Studies in Fetal Wound Healing

V. A Prolonged Presence of Hyaluronic Acid Characterizes Fetal Wound Fluid

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Midgestation fetal wound healing is characterized by healing without fibrosis or scar formation. The mechanisms that underlie this remarkable process are mediated in part through a fetal wound extracellular matrix rich in hyaluronic acid. In this study a newly developed assay was used to determine the hyaluronic acid levels in fetal and adult wound fluid. Adult wound fluid had a rapid increase in hyaluronic acid, which peaked at 3 days and decreased to 0 by 7 days. In contrast levels of hyaluronic acid in fetal wound fluid increased rapidly and remained significantly elevated for 3 weeks. This prolonged presence of hyaluronic acid in the matrix of fetal wounds creates a 'permissive' wound environment that promotes fetal fibroblast movement and proliferation and inhibits cytodifferentiation. Such a matrix environment promotes healing by regeneration rather than by scarring. This observation has therapeutic implications. The prolonged application of hyaluronic acid or hyaluronate protein complexes to wounds in children or adults may modulate healing in a manner that makes the wounds more fetal-like.

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ADULT WOUND REPAIR is characterized by fibrosis, scarring, and sometimes by contracture. The results of this deforming process affect every form of surgery and can have devastating consequences. In contrast fetal wound healing proceeds without such fibrosis or scar formation.[1-19] Previously we showed that a transition from fetal- to adult-type repair occurs midway through the last trimester.[11] The observations from our initial clinical series of fetal surgery at the University of California at San Francisco (UCSF) support these experimental findings.[20,21]

Although the mechanisms that endow fetal wound healing with these unique qualities remain enigmatic, this process is mediated in part through the extracellular matrix (ECM). DePalma and coworkers[8,14] demonstrated that the fetal wound ECM is rich in hyaluronic acid (HA), a glycosaminoglycan found in high concentrations whenever rapid cell movement and proliferation occur.[22,23] The deposition of HA and its subsequent degradation by the enzyme hyaluronidase play critical roles in a number of experimental model systems during early embryogenesis.[23] Hyaluronic acid also was found to enhance postnatal wound healing and reduce adhesions after tendon repair.[24-26] Therefore we postulate that a wound ECM rich in HA provides a permissive environment for repair, free of scar formation, and that HA is one of the ECM components that endow fetal wound healing with its unique properties.

Previously we demonstrated a mechanism for HA deposition in fetal wounds; an elevated HA-stimulating activity (HASA) has been identified in fetal wound fluid.[19] The HASA remained elevated from 1 to 14 days in fetal wound fluid, whereas it was not detected at any time in adult wound fluid samples. This observation, together with that of DePalma et al.,[8,14] led us to postulate that there may be a prolonged presence of HA in the fetal wound ECM. Herein, using a wire-mesh cylinder model, we demonstrate that fetal wound fluid indeed has a prolonged presence of HA, from 1 to 21 days after wounding. In contrast HA was present only initially in adult wound fluid. Thus the prolonged presence of HA is unique to fetal wound fluid and may provide a component of the ECM environment that underlies a process of wound repair that occurs without fibrosis or scarring.

Materials and Methods

Wound Cylinders

Sterile wire mesh cylinders were used as described by Schilling and Hunt.[27,28] The cylinders were prepared by

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cutting a rectangle from No.40 mesh, 36-gauge stainless steel. Cylinders were rolled into a tube and the ends folded closed. Fetal cylinders measured 1 × 3 cm and the adult cylinders measured 3 × 6 cm.

Animals

Seven time-dated, pregnant ewes from Torrel Farms (Ukiah, CA) were transferred to the UCSF animal care facility and fed food and water *ad libidum*. The animals were fasted for 48 hours before surgery.

Fetal Wound Fluid

At 100 days' gestation (145 days = term), the animals underwent general halothane/oxygen anesthesia using techniques developed specifically for fetal lamb surgery as previously described.[29] Ewes 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 #10 oral endotracheal tube. A 16-gauge intravenous catheter (Deseret, Sandy, UT) was inserted percutaneously into the single jugular vein and 500 mL of Lactated Ringer's solution (Baxter Health Care Corp., 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 maternal abdomen was prepped and draped using sterile techniques and a midline laparotomy was performed. Using a GIA (U.S. Surgical, Norwalk, CT) stapler, the fetal lamb was exposed by hysterotomy. The fetal position was manipulated to expose only one limb at a time and a warm moist towel was wrapped around the uterus to help keep the fetus warm. Two-centimeter incisions were made in both the groin and axillary creases at sites where excess skin folds were observed. A small, subcutaneous pocket was created by blunt dissection and a single wound cylinder was placed in each pocket. The wounds were closed in one layer using interrupted 4–0 silk sutures (Ethicon, Somerville, NJ). The fetus was returned to the uterus and amniotic fluid volume was restored with sterile saline. The hysterotomy was closed, including the membranes, with a TA-90 (U.S. Surgical) stapler. The laparotomy was closed in layers and the ewe was returned to her stall.

Subcutaneous wound cylinders were placed in seven fetal lambs. A separate lamb was used for each time point of fluid harvest. At the designated time of harvest (1, 2, 3, 7, 10, 14, or 21 days after implantation), the maternal ewe underwent general anesthesia as described above. Following laparotomy and hysterotomy, the fetus was exposed. Fetal wound fluid was harvested from wound cylinders (four cylinders per animal) by a single percutaneous aspiration under sterile technique during reoperation using an #18-gauge needle and a 12-mL syringe. The fluid was frozen immediately and stored at −70°C until analysis.

Adult Wound Fluid

Maternal ewes were used for the adult data points. At the conclusion of the fetal procedure, four large wound cylinders were placed in widely separated subcutaneous pockets along the maternal flank. Wounds were closed with interrupted 2–0 silk sutures (Ethicon). Adult wound fluid was harvested at the same time points as fetal fluid by aspirating the wound cylinders, again using sterile techniques. The fluid was frozen immediately and stored at −70°C until analysis.

[^{35}S] Streptavidin Hyaluronic Acid-binding Protein Assay

Following thawing, all samples were passed through a 22-μm filter (CoStar, Cambridge, MA) and assayed using a newly developed [^{35}S] streptavidin HA-binding protein (HABP) assay modified from Tenglbad.[30] Briefly, 100-μl samples of equilibrated digests were aliquoted into the upper chambers of CoStar Spin-X filter units (CoStar, Cambridge, MA). Aliqouts (150 μl) of HABP diluted 1:50 in assay buffer were then added. The solutions were mixed and rotated on an American Rotator V (American Scientific, McGaw Park, IL) at 160 rpm for 30 minutes at room temperature to allow the HA in the tissue extract to bind to HABP. Next 60 μl of HA gel diluted 1:15 as above was added. The solutions were mixed and then rotated as above for 60 minutes to allow the free HABP to bind to the gel. Samples were spun at 11,000*g* for 5 minutes and the filtrate then passed into the filter units' lower chamber. The HA gel was resuspended in 200 μl assay buffer as a wash and spun for 5 minutes as above. Next 200 μl of [^{35}S] streptavidin (1000 Ci/mmole, 100 μCI/mL; Amersham, Arlington Heights, IL) diluted 1:200 in phosphate-buffered saline–calcium magnesium free (PBS-CMF) were added, the pellet was resuspended and rotated for 30 minutes, and spun for 5 minutes as above. As a final wash the pellet was resuspended in 200 μl of PBS-CMF and spun as above. The HA-gel pellet was transferred to counting vials with distilled water and Optifluor® scintillation fluid (Packard, Downers Grove, IL) was added. Nonspecific streptavidin binding was determined by assaying samples without HABP. This was subtracted from all values. A standard curve was constructed using known concentrations of HA and expressed as a percentage of control value. Values from control samples without added HA were considered to be 100% bound. All samples were assayed in triplicate. The data for each sample, run in triplicate, were expressed as the mean value ± standard deviation.

Results

All seven ewes and fetuses survived the experimental protocol. No animal developed infection.

Adult Wound Fluid

Wound fluid from the seven maternal ewes was aspirated sterilely and assayed for HA levels. The volume of wound fluid aspirated per cylinder tended to increase from an average of 1 to 2 mL at 1 day to 4 to 5 mL at 21 days. Previous attempts to harvest wound fluid earlier than 1 day yielded less than 1 mL. The HA levels increased sharply during the first 3 days, reaching a peak level of 7.0 ± 1.2 μg at day 3. By 7 days the HA level had decreased to 0.0 ± 0.9 μg and remained undetectable thereafter to 21 days, the last time point to be determined (Fig. 1).

Fetal Wound Fluid

Fetal wound fluid also was obtained by sterile needle aspiration and assayed for HA levels. The volume of wound fluid aspirated per cylinder tended to increase from an average of 1.5 to 2 mL at 1 day to 3 to 4 mL at 21 days. As with adult cylinders, previous attempts to harvest wound fluid earlier than 1 day yielded less than 1 mL. The level of HA increased in a pattern similar to adult wound fluid during the first 3 days, reaching a peak of 4.6 ± 1.4 μg at day 3 (Fig. 1). In contrast to the adult, HA levels remained elevated throughout the 21 days. The differences between fetal and adult HA levels were statistically significant at $p < 0.01$ for the 10-, 14-, and 21-day data points using the Student's t test.

Discussion

The ECM of fetal wounds is rich in glycosaminoglycans, particularly HA.[8,14] Hyaluronic acid is a large molecule that plays a prominent role in the structure of the ECM and is found whenever rapid cell proliferation, motility, and dedifferentiation occur. This molecule also suppresses differentiation.[22] We postulated that HA is deposited early in the course of both fetal and adult wound healing, but its deposition is sustained throughout the course of fetal wound healing. Thus a matrix with a prolonged presence of HA is a characteristic of fetal wound healing.

The purpose of this study was to examine this hypothesis by analyzing fetal and adult wounds for their level of HA. We used the Schilling–Hunt wire-mesh cylinder model[27,28] to sample wound fluid from 1 to 21 days after implantation. We previously showed that incisional wounds in 100-day gestation fetal lambs (the same gestational age that the cylinders were implanted in this study) healed without scar formation during the 14 days of observation.[11] By harvesting fluid at intervals from 1 to 21 days, we sampled a full spectrum of fetal wound healing, from early to late.

Fetal wound cylinder implantation sites of the groin and axilla were chosen because the excess skin folds and subcutaneous space in these locations most easily accepted the fetal cylinder from a technical standpoint. Flank implantation sites in the adult ewe were chosen from ex-

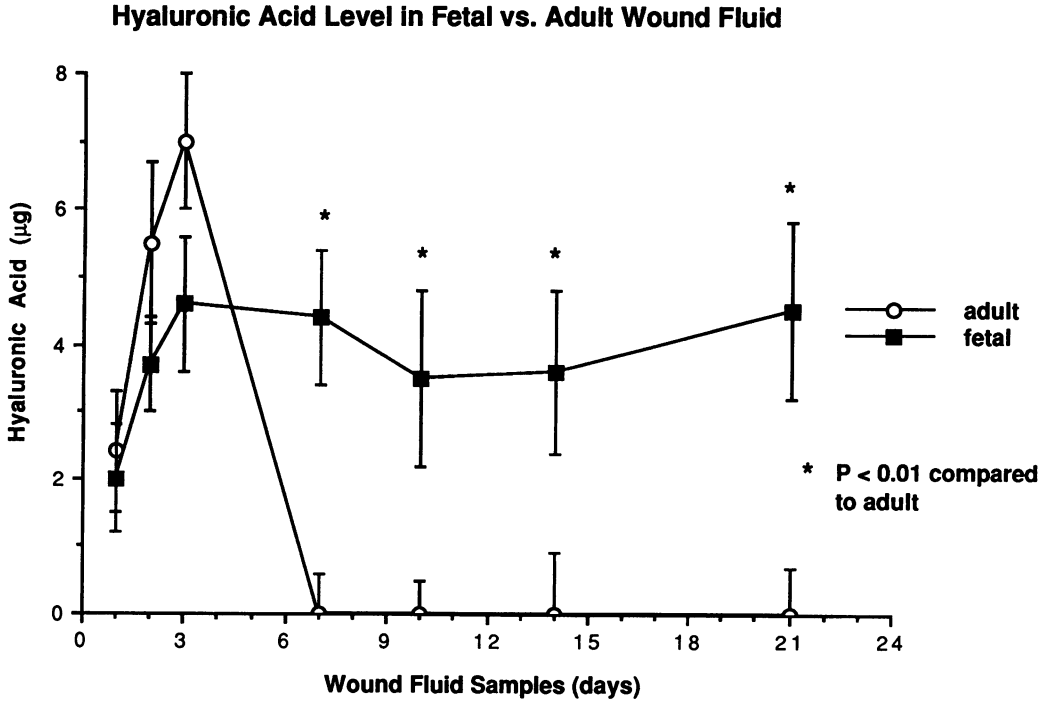

FIG. 1. HA levels in adult and fetal wound fluid. As detailed in Materials and Methods, 100 μL of wound fluid at each time point was assayed using a newly developed HABP assay. Error bars represent the standard deviation of each value run in triplicate. Student's *t* test was used for statistical analysis.

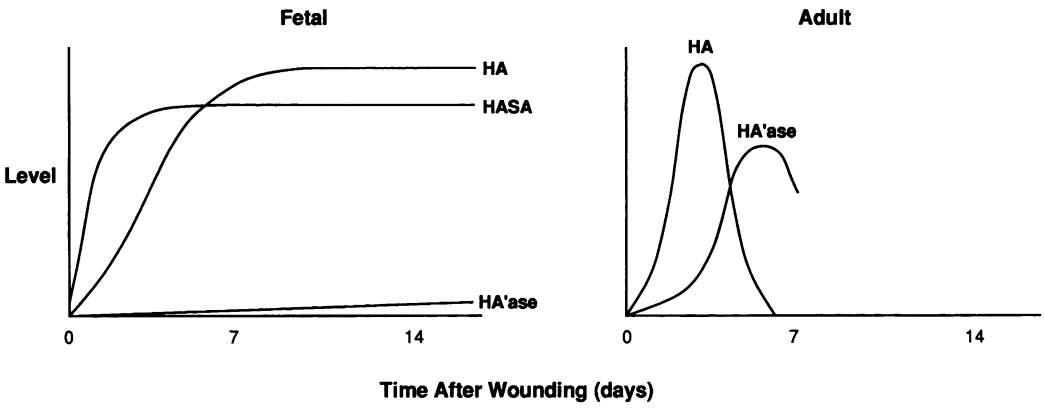

FIG. 2. Conceptualized drawing for the mechanism of HA deposition in wounds. In fetal wounds, HASA increases before HA and the sustained elevation of HASA underlies the prolonged presence of HA. In contrast HASA is not present in adult wounds at any time. HA is present only initially and is subsequently degraded by hyaluronidase.

perience and convenience. Adult sheep spend a great deal of time in the prone position and crush wound cylinders implanted in the groins, axilla, or abdomen. The wound cylinder must maintain its shape to create dead space and the flattened cylinders failed to yield wound fluid. The subcutaneous space over the rib cage is not well developed and it is technically difficult to implant the large adult cylinder into it. Finally implanting wound cylinders into the flanks of ewes was technically easy and allowed us to avoid the problems associated with the other potential sites.

The fetal and adult data differed dramatically. Hyaluronic acid was present early in both fetal and adult fluid. However after 3 days HA essentially was undetectable in the adult samples, whereas it remained significantly elevated in fetal samples between 7 and 21 days.

We recently documented a mechanism for the modulation of HA deposition in the fetus. Fetal serum, amniotic fluid, wound fluid, and a number of fetal tissues all contain HASA.[15,16,19,31,32] Fetal wound fluid contained increased HASA levels in samples harvested from 1 to 14 days after wounding.[19] The prolonged elevation of HASA is one likely mechanism for the persistent presence of HA in fetal wound fluid. Based on these studies we developed a conceptualized model for prolonged HA deposition in fetal wounds (Fig. 2). Our model suggests that hyaluronidase activity increases in the adult wound after 3 days. Studies are in progress to evaluate the activity of hyaluronidase and its inhibitors in fetal and adult wound fluid.

The prolonged presence of HA in fetal wounds provides a unique environment that promotes the highly ordered matrix observed in the ECM of fetal wounds. We previously showed that collagen is deposited rapidly and in a pattern indistinguishable from unwounded fetal skin in 100-day gestation lambs.[11] The signals for this type of organization may be mediated through a wound matrix rich in HA. Hyaluronic acid is known to affect collagen synthesis. Chandrakasan et al.[33] demonstrated that the deposition of type III collagen by fibroblasts is elevated by exogenous HA added to the media. Normal fetal tissue and granulation tissue have a much higher content of type III collagen that does normal adult tissue.[34] Scott and Hughes[35] noted that early in development, fetal collagen fibrils were smaller in diameter when HA was more abundant. Furthermore the ratio of type I to type III collagen determines the diameter of collagen fibrils.[36,37] These studies provide convincing evidence that collagen organization in the process of fetal wound healing is modulated by the presence of HA in the ECM.

Our previous work demonstrated a 'spectrum' of fetal incisional wound healing patterns, with a transition to adult-type healing and scar formation midway through the last trimester in fetal lambs.[11] Wound fluid from cylinders implanted late in gestation (*i.e.*, at 120 days) may show an adultlike pattern of HA deposition. If this is true, the pattern of HA in wound fluid can be used as a marker for 'fetal' or 'adult' phenotype in wound repair. Studies are underway to investigate this possibility.

The finding of a prolonged presence of HA in fetal wound fluid and its association with scar-free healing has major clinical implications. Hyaluronic acid has been demonstrated to enhance postnatal healing following tympanic membrane perforation and scratch incision.[24,25] Hyaluronic acid also was shown to decrease adhesions after tendon repair.[26] Thus it may be possible to modulate wound healing in children and adults to become more fetal-like, by the prolonged application of HA or HA-protein complexes to the healing wound.[38]

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