Adult Skin Wounds in the Fetal Environment Heal with Scar Formation

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Objective

This study investigated the influence of the fetal environment on the healing characteristics of adult skin.

Summary Background Data

The remarkable ability of the fetus to heal without scarring is poorly understood. The unique qualities of fetal wound healing may be caused by the fetal environment, the fetal tissues, or a combination of both. There are numerous differences between the prenatal and postnatal environments that may play a role in the unique fetal response to injury.

Methods

Full-thickness adult sheep skin was transplanted onto the backs of 60-day-gestation fetal lambs (term, 145 days of gestation). The adult skin grafts were thus perfused by fetal blood and bathed in amniotic fluid. Previous work has demonstrated that, before midgestation, fetal lambs do not reject allogeneic skin grafts. Forty days later (100 days of gestation), incisional wounds were made on both the adult skin graft and the adjacent fetal skin. The wounds were harvested 14 days postwounding and analyzed by both light microscopy and immunohistochemical testing using antibodies to collagen types I, III, and VI.

Results

The wounds in the adult skin grafts healed with scar formation. This observation contrasts strongly with the scarless healing of the incisional fetal skin wounds.

Conclusions

This study suggests that scarless fetal skin healing properties are intrinsic to fetal skin and are not primarily the result of the fetal environment.

Fibrosis and scarring after injury or surgery, with their limitations on function, growth, and appearance, carry potentially devastating consequences in all organ systems. The fetus is uniquely capable of healing skin wounds without scar formation and provides a model of ideal tissue repair. Understanding the biology of this process may allow us to modulate wound healing in children and adults to become more fetallike. This possibility has spawned a recent research interest in fetal wound healing. These studies have described the scarless na-

ture of fetal repair in several species, and recent experience with both human fetal surgery^{5.6} and human fetal skin wound repair⁷ has supported these experimental findings. However, the mechanisms controlling scarless fetal healing remain largely unknown.

Scarless fetal skin healing may be unique to the fetal tissues, the fetal environment, or a combination of both. This study focuses on the effect of the fetal environment on adult and fetal tissue repair. The fetal wound environment is different from the wound environment after adult tissue injury, that is, the fetus is relatively hypoxemic⁸ and is immersed in warm, sterile amniotic fluid known to be rich in growth and trophic factors.9 This unique fetal milieu may play an important role in supporting the scarless fetal skin healing that occurs in fetal lambs. The purpose of this study was to address the role of the fetal environment in scarless wound healing. Can transposing adult skin into the fetal environment modulate its wound healing to become more fetallike? To pursue this, we transplanted either maternal or late-gestation fetal sheep skin onto early-gestation fetal lambs, subsequently wounded the grafts, and then examined the effect of the fetal environment on adult and late-gestation skin repair.

We hypothesized that adult wound healing may become more fetallike by placing the wounded adult tissue within the fetal environment. Initial attempts to place adult tissue into the fetal milieu, using a maternal Rouxen-Y small bowel loop or a maternal myocutaneous flap placed inside the uterus, failed because of amniotic fluid leaks, infection, and abortion. Consequently, we decided to take advantage of the immature fetal immune system. Previous work has shown that adult allogeneic skin grafts placed onto fetal lambs before 77 days of gestation are not rejected. 10-12 We therefore placed maternal (adult) skin grafts onto fetuses at 60 days of gestation, and 40 days later (100 days of gestation), incisional wounds were made in the adult skin graft and in the surrounding fetal skin. The epidermis of postnatal skin functions principally as a barrier (preventing dehydration and microbial invasion), and this barrier may obstruct any potentially beneficial effects of the amniotic fluid on the adult dermal wound healing. At 120 days of gestation, fetal skin may not act as a complete barrier. To determine if the early fetal environment could modify the healing characteristics of late-gestation fetal skin-which heals with scarring¹³—we transplanted 120-day-gestation fetal skin

onto 60-day-gestation fetal lambs and wounded them 40 days later, as described earlier. As a control for the effect of skin graft excision and transfer on wound healing, we also performed fetal autografts.

MATERIALS AND METHODS

Ten time-dated pregnant ewes (8 at 58 days and 2 at 118 days of gestation) were transported from Torrel Farms (Ukiah, CA) to the animal care facility and fed food and water *ad libitum*. The animals were fasted for 48 hours before surgery.

Skin Graft Transfer and Graft Wounds (Figs. 1 and 2)

The ewes at 60 days of gestation were immobilized by 1000 mg of ketamine (Vetalar, Parke-Davis, Morris Plains, NJ) and underwent induction of general halothane/oxygen anesthesia by mask, followed by placement of a number 10 oral endotracheal tube. A 16-gauge intravenous catheter (Deseret, Sandy, UT) was percutaneously inserted into the single jugular vein, and 500 mL of lactated Ringer's solution (Baxter Health Care, Deerfield, IL) containing 4×10^6 units of penicillin G (Pfizer, New York, NY) and 400 mg of kanamycin (Kantrim, Bristol-Meyers, Syracuse, NY) was infused during the procedure. The abdomen was shaved, aseptically prepared, and a 4 × 2-cm full-thickness maternal skin graft was harvested from the abdomen, defatted, and kept moist. Skin for grafting was always removed from the abdomen of the donor because its softer nature facilitated fixation to the fetal recipient. After a midline laparotomy to expose the uterus, a 5-cm hysterotomy was made with careful attention to secure all layers of the uterine wall and amniotic membranes. The fetal back was delivered into the operative field, and a 4×2 -cm area of fetal skin was excised to create the recipient graft bed (Fig. 3A). The maternal graft was copiously rinsed in warm, sterile saline and secured onto the fetal back using 6-0 Surgilene suture (Davis & Geck, Danbury, CT). A 2 × 2-cm piece of fetal skin was also autotransplanted onto the fetal back as a control for the effect of transplantation on wound healing (Fig. 3B). Amniotic fluid was restored, and the uterus was closed using a TA-90 stapler (US Surgical, Norwalk, CT). The laparotomy was closed in layers, the maternal graft site was closed primarily, and the ewe was returned to her stall.

Two 120-day-gestation fetuses were used as donors for the skin grafts. This was accomplished by fetal surgical techniques as described earlier. A 4×2 -cm full-thickness skin graft was removed from the 120-day fetal back after hysterotomy. These grafts were transplanted onto 60day-gestation fetuses using the techniques described.

Supported by an American College of Surgeons Fellowship, and National Institutes of Health grants HD 25505 and GM 27345.

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Accepted for publication June 11, 1993.

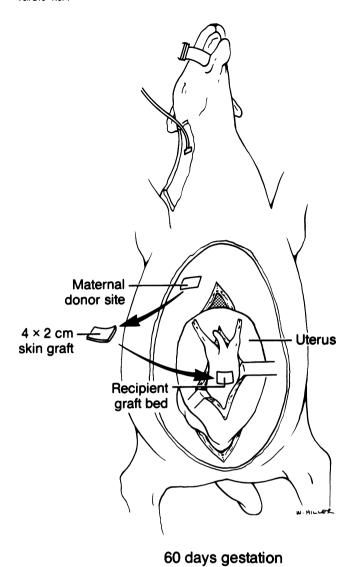


Figure 1. Line drawing of the skin transplant model. At 60 days of gestation, maternal skin was harvested from the abdomen and transplanted onto the exposed fetal back recipient graft bed.

Forty days later (100 days of gestation), the maternal ewes underwent general anesthesia as described earlier. After laparotomy and hysterotomy, the fetal hindquarters were exposed. The fetuses had grown in size approximately 300% during the 40-day interval. The wool-bearing maternal and 120-day-gestation fetal grafts were easily distinguishable from the surrounding fetal skin. By contrast, the fetal autografts were indistinguishable from the surrounding fetal skin and were marked only by four sutures (Fig. 3C). A 2-cm full-thickness incisional wound was made in both the maternal and fetal skin grafts and into the adjacent fetal skin, and the wounds were sutured closed with interrupted 6-0 Surgilene (Fig. 3D). The maternal and 120-day-gestation fetal grafts appeared healthy and bled when incised. There was no gross evidence of graft rejection or graft-versus-host disease. Am-

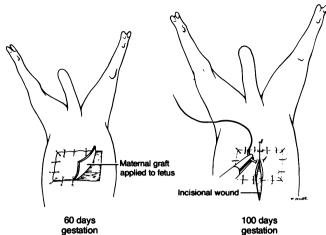


Figure 2. Line drawing of skin graft wounding. At 100 days of gestation, the fetus underwent reoperation, and both the adult and fetal skin grafts were incisionally wounded.

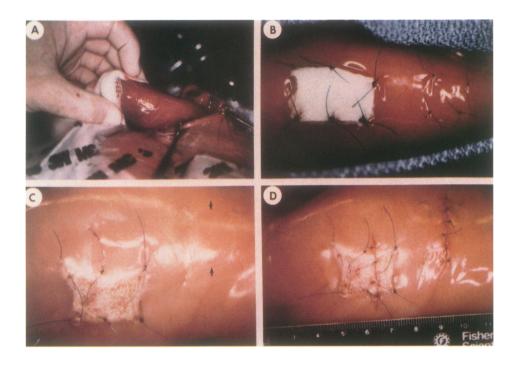
niotic fluid volume was restored with warm saline, and the hysterotomy and laparotomy wounds were closed. The ewe was returned to her stall.

Wound Harvest

Fourteen days after wounding, the maternal ewe underwent anesthesia, laparotomy, and hysterotomy. The fetuses were delivered into the field and killed by intracardiac Beuthanasia-D (Schering, Kenilworth, NJ) overdose. The wounds in both the adult and fetal skin grafts and surrounding fetal skin were excised intact. One half of each bisected adult and fetal wound was immediately snap frozen in precooled isopentane and stored at $-80 \, \text{C}$ for immunohistochemical analysis. The other half of each wound was formaldehyde-fixed and paraffin-embedded for histologic analysis.

Wound Analysis

Sections from the paraffin-embedded tissue were stained with either hematoxylin and eosin (H & E) or Masson's trichrome. A more detailed analysis of the collagenous architecture of the wounds was carried out using an indirect immunohistochemical technique. The frozen tissues were embedded in Tissue Tek (Miles Laboratories, Elkhart, IN), and 7- μ m sections were cut on a cryostat at -20 C. The sections were briefly fixed in acetone, air dried, and stained using affinity-purified polyclonal antibodies for collagen types I, III, and VI and fluorescein isothiocyanate-conjugated (FITC) secondary antibodies. The sections were covered with primary antibody in a humid environment for 1 hour at room temperature and then washed three times (for 5 minutes each) with phosphate-buffered saline (PBS). Next, the



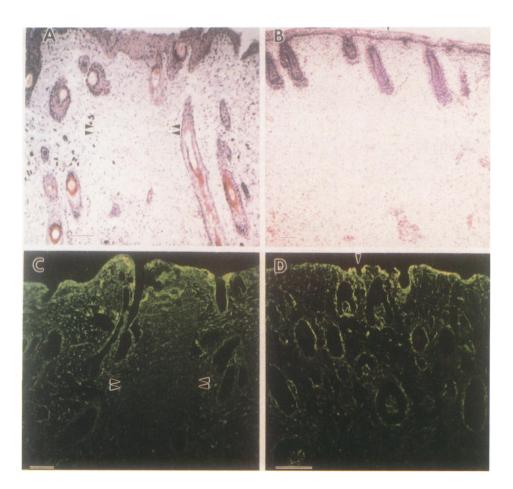


Figure 3. Photographic description of surgery. (A) Hindquarters of 60-day-gestation fetal lamb exposed and skin excised, creating a recipient graft bed. (B) Maternal (left) and autotransplanted fetal (right) skin grafts sutured onto the fetal back. Note the obvious differences in the adult and fetal skin phenotypes. (C) At 40 days posttransplantation (100 days of gestation), the adult skin (left) had maintained its phenotype and is easily distinguished from surrounding fetal skin. The fetal autograft (right) is almost indistinguishable, marked only by the four sutures used to secure the graft (arrows). No scar is evident at the graft edges. (D) Incisional wounds were created across both adult and fetal grafts. The wounds were closed with interrupted sutures.

Figure 4. Adult and fetal wounds at 14 days. Bar = 100 μ m. (A) Adult wound stained by H & E. The wound site, marked by the arrow, has healed. The epidermis is thickened, and a band of scar tissue (between double arrows) is present. The parallel packed collagen fibers and fibroblasts in this area are oriented perpendicularly to the original wound surface. Note the lack of hair follicles within the scar. (B) Fetal wound stained by H & E. The wound site (arrow) has healed and is indistinguishable from the surrounding fetal skin. (C) Adult wound (arrow) stained for type VI collagen. Note the broad band of densely staining collagen within the wound (double arrows). Normal skin, containing hair follicles, can be seen on either side of the scar. (D) Fetal wound stained for type VI collagen. The staining pattern at the site of the wound (arrow) is exactly like that of the surrounding skin. The normal pattern of tissue architecture, including hair follicles has been restored.

sections were covered with a secondary antibody for 1 hour at room temperature, washed three times with PBS, and mounted in nonfading medium (1,4-diazobicyclo (2,2,2)-octane, 1g; glycerol, 9 mL; PBS, 1 mL). The sections were analyzed and photographed using a Leitz fluorescence microscope and Ektachrome 160ASA film (Eastman Kodak, Rochester, NY). The antibodies had been previously characterized for specificity using preabsorption with the antigen before staining.³ Initially, for each primary antibody, control sections were processed in the same manner, substituting preimmune serum for the primary antibody. These controls gave uniformly negative results, and further control sections were processed substituting PBS for the primary antibody.

Antibodies

We used the following:

- 1. Goat antitype I collagen (Seralab, Manchester, England), diluted 1:100 in PBS.
- 2. Goat antitype III collagen (Seralab), diluted 1:100 in PBS.
- 3. Rabbit antitype VI collagen (Dr. S. Ayad, University of Manchester, Manchester, UK), diluted 1:100 in PBS.
- 4. Rabbit antigoat FITC-conjugated immunoglobulin G (Northeast Biomedicals, Oxford, England), diluted 1:40 in PBS.
- 5. Sheep antirabbit FITC-conjugated immunoglobulin G (Serotec, Manchester, England), diluted 1:160 in PBS.

RESULTS

Seven fetuses survived skin grafting, and three fetuses were aborted. This survival rate is similar to previously published data for fetal surgery on similarly aged fetal lambs. ¹⁴ All the maternal grafts on the surviving fetuses were viable. There were no signs of graft-versus-host disease in any of the fetuses. Histologic examination of the adult skin grafts revealed no evidence of rejection, that is, no mononuclear cell or giant cell infiltrate.

Adult Wounds

Grossly, the surface of the adult skin grafts was covered by matted wool and debris. Beneath this layer, the wounds on the adult skin graft were visible as a scar. H & E and trichrome staining of the adult wounds at 14 days (54 days posttransplantation) showed a well-healed wound. The epidermis was thickened at the wound site, and a distinct band of scar tissue was present, with an absence of hair follicles within the scar (Fig. 4A).

The interstitial collagen types I, III, and VI are widely distributed in the extracellular matrix of normal dermis. All three collagen types showed a similar staining pattern in normal dermis and at the site of the wound. Within the wound, densely packed, parallel bundles of collagen were deposited that lay perpendicular to the wound surface. This differed from the reticular pattern of fibrils in the adjacent normal dermis and is illustrated for type VI collagen in Figure 4C. A virtually identical pattern was seen for both type I and type III collagens (data not shown), confirming previously described findings in fetal monkey, fetal sheep, and fetal mouse incisional wounds.^{3,4,13}

Fetal Autograft and Fetal Skin Wounds

Grossly, the wounds on the fetal autograft and surrounding fetal skin were not discernible at 14 days. With H & E, trichrome, and immunohistochemical analysis, the structure of the fetal autograft appeared identical to the normal fetal dermis. H & E staining of the fetal wounds at 14 days showed complete healing, with a normal tissue architecture indistinguishable from the surrounding unwounded fetal skin (Fig. 4B). Collagen types I, III, and VI all showed a reticular staining pattern within the fetal wounds identical to that of the unwounded fetal dermis (Fig. 4D).

Late-Gestation Fetal Skin Wounds

At 14 days postwounding (54 days posttransplantation), the 120-day fetal skin was easily identifiable because it was wool bearing and of an adultlike skin thickness. Histologically, the 120-day fetal skin had differentiated from the time of grafting and looked similar to the adult skin. Scar formation was clearly visible at the site of the wound.

DISCUSSION

We defined a scar morphologically as the lack of tissue organization compared with the surrounding normal tissue architecture. Wounds in the adult skin graft healed with scar formation in a similar manner to normal adult wounds. By contrast, wounds in the fetal skin grafts and surrounding fetal skin both healed without scar formation. This is in accord with the results of a previous study of incisional wounds in fetal lambs at the same gestational age and at the same 14-day time point postwounding. At the time of wounding, the adult skin graft had retained its adult phenotype with an easily recognizable wool-bearing surface, whereas the fetal autograft could not be distinguished from the surrounding fetal skin except for the sutures used to secure the graft.

These findings demonstrate that the more differentiated cells of adult skin cannot be modulated to heal in a scarless manner simply by exposure to the fetal environment. Neither an amniotic fluid environment nor perfusion by fetal blood prevented scar formation in the wounded adult skin graft. Therefore, the fetal environment alone does not support scarless repair. However, it is possible that the fetal environment, although not producing scarless healing, may alter adult wound healing, either in the rate of the healing or in the extent of scar formation. The gross and microscopic appearances of these adult wounds were similar to those seen in adult wounds healing in the normal adult environment, 13 but determining such possible subtle effects of the fetal environment will require more extensive studies to allow for the normal variation in adult wound healing.

It is possible that the structure of adult skin, particularly the epidermis, prevented it from absorbing important factors from the amniotic fluid and was thus unable to take advantage of the unique fetal environment. To investigate this possibility, 120-day-gestation fetal lamb skin was transplanted onto 60-day-gestation fetal lambs. Skin at 120 days of gestation heals by scar formation. By transplanting it onto early-gestation fetuses, it was not possible to "turn back the developmental clock" and alter wound healing in late-gestation fetal lamb skin. This finding suggests that the state of differentiation of fetal skin at the time of wounding is an important parameter in whether or not the healing is scar-free.

The findings of this study are in broad agreement with the conclusions from investigations of wound healing in newborn and adult marsupials in which the immature pouch youngsters heal without scarring (just like eutherian mammalian embryos) despite the absence of amniotic fluid and the presence of highly keratinized skin. Similarly, human fetal skin heals without scar formation when it is transplanted to a subcutaneous location on an adult athymic mouse and subsequently wounded. Thus, amniotic fluid, fetal serum, and fetal blood components such as platelets are not required for scarless repair.

Why, then, does the adult and late-gestation fetal skin in an early fetal environment respond to wounding by scarring? Broadly, this can be considered in relation to the composition and architecture of the fetal and adult extracellular matrix and the fetal and adult cells. Fetal dermis consists of a basket-weave network of thin collagenous fibrils in a glycosaminoglycan-rich matrix. ¹⁶ In general, the adult dermis is denser, consists of larger diameter collagen fibrils, and shows less remodeling than does the fetal dermis. The fetal extracellular matrix often consists of fetal isoforms of the molecules in question, for example, fibronectin¹⁷ and laminin, ^{18,19} and there is developmental regulation of integrin receptor expression in the maturing epithelium. ^{20,21} There are also differ-

ences in dermal collagen; the presence of aminopropeptides of type I collagen in heterogeneous collagen fibrils is more common in the fetus compared with the adult.²² These differences between the fetus and adult may be important in modulating the nature of the wounding response. Interestingly, smaller diameter collagen I fibrils with a high turnover and a similar configuration to fetal collagen (in terms of attached type III collagen and the presence of type I aminopropeptide) occur adjacent to the epidermal-dermal junction in adult skin.²² This is also the region that shows minimal scarring on wounding.²³ Moreover, there may also be differences between the amounts of growth factors bound to the extracellular matrix in fetal and adult skin²⁴ and their subsequent release in an active form in response to the tissue changes after wounding, for example, pH and hypoxia.

There are at least two possible explanations for our findings. First, there may have been a transfer of mature tissue macrophages with the adult and late-gestation fetal graft. Previous studies have correlated the absence of scarring in fetal wounds with the sparse inflammatory response, as evidenced by reduced macrophage and monocyte infiltrates, 15 absence of endogenous immunoglobulins at the wound site,³ and low levels of peptide growth factors.²⁴ These studies suggest that immature fetal immune cells do not respond to the wounding stimulus in a similar fashion to that of adult cells. Perhaps, transfer of mature resident tissue macrophages and monocytes with the adult and late-gestation fetal grafts resulted in an adultlike response to wounding; the absence of such cells in the early fetal skin and graft wound thus resulted in scar-free healing. Second, the differences in the healing response of the adult and fetal tissues may reflect intrinsic differences between fetal and adult cells and tissues, such as a more rapid and ordered deposition and turnover of fetal tissue components and a less differentiated state of the wounded fetal tissues. The early fetal cells and tissues are in general dividing faster and have higher biosynthetic levels, not only of extracellular matrix molecules, but also of their degradative enzymes. This may alter the response of adult and fetal cells to the stimuli in the wound environment. It is well known that many growth factors and their receptors are differentially regulated; their numbers, affinity, and isoform type change with their developmental stage.²⁵ For example, the growth of early human embryo fibroblasts is stimulated by transforming growth factor- β , whereas the growth of later stage human fetal fibroblasts is inhibited.26 Such differences may reflect differences in receptor number or receptor affinity. The developmentally regulated epidermal response to wounding in the chick embryo is probably the result of the underlying regulation of epidermal growth factor receptor expression and endogenous epidermal growth factor production.²⁷ Embryonic and fetal cells often undergo an isoformic transition during development, for example, in their migratory behavior, 28 in the secretion of motility factors, 29 and in hyaluronic acid biosynthesis. 30,31 Such phenotypic changes may result in different types of cell behavior and biosynthesis by early fetal, late fetal, or adult cells in response to the same cytokine or physicochemical stimulus at the wound site. Moreover, a differential synthesis of growth factors occurs during development, for example, the A or B chain of platelet-derived growth factor^{32,33} or insulinlike growth factors I and II. 25,34 Because the effects of many growth factors on cell division and matrix biosynthesis are highly dependent on the precise combination of factors present in the tissue, these differences in the endogenous synthesis of different factors by fetal or adult cells may result in different phenotypic effects, for example, more hyaluronic acid biosynthesis by fetal cells. Furthermore, early fetal tissues often have high endogenous levels of transcription factors, for example, homeobox genes. 35 Because it is known that such transcription factors (e.g., a regeneration-specific homeobox gene³⁶) are expressed during regeneration of newt limbs, it may be that their endogenous presence in early fetal cells has a marked effect on the response to wound stimuli.

The relative contributions of the fetal matrix and cells and the adult matrix and cells are now being experimentally tested by examining the healing interface of the adult skin graft and fetal recipient skin at various time points after adult skin graft placement. We are also using grafts of irradiated or proliferation-arrested adult tissues sutured onto fetal sheep and placing cross-species grafts with analysis using species-specific antibodies against components of the extracellular matrix.

This study highlighted the multiple complex differences between the fetal and the adult response to wounding and dramatically demonstrated that the scar-free fetal healing phenotype is not simply the result of being bathed in warm, sterile, growth factor-rich amniotic fluid. These experiments have led to the hypothesis that fetal healing must involve different cellular and matrix events than adult repair and that this process may be independent of the unique fetal environment.

Acknowledgments

The authors thank Dr. Shirley Ayad for the gift of the anticollagen type VI antibody.

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