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ARACHIDONIC ACID METABOLITES: Effects on Inflammation of Fetal Rabbit Excisional Wounds[1]

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Abstract—Uncovered fetal rabbit excisional wounds do not exhibit any classic signs of healing; wounds covered with an impermeable cover do contract, reepithelialize, and exhibit inflammation. Prostaglandin E_2 (PGE_2) is elevated in amniotic fluid, acting as an immunosuppressant at the maternal–fetal interface. Full-thickness excisional wounds were made on 25-day gestational age rabbit fetuses. Half the wounds were covered with an impermeable cover. Tissue from covered, uncovered, and nonwounded fetuses was examined 72 h after wounding for arachidonic acid metabolites. Uncovered wounds had significantly ($P \leq 0.05$) elevated levels of PGE_2, $PGF_{2\alpha}$, and 12-HETE versus covered wounds and control tissue. Covered wounds had significantly elevated levels of 15-HETE compared to uncovered and control tissue. The elevated PGE_2 in uncovered wounds may act as a fetal immunosuppressant; covered wounds (lower PGE_2) developed cellular inflammation. Further investigations of these interactions may permit modulation of adult inflammation.

INTRODUCTION

Fetal wounds exhibit significantly different healing characteristics than postpartum wounds. Sutured incisional fetal wounds heal with no visible scar formation,

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while excisional fetal rabbit wounds exposed to amniotic fluid apparently do not heal, displaying no inflammation, contraction, or reepithelialization (1–6). However, coverage of fetal rabbit excisional wounds with an impermeable membrane that excludes amniotic fluid does allow a cellular inflammatory response, wound contraction, and reepithelialization (7–9).

Amniotic fluid contains elevated levels of arachidonic acid metabolites, particularly prostaglandins (PG), which are known to be potent inflammatory mediators (10–12). The elevated levels of PGE_2 found in amniotic fluid are thought to act as immunosuppressants at the maternal–fetal interface to prevent rejection (13–18). This study was designed to examine the levels of various arachidonic acid metabolites (eicosanoids) in fetal tissue surrounding covered and uncovered excisional wounds, correlating the data to cellular inflammation.

MATERIALS AND METHODS

Surgery and Sample Procurement. Time-dated pregnant New Zealand White rabbits were procured on day 20 of gestation (term = 31–32 days). The rabbits were allowed to recover from the stress of transport. On day 25, the does were anesthetized by intramuscular administration of xylazine (Rompun, 12 mg/kg), ketamine (60 mg/kg), and acepromazine (0.6 mg/kg). One percent halothane was administered by mask for maintenance and uterine relaxation during the procedure. Halothane exposure was limited to 1 h to minimize fetal mortality.

The bicornate uterus was exposed through a midline laparotomy, and the distal (ovary end) two fetuses were identified in each horn for surgery. The tail and dorsal hindquarters of the fetus were gently exposed through individual purse string hysterotomies, with the hind legs remaining within the confines of the uterus. A 4-mm-diameter punch wound, inclusive of the panniculus carnosus, was created on the dorsal midline immediately cranial to the tail of the fetus. Following creation of the defect, fetuses were divided into one of two groups: group 1 fetuses were returned to the uterus with no further manipulation (uncovered control) ($N = 8$); group 2 fetuses were stabilized to allow a 6-mm-diameter impermeable silicone cover to be sutured over the defect with six 7-0 prolene sutures before being returned to the uterus (covered) ($N = 8$). Six-millimeter-diameter covers were utilized, as covers larger than 6 mm buckled on the curved dorsum of the animal, creating a conduit to the wound. Sham controls included a nonwounded fetus exposed to air and returned to the uterus, a nonwounded fetus that had a cover sutured in place, and a fetus in which the silicone cover was placed subcutaneously and the incision sutured closed with 7-0 prolene. Sterile saline (37°C) was added to replace lost amniotic fluid (approx. 1–2 ml). The does were returned to their cages and allowed to recover. Food and water were supplied ad libitum.

Fetuses were harvested by cesarian section at 72 h after wounding. The wounds were photographed and the surface area of the wounds was calculated by planimetry. The dorsal operated area was excised; half the fetuses were fixed in 10% buffered formalin for histologic analysis and half were flash frozen in liquid nitrogen and stored at −70°C for determination of inflammatory mediators. Dorsal skin for nonexposed, nonwounded fetuses was excised and flash frozen to serve as control samples for eicosanoid analysis ($N = 4$). The frozen tissue from the initial two fetuses in each group were combined to ensure measurable levels of eicosanoids. The remaining two fetuses from each group were processed individually. Amniotic fluid from around two nonoperated fetuses were collected at harvest, pooled, and analyzed for eicosanoid levels.

Arachidonic Acid Metabolites (Eicosanoid) Quantification. Presence and quantities of

inflammatory mediators prostaglandins (PGE_2, $PGF_{2\alpha}$), leukotrienes (LTB_4, LTC_4, and LTD_4), and hydroxyeicosatetraenoic acids (12-and 15-HETE) were determined following the procedure of Duell et al. (19). Briefly, the frozen sections were weighed and then powdered under liquid nitrogen in a precooled mortar and pestle. While still frozen, the powder was transferred to a polypropylene centrifuge tube containing 4 ml of extraction solution (8 parts ethanol to 2 parts 0.01 M phosphate buffer, pH 6.4). The extraction solution prevents further synthesis or hydrolysis of the eicosanoids. The samples were stirred on ice for 30 min, followed by centrifugation at 2500 *g* for 10 min. The supernatant was saved. The pellets were reextracted for 30 min with 2 ml of ethanol–methanol–buffer (4:4:2) and recentrifuged. The supernatants were combined, and buffer added to reduce alcohol content to 15%.

The combined supernatant fractions and amniotic fluid were partially purified with conditioned Sep-pak cartridges, washed with hexane, and the eicosanoids eluted with methyl formate and methanol. The methyl formate and methanol fractions were evaporated to dryness under nitrogen and resuspended in a final volume of 30 μl (8:2) of acetonitrile and H_2O containing 0.2% H_2PO_4 adjusted to pH 3.9 with NH_4OH.

The eicosanoids were separated using a modification of the HPLC method of Van Rollins et al. (20). Briefly, HPLC separations were done using a Hewlett-Packard 1090 M unit equipped with a ternary solvent system, diode array detector, and sample oven. Twenty-five microliters of each sample were injected onto a Hewlett-Packard C_{18}-ODS microbore column (100 mm × 2.1 mm). A flow rate of 0.3 ml/min at 38°C was used for the 30-min analysis. The initial solvent was 30 parts acetonitrile–70 parts water containing 0.2% phosphoric acid adjusted to pH 3.9 with ammonium hydroxide. After 6 min of isocratic elution, an acetonitrile gradient was initiated that reaches 50% at 15 min. During the next 5 min the acetonitrile concentration was increased to 55%, followed by an increase to 85% in the next 3 min. At 25 min the acetonitrile concentration was lowered to 65% to prevent precipitation of ammonium phosphate at high acetonitrile concentrations and still clear the column of noneicosanoid lipids. The fractions were evaporated to dryness under a stream of nitrogen and the residues resuspended in 200 μl of the appropriate buffer.

The mediators (PGs, LTs, HETEs) were quantified by RIA. The HPLC HETE samples were assayed at three dilutions in 0.01 M phosphate buffer, pH 7.6, 0.9% NaCl, 0.1% BSA, and 0.1% sodium azide. Appropriate tritiated mediator was added as labeled antigen. Total volume of the assay was 300 μl. The standard curve ranged from 10 to 400 pg/tube. The samples were incubated at room temperature for 2 h. The assay was terminated by addition of 700 μl of ice-cold dextran-coated charcoal. Samples were kept on ice for 10 min, then centrifuged at 2500 *g* for 10 min. The supernatant fractions were transferred to scintillation vials and counted. The results are expressed as picograms per milligram of protein or picograms per milligram of DNA. The assay for LTs and PGs was similar, utilizing a slightly different pH and was run overnight at 4°C.

Protein levels of the tissue were determined by the methods of Lowry et al. (21). DNA concentration in the tissue was determined by the method of Burton (22).

Statistical Analysis. A one-way analysis of variance (ANOVA) was performed to determine significance between uncovered, covered, and control (nonoperated) tissue levels at $P \leq 0.05$.

RESULTS

Uncovered wounds exhibited a very sparse cellular inflammatory response. Silicone-covered wounds exhibited a marked, mixed cellular inflammatory response of histiocytes, monocytes, macrophages, and polymorphonuclear leukocytes. The inflammatory cells were present throughout the subcutaneous tissue

under the wound and in the coagulum under the cover. Inflammatory cells were rarely found in the subcutaneous tissue under the nonwounded tissue surrounding the defect. Using the edge of the dermal tissue as reference, all covered wounds exhibited varying degrees of contraction (mean = 69% original size), while uncovered wounds increased in surface area (mean = 139% original area).

Examination of the sham controls showed no gross or histologic changes when compared to skin of normal, nonexposed, nonoperated rabbit fetuses of the same gestational age. A few inflammatory cells were noted around the suture used to affix the silicone cover to the fetus. A very mild foreign-body reaction was noted around the silicone cover placed subcutaneously.

Analysis of tissue samples encompassing uncovered wounds showed significantly elevated levels of PGE_2, $PGF_{2\alpha}$, and 12-HETE compared to covered wounds and control tissue (Table 1, Figure 1). LTB_4 levels in uncovered wounds were significantly higher than covered wounds and nonoperated skin. Covered wounds exhibited significantly elevated levels of 15-HETE compared to uncovered and control samples, and significantly elevated levels of $PGF_{2\alpha}$ compared to control tissue. Overall, the leukotriene pathway of the eicosanoid biosynthetic pathway was suppressed for all tissue samples. Amniotic fluid levels of eicosanoids were elevated for all eicosanoids with the exception of LTD_4, which was at background levels.

DISCUSSION

The response of the fetus to surgical insult is becoming increasingly important due to the expanding number of invasive procedures performed on fetuses. These procedures range from accidental trauma (misguided amniocentesis sticks) to in utero procedures (fetal blood transfusions, biopsies, and skin sampling) to direct surgical intervention for life-threatening anomalies (distal urinary tract obstructions and diaphragmatic hernia) (23–28). The sequelae to these procedures are represented in this study: uncovered wounds are similar to needle sticks and fetal skin sampling; covered wounds are similar to diaphragmatic hernia repair, in which a Gortex patch is sutured to the abdominal wall and prevents amniotic fluid from entering the abdominal cavity.

The lack of a cellular inflammatory response for uncovered (amniotic fluid-exposed) fetal excisional wounds initially appears paradoxical, as fetuses are capable of mounting a cellular inflammatory response to bacterial and chemical challenges and also to thermal burns (29–34). However, the situations in which the fetus can mount an inflammatory response (bacterial and chemical challenges, burns with intact eschar) are closed insults, protected from amniotic fluid. This is in contrast to open, uncovered excisional wounds, which are

Table 1. Levels of Eicosanoids in Tissue Samples from Uncovered, Covered, and Control (Nonoperated) Fetuses 72 Hours after Wounding (Mean ± *SEM*)

Eicosanoid	Level (pg/μg DNA)			Amniotic fluid (pg/ml)
	Uncovered	Covered	Control	
PGE_2	14.20 ± 7.39[a]	4.43 ± 1.29	3.37 ± 1.55	565.6
$PGF_{2\alpha}$	4.68 ± 2.46[a]	1.58 ± 0.65	0.67 ± 0.16	457.8
LTB_4	0.66 ± 0.38[a]	0.37 ± 0.18	0.05 ± 0.03	92.41
LTC_4	[b]	[b]	[b]	332.8
LTD_4	[b]	[b]	[b]	[b]
15-HETE	17.95 ± 5.63	28.35 ± 4.62[c]	11.99 ± 3.37	2420.0
12-HETE	18.25 ± 5.67[a]	5.38 ± 2.22	7.60 ± 5.02	706.5

[a] Significantly higher than covered at $P \leq 0.05$.
[b] Background levels.
[c] Significantly higher than uncovered at $P \leq 0.05$.

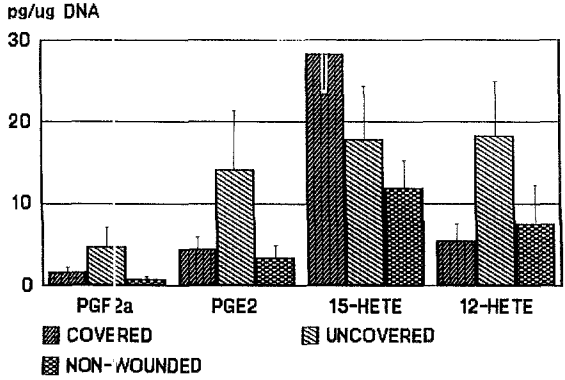

Fig. 1. Bar graph of levels of $PGF_{2\alpha}$, PGE_2, 15-HETE, and 12-HETE in uncovered and covered fetal wound tissue and in nonoperated control tissue.

continuously bathed by amniotic fluid and do not mount an inflammatory response. The fetal inflammatory response seen here for covered wounds confirms previously reported results (9).

Amniotic fluid contains elevated levels of PGE_2, which functions at the maternal–fetal interface to prevent maternal (host) rejection of the foreign fetal tissue (13–18). PGE_2 has also been shown to inhibit contraction of fibroblast-populated collagen lattices in culture (35). Thus the PGE_2 in amniotic fluid may also inhibit contraction of fetal wounds, with the covers excluding PGE_2 and resulting in wound contraction.

Additional arachidonic acid metabolites are present in amniotic fluid and are known to be potent inflammatory and contractile mediators (17). The sig-

nificantly elevated levels of PGE_2 in the tissue surrounding uncovered fetal rabbit wounds may suppress the fetus' ability to mount an inflammatory response, while the lower levels in covered wounds may permit a response. These quantitative data, along with the histologic evidence, supports the theory that inhibitory or suppressive factors are present in amniotic fluid and can affect the fetus' response to insult.

The suppression of the leukotriene portion of the arachidonic acid metabolic pathway deserves further comment. The HPETE and HETE families of molecules have potent chemotactic activity for eosinophils and neutrophils (10). As such, the marked cellular inflammation of covered wounds is not unexpected when the elevated levels of 15-HETE in covered wounds is considered. The leukotrienes are chemotactic for polymorphonuclear leukocytes and cause release of lysosomal enzymes from PMNs (36). Additionally, LTs are closely related (or identical) to "slow-reacting substance" (SRS) (which possesses smooth muscle contracting activity), with LTD_4 being the most potent (10). The general suppression of the leukotriene family may be related to the lack of wound contraction in uncovered wounds, but the lack of leukotrienes (especially LTD_4) in covered wounds and nonwounded skin seems to refute any role leukotrienes may play in covered fetal wound contraction. It is possible that there are unidentified factors present in the fetal milieu that suppress leukotriene production, or perhaps fetuses of this age have not developed the capability to synthesize leukotrienes.

Further investigations into the relationship between the fetal response following excisional wounding and factors present in amniotic fluid may elucidate the roles these factors play in inflammation and healing. This knowledge has significant ramifications regarding modulation of the inflammatory response, future fetal surgery, and potentially may be used to modify abnormal adult inflammation and healing.

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