated grafts supported a sparse population of fibroblast-like-looking cells. Unlike other studies investigating SIS as a replacement for musculoskeletal tissues,<sup>30,58</sup> the SIS implants in this model were not sutured into place and animals were not immobilized. It is possible that mobility of the implant within some animals may have prevented

host-implant adherence. Our results suggest that adherence of the SIS to native tissue is necessary to stimulate the normal healing response.

There was a trend toward a more complete defect fill with SIS at the lower doses of radiation at 6 weeks, with the 12 Gy group showing a complete defect fill in all specimens. This study utilized five individually layered SIS sheets as an implant construct, which may not have been sufficient to fully fill the defect and allow implant contact with the surrounding tissues after wound closure. Previous work utilizing SIS as a wound dressing in full-thickness rodent wounds showed a decrease in wound contraction compared with controls.<sup>59</sup> SIS may therefore inhibit tissue contraction and prevent adherence. At 12 weeks postoperatively, the SIS remained although there appeared to be some breakdown of the material. It is not clear why regeneration of normal tissues did not occur in the animals in our study. Radiation may have certainly played a role but this phenomenon was also seen in the unirradiated animals. Although implantation of SIS did not result in regeneration of normal tissue, our results suggest that it has the potential to enhance repair of irradiated deep wound but further study is required to identify the appropriate conditions that would favor tissue regeneration.

The presence of an inflammatory reaction to the implanted SIS was not unexpected. Studies of SIS implants for other biologic tissue repair report a similar mononuclear infiltrate within 2 weeks of implantation, diminishing by 4–6 weeks with subsequent resolution of the inflammatory response.<sup>60</sup> In this study, the inflammatory response had completely resolved by 12 weeks, making it unlikely that chronic inflammation would occur in the clinical situation.

In summary, we have developed a reproducible radiation healing-impaired deep wound animal model. SIS was shown to stimulate a favorable deep wound-healing response in irradiated tissue, confirming that this model can be used to evaluate agents or materials that have the potential to enhance wound healing. An implant that can fill a deep wound defect and stimulate healing would be useful in the combined management of soft tissue sarcomas and other solid tumors.

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