TGF-β1 Alters the Healing of Cutaneous Fetal Excisional Wounds

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Background/Purpose: In a number of species, fetal wound healing differs from the adult in the absence of inflammation, fibrosis, scar formation, and excisional wound contraction. The lack of inflammation also may explain the relative absence of any cytokine levels at the wound site, such as transforming growth factor (TGF)-β, and therefore the unique characteristics of fetal wound healing. The authors hypothesized that exogenous TGF-β1 would induce contraction, inflammation, fibrosis, and scar formation in cutaneous excisional wounds in the fetal rabbit.

Methods: Cellulose discs (3 mm in diameter) were formulated with either 1.0 μg TGF-β1 (n = 6) or bovine serum albumin (BSA; n = 7), as a control, for sustained-release over 3 days. Each disc was implanted into the subcutaneous tissue on the backs of fetal New Zealand White Rabbits in utero on day 24 of gestation (term, 31 days). A full-thickness, 3-mm excisional wound (7.4 mm^2) was then made next to the implanted cellulose disc. All wounds were harvested 3 days later.

Results: At harvest, the excisional wounds in the TGF-β1 group had contracted (5.6 ± 2.0 mm^2), whereas those in the control group had expanded (13.5 ± 1.2 mm^2, $P < .01$). The surrounding dermis in the TGF-β1 group had 16.3 inflammatory cells per grid block compared with 12.4 cells in the control group (not significant). In addition, a greater amount of fibrosis was induced by the TGF-β1 implant (1.7 ± 0.3) than the control implant (0.4 ± 0.2) on a scale of 0 to 3, $P < .01$. In situ hybridization analysis showed an increase in procollagen type 1α1 gene expression in the surrounding dermis of the TGF-β1 group (36.7 ± 3.6 grains per grid block) compared with the control group (7.1 ± 0.9 grains per grid block, $P < .001$).

Conclusions: These results demonstrate that the cytokine TGF-β1 can induce fetal excisional wounds to contract, stimulate fibrosis, and increase procollagen type 1α1 gene expression. These findings further suggest that the absence of TGF-β1 at the wound site may be responsible in part for the lack of a postnatal healing response.

INDEX WORDS: Fetal wound healing, wound contraction, TGF-β1.

IN MANY SPECIES, early and midgestational wound healing in the fetus differs from the neonate and adult in the absence of inflammation, fibrosis, and scar formation.[1,2] In addition, excisional wounds in these fetuses expand in utero, which is not caused completely by the growth of the fetus.[3,4] The mechanisms underlying these unique characteristics of fetal wound healing may be caused by the relative lack of effective levels of specific cytokines present at the wound site.

Transforming growth factor (TGF)-β1 is a multifunctional regulator of cell growth, differentiation, and extracellular matrix formation.[5] It is thought to play a key role in determining the quality of healing in postnatal wounds, and levels have been correlated with the amount of fibrosis and scar formation. In fact, supraphysiologic doses of TGF-β produce excessive fibrosis and scar formation in adult tissue, and elevated TGF-β levels have been demonstrated in many fibrotic conditions.[6-8] Although prominent in neonatal and adult wounds, it appears to be present in significantly lower amounts in the fetal dermis and fetal wounds.[9-12] However, fetal incisional wounds will respond to exogenous TGF-β with a postnatal healing response.[13,14] Additional in vitro and in vivo studies have corroborated these findings and demonstrated that fetal tissues are capable of producing fibrosis and scar formation when given the appropriate stimulus. Also, fetal fibroblasts will contract collagen lattices in the presence TGF-β1.[15]

Beyond the initiation of an inflammatory response and subsequent cytokine release, the specific type, amount, and duration of selected cytokines present at the wound site play a critical role in determining the quality of the

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healing response. Levels of TGF-β1 are maintained at adult wound sites through autocrine and paracrine mechanisms.[13,16] Therefore, this study was designed to test the hypothesis that administering TGF-β1 in a sustained manner would induce contraction, inflammation, fibrosis, and an up regulation of collagen gene expression in cutaneous excisional wounds in the fetal rabbit.

MATERIALS AND METHODS

Animals

Time-dated pregnant New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, TN) were maintained in the animal facilities at the Medical College of Virginia and allowed to be accustomed to environment for 9 to 10 days before surgery. The rabbits were provided rabbit chow and water ad libitum and evaluated on a daily basis by a staff veterinarian. Approval for this study was obtained from the Institutional Animal Care and Use Committee.

Implants

Cellulose discs (Innovative Research of America, Sarasota, FL) also are composed of stearates, lactose, phosphates, and cholesterol and are designed for sustained release. The discs measured 1 mm in height and 3 mm in diameter and were formulated with TGF-β1 and its carrier bovine serum albumin (BSA). Additional discs were formulated with only BSA as a control.

Fetal Surgery

At day 24 of gestation (term, 31 days), each doe underwent laparotomy and hysterotomy after initially receiving 1 mL of an acepromazine and ketamine mix (12.5:1) intravenously and induction of mask isoflurane anesthesia. Using standard surgical techniques, a cellulose disc formulated with either 1.0 μg of TGF-β1 (n = 6) or BSA (n = 7), as a control, was implanted in a subcutaneous pocket in the midline on the back of each fetus.[17] A single interrupted 6-0 Maxon suture was used to close the incision made to insert the disc. Next to the implanted disc and opposite the insertional incision, a full-thickness, 3-mm excisional wound was made with a disposable punch biopsy tool. The amniotic fluid was replaced with 6 mL of warmed plasmalyte solution (Baxter Healthcare Corporation, Deerfield, IL) before closing the hysterotomy with a 3-0 silk purse-string suture. Up to four fetuses were operated on per doe. The fascia was then reapproximated with a running 2-0 Dexon suture and the skin closed with buried, interrupted 3-0 Dexon sutures. Each doe was given an intramuscular injection of 0.15 mg of buprenorphine and placed in a warm recovery area. Once the doe had recovered fully, it was returned to its cage. Each animal was checked on a daily basis for evidence of wound infection or abortion.

Wound Evaluation

At 3 days postoperatively, does were killed with an overdose of intravenous sodium pentobarbital, and each fetus was immediately decapitated. The excisional wounds were photographed with a Nikon (Tokyo, Japan) AF8008S camera with a 105-mm macro lens next to a standard reference. Wound areas were determined from the scanned images using Image Pro Plus (version 1.3.2; Media Cybernetics, Silver Spring, MD). Fetuses were fixed in 10% formalin for 2 days before wound tissue was processed and placed into paraffin blocks. Sections stained with H&E were viewed in a blinded fashion by a dermatopathologist. Inflammatory cells were counted at several random areas around each wound. The degree of fibrosis around the implants and wound site was determined based on the extent of mesenchymal cell infiltration and graded on a 0 to 3 scale with 0 = none, 1 = mild, 2 = moderate, and 3 = severe.

In Situ Hybridization

In situ hybridization was performed essentially as described by Wilcox.[18] Sense and antisense probes labeled with ^{35}S-UTP (New Life Science Products, Boston, MA) were synthesized from linearized plasmids for procollagen type 1α1 and hybridized to sections on glass slides. Slides were then washed and immersed in 20 μg/mL Rnase A for 30 minutes at 37°C to reduce the nonhybridized background. After coating with Kodak NTB2 nuclear emulsion, the slides were kept in the dark at −80°C for 4 days before development. Using darkfield microscopy and a gridded ocular, multiple random counts were performed in a blinded fashion around each implant and wound site and recorded as grains per grid block.

Statistics

Data are presented as mean ± SEM. Statistical analysis of data was performed using either Student's t test or Mann-Whitney Rank Sum test, and a P value of less than .05 was considered significant.

RESULTS

At the time of harvest, 3 days after the initial surgery, the excisional wounds in the control group did not demonstrate any gross evidence of contraction, whereas those in the TGF-β1 group demonstrated thickening of the wound edges and wrinkling of the surrounding dermis, (Figs 1A and B). At the time of measurement, the excisional wounds in the TGF-β1 group had contracted (5.6 ± 2.0 mm^2) from the original size (7.4 mm^2), whereas those in the control group had expanded (13.5 ± 1.2 mm^2; $P < .01$; Fig 2). Hematoxylin and eosin–stained tissue sections were evaluated in a blinded fashion by a dermatopathologist for degree of inflammation and fibrosis around the implants. The TGF-β1 group had a mean number of 16.3 inflammatory cells per grid block compared with 12.4 cells in the control group (not significant; Figs 3A and B). The inflammatory cells were present in the dermis around the implants and under the excisional wound and appeared to be predominantly mononuclear cells with a few scattered polymorphonuclear cells. In addition, a greater amount of fibrosis was seen in the TGF-β1 group (1.7 ± 0.3) than the control (0.4 ± 0.2) on a scale of 0 to 3 ($P < .01$). A thick fibrous capsule could be identified around the implants formulated with TGF-β1 along with extensive fibrosis at the edges of the excisional wounds (Fig 3B). However, fibrosis was not seen in the dermis around the implants or under the excisional wounds in the control group (Fig 3A). In situ hybridization of tissue sections from each group were performed with antisense and sense probes for procollagen type 1α1 and showed a significant increase in gene expression in the TGF-β1 group (36.7 ± 3.6 grains per grid block) compared with the control group (7.1 ± 0.9 grains per grid block; $P < .001$; Figs 4A and B and Fig 5).

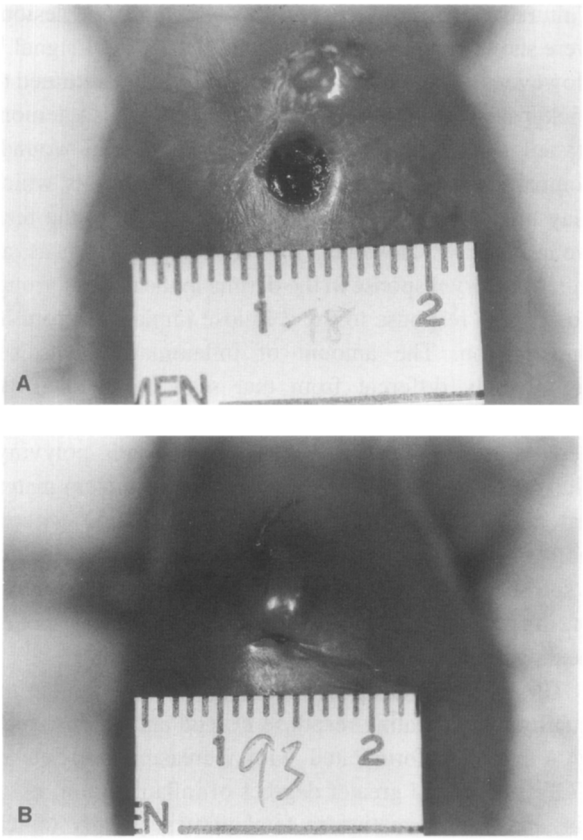

Fig 1. Photographs of representative excisional wounds from fetuses from the control (A) and TGF-β1 (B) groups at the time of harvest, 3 days after wounding. The wound in the control group has expanded, whereas the wound in the test group has contracted and almost closed.

DISCUSSION

Wound contraction is an important component of the healing of excisional wounds in the neonate and adult. However, the mechanisms underlying this process have not been elucidated completely. Myofibroblasts are mesenchymal cells with functional and structural characteristics in common with fibroblasts and smooth muscles cells and are thought to play a critical role in wound closure.[19,20] The matrix glycoproteins fibronectin and tenascin also may be important. Fibronectin promotes adhesion and migration of inflammatory cells and fibroblasts into the wound region while tenascin is expressed at the site of epithelial migration over healing wounds.[21,22] In an effort to learn more about the mechanisms responsible for wound contraction, an in vivo contractile model in the fetal rabbit was developed. Using this normally noncontractile model, it was possible to demonstrate contraction of excisional wounds and the initiation of a postnatal healing response after sustained administration of TGF-β1.

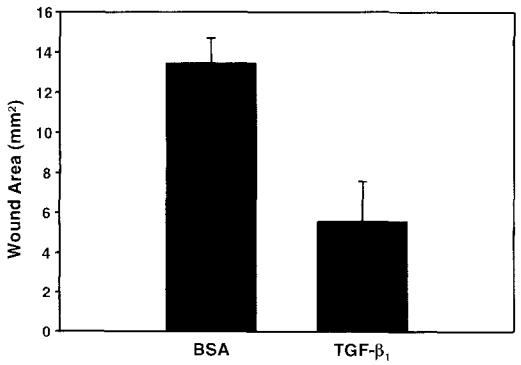

Fig 2. Bar graph shows area of excisional wounds at the time of harvest in the control and TGF-β1 groups. The wounds in the control group were significantly larger than those in TGF-β1 group. Original size of excisional wounds was 7.4 mm², $P < .01$.

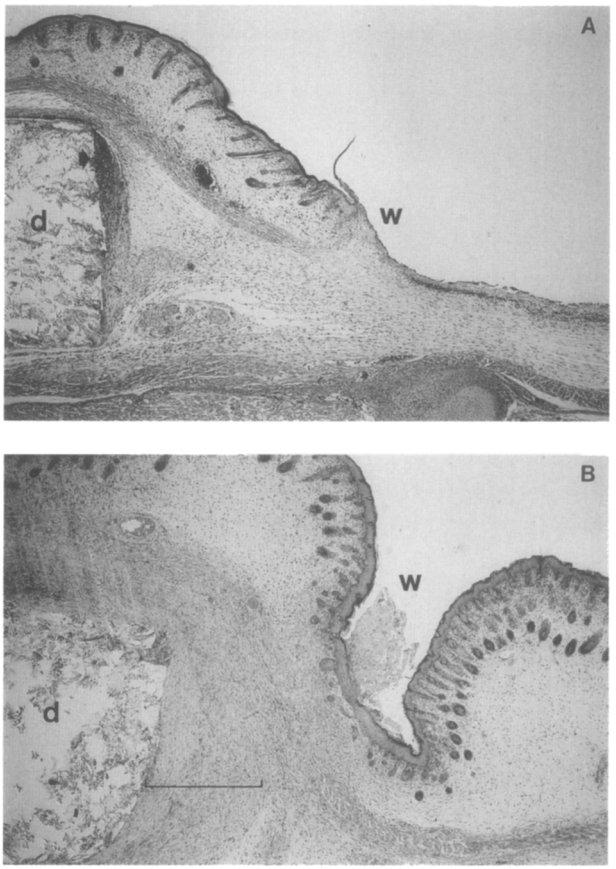

Fig 3. Photographs of H&E–stained tissue sections from the control (A) and TGF-β1 (B) groups viewed with light microscopy (original magnification ×40). There are inflammatory cells (arrow in photograph A) distributed in the dermis around the cellulose discs (d) and under the excisional wounds (w) in both groups. However, there is a significant degree of mesencymal cell infiltration only around the TGF-β1 implant (bracket in photograph B).

TGF-β is chemotactic for fibroblasts and macrophages, induces angiogenesis and modulates expression of collagen and collagenase.[23,24] It has been proposed that TGF-β1 is involved in the early inductive events of epithelial-mesenchymal interactions and formation of the extracellular matrix components important for cell migration and cell-cell interaction.[25] Incisional wounds have

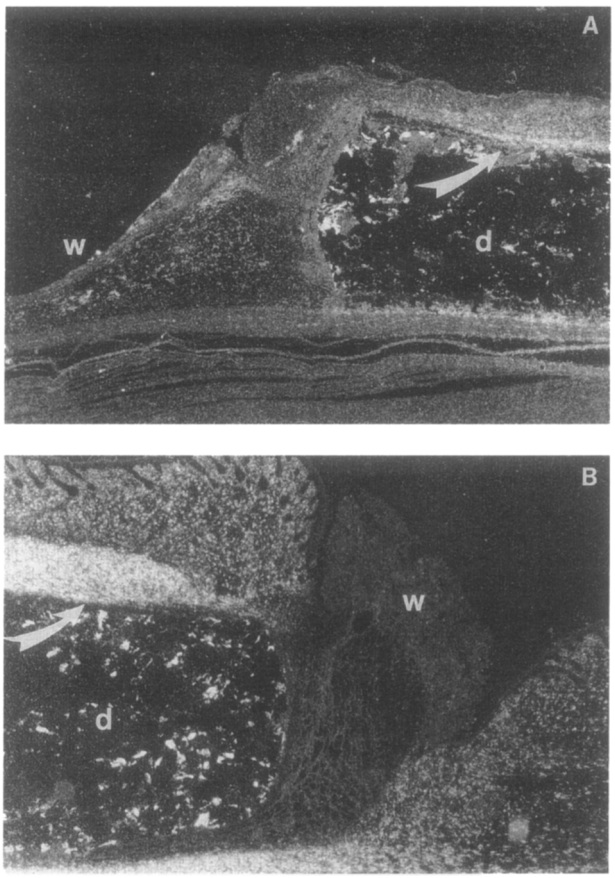

Fig 4. Photographs of tissue sections from the control (A) and TGF-β1 (B) groups hybridized with an antisense probe for procollagen type 1α1 and viewed with darkfield microscopy (original magnification ×40). There is minimal gene expression in the dermis around the control implant (arrow in photograph A), yet significant expression in the dermis around the TGF-β1 implant (arrow in photograph B) and excisional wound (w).

significantly elevated levels of TGF-β1 and TGF-β2 in the adult rabbit.[11] In contrast, levels of TGF-β1 and TGF-β2 are minimal in the fetal rabbit dermis, and there is no increase of either isoform after incisional wounds.[11]

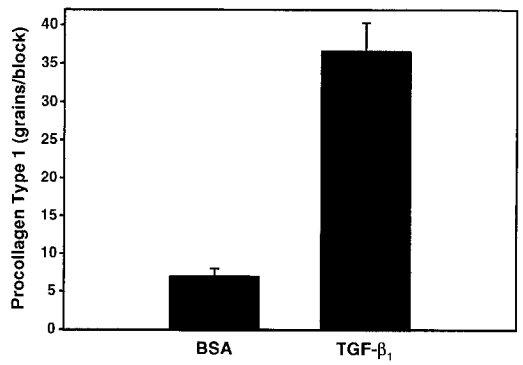

Fig 5. Bar graph shows procollagen type 1α1 gene expression at the time of harvest in the control and TGF-β1 groups. There was a significantly greater gene expression in the surrounding dermis in response to TGF-β1 than BSA, $P < .001$.

Cultured E11.5 murine embryos with limb bud lesions were shown to have a rapid elevation of TGF-β1 signal.[10] However, by 18 hours the transcript levels had returned to background levels. Additional studies have also demonstrated a relative absence of TGF-β in fetal wounds compared with the elevated levels in adult wounds, which may be a result of the lack of inflammation at the fetal wound site.[9,12] However, in this study, there was an inflammatory response in the dermis in the control group, possibly in response to the cellulose implant or from its implantation. The amount of inflammation was not significantly different from that seen in the TGF-β1 group, yet no additional characteristics of postnatal healing were noted. Previous studies with polyvinyl alcohol sponge (PVA) implants transplanted from maternal doe to fetus also produced inflammation without fibrosis or scar formation.[26] This suggests that the lack of a specific cytokine, such as TGF-β1, and not the absence of inflammation is responsible for a prenatal wound healing response.

The level of TGF-β has been shown to determine the quality of the healing response in fetal and adult tissues. PVA implants formulated with increasing amounts of TGF-β produced greater degrees of inflammation, fibrosis, and scar formation in fetal tissues.[14] Daily topical administration of TGF-β1 to adult porcine excisional wounds resulted in increased granulation tissue and a dose-dependent upregulation of collagen types 1 and 3.[27] In fact, elevated levels of this cytokine have been demonstrated in fibrotic conditions such as pulmonary fibrosis, cirrhosis, and glomerulonephritis.[7,8,28] Furthermore, neutralizing antibodies to TGF-β1 and TGF-β2 have resulted in less fibrosis and scar formation.[29] In the neonate and adult, autocrine and paracrine effects contribute to sustained levels of TGF-β.[16] TGF-β1 induction also was demonstrated in fetal incisional wounds exposed to exogenous, sustained-release TGF-β1.[13] These wounds healed in an adultlike manner. Earlier studies in our laboratory demonstrated a reduction in the expansion of fetal excisional wounds with a single topical application of TGF-β1.[30] Using sustained-release implants of TGF-β1 in fetal rabbits, we have been able to induce a dose-dependent degree of wound contraction including closure of 3-mm cutaneous excisional wounds with 10 μg at 5 days (unpublished observations). This suggests that we may be able to reduce the extent of contracture and scar formation by inhibiting the autoinduction of TGF-β at the adult wound site.

Adult porcine excisional wounds showed an upregulation of procollagen type 1 signal in response to topical TGF-β1.[27] Also, procollagen type 1α1 expression is increased in fetal fibroblasts with TGF-β1, in vitro.[31]

Fetal incisional wounds have been shown to have an elevated signal for procollagen type 1 by mesenchymal influx using in situ hybridization.[32,33] However, by day 7, this signal had returned to baseline levels. In this study, we have found a significant increase in procollagen type 1α1 transcripts in the fibrous capsule surrounding the sustained-release implants formulated with TGF-β1. Some of this increased signal may be caused by fibroblasts migrating into the wound area as was suggested in the studies by Nath et al.[32,33] However, there was increased expression up to 4 mm from the implant and wound before tapering off. The control implants formulated with BSA had only minimal signal immediately around the disc. These results suggest that given a sufficient stimulus, such as TGF-β1, fetal wound fibroblasts are able to respond with a significant up regulation of procollagen type 1α1 transcripts.

These studies demonstrate that sustained levels of TGF-β1 induce contraction of excisional wounds as well as inflammation, fibrosis, and an up regulation of procollagen type 1α1 transcript in the surrounding dermis in the fetal rabbit. These studies further demonstrate that the pericellular environment is critical in determining the quality of the fetal wound healing response. Future studies hopefully will show which transcription factors and cytokines are responsible for dictating a prenatal response so that they may be incorporated into the strategies for modifying the healing of abnormal neonatal and adult wounds.

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Discussion

T.M. Crombleholme (Philadelphia, PA): I have two questions for Dr Lanning. First, because your most important observation is the effect of TGF-β1 on the contraction of these excisional wounds, did you perform any alpha smooth muscle actin staining to examine the effect of TGF-β1 on myofibroblast differentiation, as this might be correlated with the degree of contraction you observed? It has been demonstrated in excisional wounds in fetal sheep that you will get induction of myofibroblast differentiation if you have a sufficiently large excisional wound in that animal model.

Second, you stated in the manuscript that the effect of the fibrosis and the wound contraction was specifically related to the TGF-β1. There seemed to be a significant increase in the amount of inflammatory cell infiltrate that was observed with the TGF-β1 loaded discs. Was there any specific staining done to characterize the nature of the inflammatory infiltrate in any experiments considered to try to tease apart the issue of macrophages that might be recruited to the wound and would induce secondary growth factor and cytokine expression that might have confounded the effects of the TGF-β1 disc that you inserted?

D.A. Lanning (response): Certainly there are a number of projects that we have planned from this preliminary work. Alpha smooth muscle actin is something critical to look at, and we are studying its relationship to wound contraction.

We are also doing more definitive studies utilizing immunohistochemistry to identify the cell types that are in these wounds and how they differ in the control and the test groups. The function of these cells may be very important.

G.K. Gittes (New York, NY): Can you expand a little on what is going on without any perturbation of the fetal wound in terms of TGF-β1 or other isoforms? For example, how does the introduction of this recombinant protein correlate with the levels that are normally endogenously there?

Is this a pharmacological effect you are seeing? Also, what is the relationship with other species?

D.A. Lanning (response): As mentioned earlier, there are minimal levels of the TGF-β isoforms in the fetal dermis and in fetal wounds relative to the adult. To address this in our study, tissue sections also were hybridized with antisense and sense probes for TGF-β1 and TGF-β3. However, no signal expression was identified, suggesting that endogenous levels at 3 days were minimal.

In regards to how TGF-β1 induces its contractile effect, it is not clear at this time. Previous in vitro studies have shown that TGF-β stimulates collagen lattice contraction to a greater extent than other cytokines. Furthermore, preliminary studies in our lab have demonstrated a dose-dependent degree of excisional wound contraction in our in vivo model, suggesting a physiological role. However, since TGF-β is a multifunctional cytokine, which stimulates chemotaxis of various inflammatory and mesenchymal cell types, the effect of these migrating cells on the wound is unknown and is the focus of ongoing studies.

The absence of wound contraction is seen in the prenatal period in a number of other species. However, in the later gestational periods, such as in the sheep, there is a transition to a postnatal pattern of healing where excisional wounds contract. To date, we have not used other animals in our studies.