Studies in Fetal Wound Healing

IV. Hyaluronic Acid-Stimulating Activity Distinguishes Fetal Wound Fluid from Adult Wound Fluid

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Recent clinical and experimental evidence suggests that the fetus responds to injury in a fashion fundamentally different from that of the adult. Our initial experience with human open fetal surgery reinforces experimental observations that the fetal wounds heal without the scarring, inflammation, and contraction that often accompany adult wounds. In this study we examine fetal wound fluid in an attempt to elucidate the control mechanisms that endow the fetus with unique healing properties. The extracellular matrix of fetal wounds is rich in hyaluronic acid, a glycosaminoglycan found in high concentrations whenever there is tissue proliferation, regeneration, and repair. We establish that wound fluid from the fetus contains high levels of hyaluronic acid-stimulating activity that may underlie the elevated deposition of hyaluronic acid in the fetal wound matrix. In contrast there was no hyaluronic acid-stimulating activity present in adult wound fluid. Hyaluronic acid, in turn, fosters an extracellular environment permissive for cell motility and proliferation that may account for the unique properties observed in fetal wound healing.

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THE ERA OF OPEN fetal surgery has arrived. Experience at the Fetal Treatment Program (University of California, San Francisco) with this new surgical frontier includes twelve human fetuses, from 18 to 27 weeks gestation.[1] This clinical experience reinforces experimental observations in various animal models that the fetus responds to injury in a fashion fundamentally different from that of the adult.[2-10] Fetal wounds heal rapidly and without the scarring, inflammation, and contraction that often accompany adult wound healing.

These unique qualities of fetal wound healing may be attributed to a unique extracellular matrix (ECM). The fetal matrix has an entirely different profile of macromolecular structural constituents compared to the adult ECM. The fetal ECM is richer in glycosaminoglycans, particularly hyaluronic acid (HA).[9,10] The purpose of this study was to define the mechanisms by which HA is deposited in such abundance in the fetal wound and to compare that process with adult wound healing.

HA is found in high concentration whenever tissue proliferation and regeneration occur.[11] It is involved in the detachment process of the cell cycle that allows cells to move.[12] A burst of HA synthesis occurs before mitosis so that the dividing cells become dissociated from neighboring cells.[13-16] HA also inhibits cell differentiation, creating an environment that instead promotes cell proliferation.[17,18] HA is composed of alternating units of glucuronic acid and N-acetylglucosamine, and has a key role in the structure and organization of some ECMs that have been well studied, such as cartilage.[19]

We have previously described an HA-stimulating activity in both fetal bovine and sheep serum that has the ability to stimulate the synthesis of HA in a consistent manner.[20] We now establish that fetal wound fluid uniquely contains high levels of HA-stimulating activity, which results in elevated HA synthesis and sustained HA deposition in fetal wounds. We postulate that HA is laid down early in the ECM of both fetal and adult wounds, but HA deposition remains elevated for an extended time period only in fetal wounds. This may provide a permissive environment for healing through regeneration and growth, rather than through scarring and fibrosis.

Materials and Methods

Wound Cylinders

Sterile wire mesh cylinders were used as described by Schilling and Hunt.[21,22] The cylinders were made by cut-

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ting a rectangle from No. 40 mesh, 36-gauge stainless steel, rolling them into a tube and folding the ends closed. The fetal cylinders measured 1×3 cm, and the adult cylinders were 3×6 cm (Fig. 1).

Fetal Wound Fluid

Time-dated pregnant ewes (Torrel Farms, Ukiah, CA) at 100 days gestation underwent general halothane/oxygen anesthesia using techniques for fetal lamb surgery as previously described.[23] The ewe was prepped and draped sterilely, and a midline laparotomy was made. Using a GIA stapler a hysterotomy exposed the fetal lamb. A 1-cm incision was made in the right axillary crease where excess skin folds were observed. A small subcutaneous pocket was created by blunt dissection, and a single wire mesh wound cylinder was placed in the pocket (Fig. 2). The wound was closed in one layer using interrupted 4-0 silk sutures.

The fetus was returned to the uterus and amniotic fluid volume was restored with sterile normal saline. The hysterotomy was closed, including the membranes, with a TA-90 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 wound fluid harvest. The fetal wound fluid was harvested from the wound cylinders (one wound cylinder per animal) by a single percutaneous aspiration under sterile technique during reoperation using an 18-gauge needle and 12-cc syringe on days 1, 2, 4, 6, 8, 10, and 14 after implantation. The fluid was frozen immediately and stored at −70 C until analysis.

Adult Wound Fluid

One nonpregnant adult sheep underwent halothane/oxygen general anesthesia, the large wound cylinders were placed in separate subcutaneous pockets, and the wound was closed with interrupted 2-0 silk sutures. The adult wound fluid was harvested at the same time points as was the fetal fluid by aspirating the wound cylinders using sterile technique. The fluid was frozen at −70 C until analysis.

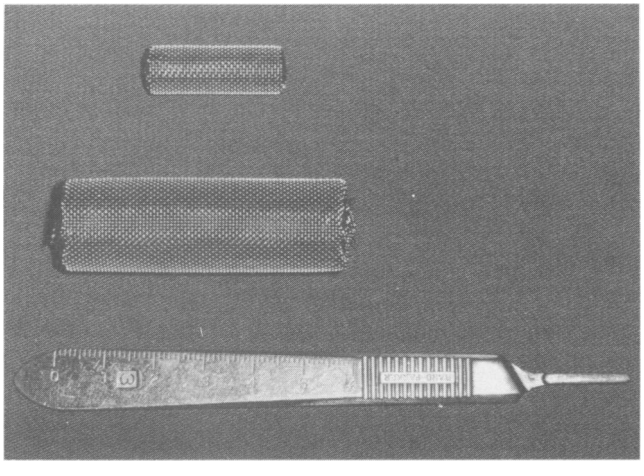

FIG. 1. Photograph of wire mesh wound cylinders. Top: fetal cylinder. Bottom: adult cylinder.

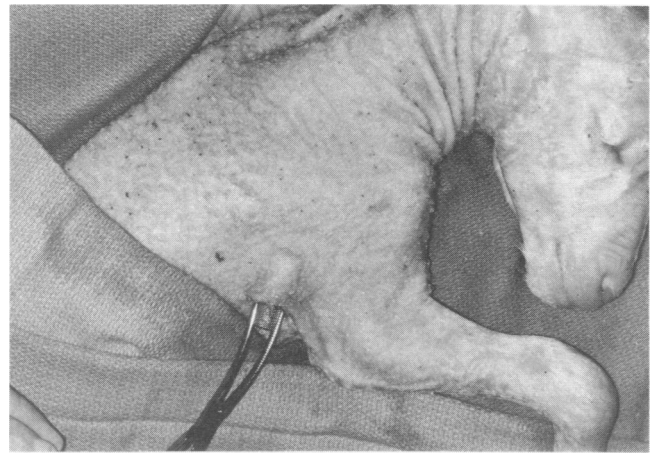

FIG. 2. Photograph of a wire mesh wound cylinder being placed into the right axillary subcutaneous pocket of a 100-day fetal lamb.

Cells and Cell Culture

Rat fibrosarcoma cells were obtained from the laboratory of Dr. Merton Bernfield (Stanford University) and were derived from a methylcholanthrene-treated rat.[24] Such cells have been demonstrated previously to synthesize copious quantities of HA.[25] These cells were cultured in Roswell Park Memorial Institute-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum, 25 mM HEPES (Gibco), 1% penicillin, streptomycin, and Fungizone (Squibb, Princeton, NJ), at 37 C in an atmosphere of 5% CO_2 and 95% air.

Measure of HA Synthesis

Rat fibrosarcoma cells were seeded routinely at a density of 2.5×10^5 cells in wells of 35-mm diameter (Costar, Cambridge, MA). Cells were 50% confluent at this density. Experiments were conducted during the logarithmic stage of growth and were generally concluded when cells were approximately 90% confluent. Cells were allowed to attach for 24 hours and the media was removed, the cells washed three times with low glucose serum-free Dulbecco's modification of minimal essential media H16 (DME H-16, Gibco), and replaced with 1.5 cc of DME H-16 containing 0.1% glucose, 5% calf serum, 1% penicillin, streptomycin, and 25 μg/mL of ascorbic acid. Next 150 μL of the sample to be tested (fetal or adult wound fluid) was introduced, and 1 hour later (^{3}H)glucosamine (44.8 Ci/mmol, New

England Nuclear Corporation) was added for 24 hours. The media, which contains 80% of the synthesized HA, was decanted. The cell layer was then washed with 1 mL calcium-and-magnesium-free phosphate-buffered saline.

To liberate glycosaminoglycans into the solution, proteins, including proteoglycan core proteins, were digested with 5 mg/mL pronase (type XIV bacterial protease, Sigma Chemical, St. Louis, MO), which was freshly prepared each time in 0.2M Tris (Schwartz/Mann), 0.02% Na azide, pH 8.0, and predigested at 37 C for 1 hour. The protease digestion was terminated by boiling the samples for ten minutes. The specific hyaluronidase from the mold *Streptomyces hyalurolyticus* (Calbiochem, La Jolla, CA) was used to digest HA for 2 hours. This enzymatic digestion was also terminated by boiling for ten minutes. The digested solution was precipitated with 7.5% cetylpyridinum chloride (CPC, Sigma Chemical) in the presence of carrier HA and chondroitin sulfate (type A, Sigma Chemical) and transferred onto GF/A glass fiber filters (Whatman, Maidstone, England) using a ten-well vacuum-assisted manifold suction apparatus (Hoefer Scientific, San Francisco, CA). The precipitate on the filter was washed with a 1.5% CPC solution, placed into scintillation vials, and allowed to dry in a 55 C oven. Optifluor^{R} (Packard, Downers Grove, IL) was then added and the filters were soaked for 24 hours before determination of levels of radioactive label in a Beckman LS 7500 counter (Beckman Instruments, Palo Alto, CA). The level of HA was derived from the difference in radiolabeling in triplicate samples digested in the presence and absence of the hyaluronidase enzyme.

Cell Counts

The duplicate cultures for cell counts were initiated at the same time and grown under conditions identical to their radiolabeled counterparts. A solution of 0.05% trypsin and 0.02% EDTA was used and cells were incubated for one minute to lift cells from the culture dishes. After dilution cells were counted in duplicate using a hemocytometer.

Statistical Analysis

Data are expressed as the mean value for the three samples at each experimental point. The standard deviation is given in each case.

Results

Indicator Cells

As previously reported, the rat fibrosarcoma cells have been established as valid indicator cells for the presence of the HA-stimulating activity.[20] These cells can synthesize large quantities of HA and respond to the serum factor in a time- and dose-dependent manner (unpublished observations). They require 5% calf serum to maintain viability during the assay period. The controls represent culture plates in which no wound fluid was added.

Adult Wound Fluid

Wound fluid obtained by sterile needle aspiration of the subcutaneously placed wound cylinders at 1, 2, 4, 6, 8, 10, and 14 days after implantation was assayed for HA-stimulating activity. Because wound fluid is known to possess mitogenic effects, all activity was expressed on a per-cell basis. At each time point assayed, the adult wound fluid had less HA-stimulating activity than did control samples (Fig. 3). In general the activity decreased, with time reaching its lowest level at 14 days, which was the last point at which a determination was made.

Fetal Wound Fluid

Fetal wound fluid was obtained also by sterile needle aspiration of the wound cylinders at 1, 2, 4, 6, 8, 10, and 14 days after implantation. The assay for HA-stimulating activity was performed, and as with the adult wound fluid, all activity was expressed on a per-cell basis. In sharp contrast to the adult fluid, levels of fetal wound fluid HA-stimulating activity were much higher than adult speci-

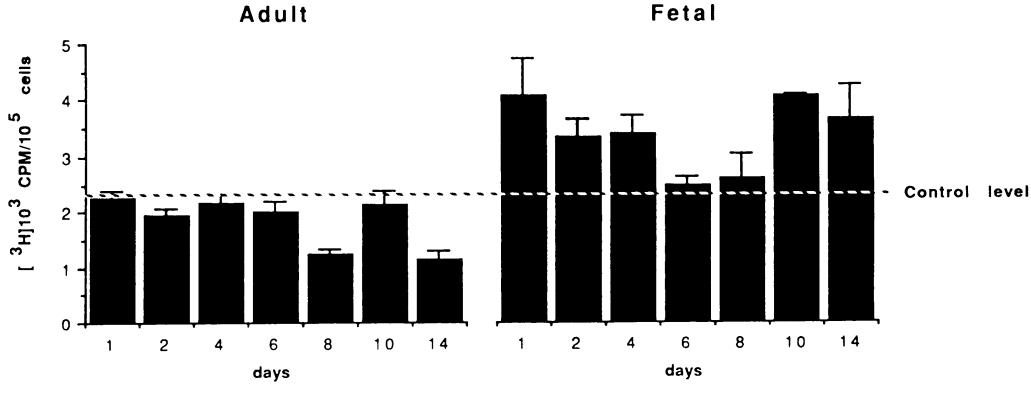

FIG. 3. HA-stimulating activity of adult *versus* fetal wound fluid. The activity was expressed on a per-cell basis as 10^{3} radioactive counts per minute per 10^{5} cells. As mentioned in Materials Methods, 150 μL of wound fluid was used with the indicator fibrosarcoma cells. The control value represents culture plates in which no wound fluid was added. Error bars represent the standard deviation of each value run in triplicate.

mens at each data point. The HA-stimulating activity appears to be bimodal with apparent maximum activity at days 1 and 10 of harvest.

Mitogenesis

We compared the mitogenic effect of fetal and adult wound fluid on these same indicator cells. As shown in Table 1, adult wound fluid had an increasing mitogenic effect with an apparent maximum at eight days. However by 14 days the fluid has an inhibitory mitogenic effect when compared to the control. By contrast the fetal wound fluid, surprisingly, was not mitogenic above control levels at any of the time points examined (Table 2).

TABLE 1. *Mitogenic Effect of Adult Wound Fluid*

Sample	Cell Count (10^5 cells)
Control	5.68
1 day	5.50
2 days	6.11
4 days	6.54
6 days	6.66
8 days	7.64
10 days	6.18
14 days	4.85

TABLE 2. *Mitogenic Effect of Fetal Found Fluid*

Sample	Cell Count (10^5 cells)
Control	5.68
1 day	3.77
2 days	4.42
4 days	4.82
6 days	4.64
8 days	4.50
10 days	3.40
14 days	4.00

Discussion

The fetus responds to injury in a fashion fundamentally different from that of the adult: the process of mesenchymal proliferation proceeds in the absence of an inflammatory process and fibrosis.[2–10] However the mechanisms that control these differences are unknown and are the objective of our ongoing studies.[20,26,27]

Krummel and DePalma[9,10] demonstrated that the ECM of fetal wounds is rich in glycosaminoglycans, specifically HA. This macromolecule is a key structural and functional component of the ECM, found whenever there is rapid tissue proliferation, regeneration, and repair. It inhibits cell differentiation, creating a permissive environment that promotes cell proliferation.[17,18] We postulate that HA is laid down early in the ECM of both fetal and adult wounds, but that sustained deposition of HA distinguishes fetal from adult wound healing. Our purpose was to determine if fetal wound fluid had the ability to stimulate and sustain HA synthesis. A prolonged presence of HA in fetal wounds may provide the mesenchymal signal for healing by regeneration rather than by scarring and fibrosis. A newly developed *in vitro* assay was used to evaluate HA synthesis in this study based on glycosaminoglycan precipitation with the detergent CPC, measuring the difference of radiolabeled precipitate in the presence and absence of specific hyaluronidase digestion. Siliconized glass fiber filters were used to catch precipitates, so that multiple samples could be assessed simultaneously, unlike other HA assays.[28,29] We used our assay to examine the observations of Krummel and DePalma and to elucidate possible underlying mechanisms.

We used the wire mesh wound cylinder model first described by Shilling and used extensively by Hunt.[21,22] These cylinders have relatively large mesh pores and separate the tissue, creating a dead space that fills with wound fluid. We harvested the wound fluid over a period of 14 days. Previous work has demonstrated that the fetus heals quickly and that sampling fluid from 1 to 14 days would suffice for the evaluation of early and late fetal wound healing.

We chose the fetal lamb because of our extensive experience with this model. We have performed more than 1000 fetal lamb operations in the past decade and have refined a reliable procedure to expose and operate on the fetal lamb.[23] Survival is approximately 90% with such procedures. In addition the long gestation (145 days) allows us to compare fetal wound healing at different gestational ages. This may be crucial because previous work in rats suggests that a spectrum of wound healing exists as a function of gestational age, with wound healing becoming more like that of adult sheep late in gestation.[30]

Our data demonstrate that the levels of HA-stimulating activity between fetal and adult wound fluid differ dramatically. The fetal wound fluid HA-stimulating activity was elevated at each data point. This may underlie the rich deposition of HA in the ECM of fetal wounds. The activity of fetal wound fluid has a bimodal distribution (Fig. 3). One explanation may be that the first peak (days 1 to 4) reflects elevated HA-stimulating activity from fetal sera. The second peak (days 8 to 14), may represent local production of HA-stimulating activity factor by fibroblasts under the influence of trophic factors such as TGF_{beta} contained in wound fluid. In contrast the level of activity in adult wound fluid was below control even at the earliest time point (one day). In general this relatively low level increased as the time points progressed, reaching a maximum at 14 days.

We have also compared the mitogenic effect of fetal and adult wound fluids at each data point. Adult wound fluid was initially mitogenic, but then decreased to below control levels by 14 days. This may be explained by the increased acidity of the adult wound environment between 10 and 15 days.[21] In contrast fetal wound fluid had an inhibitory effect on mitogenesis at all data points. Studies are in progress to measure fetal wound pH and lactate levels.

HA is deposited briefly at the earliest stages of adult wound healing.[31] We have previously demonstrated that the HA-stimulating activity of adult sheep serum is much lower than that of fetal lamb serum. Therefore it is unlikely that a circulating HA-stimulating activity is the basis for the early deposition of HA in the adult wound. A more localized mechanism may be required. Fibrinogen has been shown to contain an HA-binding site. HA may be brought to the wound site by fibrinogen from the circulation, as well as by the fibrinogen contained in alpha granules in platelets.[32–35] In the adult wound, local deposition of HA by the platelet plug and the fibrin clot obviates the need for an exogenous mechanism for stimulating local HA production. We have demonstrated that an HA-stimulating activity is present in the fetal wound for a prolonged period, a mechanism that is entirely absent in the adult wound. Our findings suggest that the mechanism for eliciting the early HA-rich response in adult wounds is entirely different from the prolonged HA-rich response in fetal wounds.

We have previously reported a glycoprotein factor present in fetal sera that stimulates HA synthesis with peak levels of activity occurring at mid-gestation.[20] Thus the HA-stimulating activity (HASA) present in fetal wound fluid may be a reflection of the relative amounts of HASA in fetal sera compared to adult sera. We have also found that fetal urine and amniotic fluid have elevated HA-stimulating activity[20] (Longaker et al., unpublished observations). Unlike fetal sera, the peak activity of amniotic fluid occurs at or near term.[20] This probably reflects the increased contribution of fetal urine to amniotic fluid volume in the second half of gestation. This may be an additional mechanism by which HA is deposited in the matrix of fetal skin wounds because the wounds are bathed in amniotic fluid that contains both an HASA and HA.[36] We are now investigating the effect of excluding fetal wounds from amniotic fluid.

We have also wondered if this HA-stimulating activity crosses the placenta. It turns out that the pregnant ewes have only a slightly higher level of activity than the nonpregnant ewes, which is minimal.[20] In addition we have examined the activity of pregnant maternal urine and have found evidence for a distinct inhibitory activity (Longaker et al., unpublished observations). We are now investigating this phenomenon that may be a protective mechanism for the pregnant ewe.

This study is the first to attempt to analyze constituents of fetal wound fluid and is part of our ongoing effort to investigate the control mechanisms that endow the fetus with unique healing properties.[20,26,27] The ultimate aim of these studies is to apply some of the special features of fetal wound healing to modulate adult wound processes and to avoid fibrosis and contracture.

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