Lower Cytokine Release by Fetal Porcine Platelets: A Possible Explanation for Reduced Inflammation After Fetal Wounding

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• Fetal dermal wound healing is unique because of its rapidity, minimal inflammation, and lack of scarring. Cytokines such as transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF) evoke an inflammatory response and scarring when applied to fetal wounds. Because adult and fetal platelet counts are comparable, the aim of this study was to test the hypothesis that the minimal inflammatory response seen in the fetus is attributable to differences in the serum content of cytokines released by fetal platelets. Using Yorkshire swine, blood was collected from 10 adults and 10 fetuses at day 60 of gestation (fullterm, 114 days). Platelets were isolated from anticoagulated blood and examined by transmission electron microscopy. Serum was analyzed for PDGF-AB and TGF-β2 by enzyme-linked immunosorbent assay (ELISA), and TGF-β1 by ^{125}I radioimmunoassay. TGF-β samples were assayed with and without prior acid activation to determine the total TGF-β and the biologically active form of the cytokine. Electron microscopy of adult and fetal platelets showed no gross structural differences. Alpha granules, which contain cytokines as well as procoagulant factors, were present in similar quantities and with the same degree of homogeneity. The cytokines analyzed were present in all the adult and fetal sera tested. However, PDGF-AB was present in significantly lower concentrations in the fetus (383 ± 72 pg/mL *v* 972 ± 185 pg/mL in the adult; $P < .05$). In addition, the fetal samples contained lower amounts of TGF-β1 (13,895 ± 1,770 *v* 29,864 ± 5,050 pg/mL; $P < .05$) and TGF-β2 (6,758 ± 734 *v* 13,407 ± 1,395 pg/mL; $P < .05$). The majority of TGF-β was in latent form; the adult sera contained significantly more active TGF-β1 and active TGF-β2 than the fetal sera. The ratios of active TGF-β1 to active TGF-β2 were similar for the adult (22.3) and fetus (18.5). However the ratio of total TGF-β1 to total TGF- β 2 was significantly lower for the fetus (2.26 *v* 7.69). The authors conclude that although no gross differences in platelet ultrastructure were noted, fetal porcine platelets release lower quantities of cytokines into serum. This lower serum cytokine content and the relative concentrations of TGF-β1 and TGF-β2 may explain, in part, the minimal inflammation and sparse fibrosis characteristic of fetal wounds. These observations provide further insight into the unique fetal response to wounding and may offer alternative avenues to modulate the postnatal wound healing response.

INDEX WORDS: Wound healing, fetal; platelet; cytokines.

FETAL DERMAL WOUNDS heal rapidly and without the marked inflammation and scarring characteristic of adult wounds.[1-3] An "adult-like" inflammatory response can be evoked in fetal wounds by the application of diverse agents such as bacteria,[4] formyl-met-leu-phe (a bacterial by-product), and cytokines such as transforming growth factor-β (TGF-β)[5] and platelet-derived growth factor (PDGF).[6] These observations suggest that the fetus is capable of evoking an inflammatory response but perhaps an earlier event in the wound healing cascade is missing. In the adult, platelets are the first cellular elements at the site of injury. They aggregate, forming a hemostatic plug and in the process release contents of their alpha granules. These alpha granules contain procoagulant factors and cytokines such as TGF-β[7,8] and PDGF,[9] which are chemoattractive and recruit inflammatory cells to the wound site.[10] In previous studies from our laboratory, it was found that although platelet counts are comparable in the fetus and the adult, significant functional differences exist.[11] Therefore, the objective of the present study was to test the hypothesis that the minimal inflammatory response observed in the fetus upon wounding (compared with the adult) is attributable to differences in serum content of the cytokines released.

MATERIALS AND METHODS

Preparation of Samples

Using Yorkshire swine, whole blood was obtained from 10 adult animals by sterile venipuncture, and from 10 fetuses (day 60 of gestation; full term, 114 days) by umbilical vein catheterization. Fetal blood was obtained during intrauterine fetal surgery on time-dated pregnant sows anesthetized with ketamine, acepromazine, and isofluorane, as described previously.[11] The blood was gently aspirated into disposable plastic syringes and immediately transferred into specimen tubes that contained sodium citrate (3.8%) or no anticoagulant. The anticoagulated samples were centrifuged at 150*g* for 20 minutes. The upper two thirds of the platelet-rich plasma supernatant was aspirated for electron microscopy. Blood samples with no anticoagulant were allowed to stand at room temperature for 30 minutes. Serum was separated by centrifugation at 3500*g*, placed in aliquots, and frozen at −20°C until analyzed.

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Preparation for Electron Microscopy

The platelets were fixed in suspension by adding an equal volume of 3% phosphate-buffered glutaraldehyde to the platelet-rich plasma, and the mixture was incubated at 4°C for 60 minutes with gentle agitation. Fixed platelets were separated by centrifugation at 3,500*g* for 10 minutes. The pellet was washed four times in phosphate-buffered saline (PBS), resuspended in 1% osmium tetroxide, and postfixed for 2 hours. The platelet pellets were further processed for electron microscopy in standard fashion, as described by Saving et al.[12] Thick 1-μm sections were screened by light microscopy to locate areas of abundant platelets. Thin 100-nm sections were then cut from this block, mounted on hexagonal copper grids, and scanned with a transmission electron microscope (JOEL 1200 EX; JOEL Ltd, Tokyo, Japan). Photographs were taken at 10,000× and 22,000× magnifications. Only one set of photographs was taken per grid square (125 × 125 μm).

Cytokine Analyses

TGF-β1, TGF-β2, and PDGF-AB were analyzed in this study. PDGF-AB was assayed by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies to human PDGF-AA and human PDGF-BB (R&D Systems Inc, Minneapolis, MN). Also using the ELISA technique, TGF-β2 was assayed with monoclonal and polyclonal antibodies to recombinant human TGF-β2 (R&D Systems Inc). TGF-β1 was analyzed by ^{125}I radioimmunoassay (Du Pont NEN Research Products, Boston, MA) using monoclonal and polyclonal antibodies to recombinant human TGF-β1. The samples for TGF-β analysis were assayed with and without prior acid activation with 1.2 mol/L hydrochloric acid for 15 minutes and neutralization with 0.5 mol/L HEPES/0.17 mol/L sodium hydroxide (NaOH) mixture just before adding the samples to the ELISA plate (TGF-β2) or adding the monoclonal anti-TGF-β1 antibody. The rationale for this treatment is that TGF-β exists both in the active and latent forms.[8] Most of the antibodies currently available against TGF-β detect only the active form of the cytokine. Samples assayed without acid activation will detect the inherently active form of TGF-β, whereas those assayed after acid activation will reflect the total TGF-β activity within the sample.[13] ELISA samples were read on a microplate spectrophotometer at a wavelength of 450 nm, with wavelength correction set at 540 nm. ^{125}I TGF-β1 radioactivity was counted with a gamma scintillation counter (Tri-Carb 1500, Packard Instrument Co, Downers Grove, IL). The unpaired two-tailed Student's *t* test was employed for statistical analysis. A *P* value of less than .05 was considered significant.

RESULTS

Electron Microscopy

Transmission electron microscopy of fetal and adult platelets showed no gross structural differences. The types and quantity of organelles appeared similar (Fig 1). However, the fetal platelets contained more glycogen pigments. This high glycogen content is typical of most fetal tissue and serves as energy stores for anaerobic metabolism. Several large fetal platelets were noted, although the average platelet size was comparable to that of the adult. Adult and fetal platelets contained similar amounts of alpha granules with similar degrees of homogeneity. Dense granules were sparse both adult and fetal platelets.

Cytokine Analyses

The cytokines tested (PDGF-AB, TGF-β1, and TGF-β2) were detected in all adult and fetal samples (Table 1). Fetal porcine sera contained significantly lower amounts of PDGF-AB compared with adult sera. As previously noted by other investigators, most TGF-β in serum was in latent form.[14] Only about 10% of TGF-β1 and 1% of TGF-β2 were in active form. Active TGF-β1 levels were significantly lower in the fetus. Similarly, the total TGF-β1 levels were lower in the fetus. TGF-β2 levels were lower in the fetus than in the adult, both in active form and total protein. Because the ratio of the TGF-β isoforms may be equally as important as their absolute values,[15] these were calculated for each animal. The average ratios of active TGF-β1 to active TGF-β2 were similar in the adult (22.3) and fetus (18.5). However, the ratio of total TGF-β1 to total TGF-β2 was 7.69 in the adult and significantly lower in the fetus 2.26 ($P < .05$).

DISCUSSION

Scarless healing occurs in a fetal wound environment where there is a minimal inflammatory response. Once acute inflammation is evoked at the site of fetal wounds, marked fibrosis results. Platelets arrive at the wound site immediately after injury. Their activation results in the release of numerous procoagulant factors and cytokines such as TGF-β and PDGF. Platelet activation is a critical step that provokes healing in the adult by stimulating clotting and initiating an inflammatory response.[16] PDGF, produced primarily by platelets, is a potent chemoattractant and inducer of growth and proliferation of mesenchymal cells, especially fibroblasts[17,18] and smooth muscle cells.[19,20] The highest concentrations of TGF-β are found in platelets and bones. TGF-β is a multifunctional polypeptide growth factor whose actions include the attraction and induction of macrophages to secrete several growth factors.[14,21] TGF-β also is a critical modulator of collagen metabolism. It induces the production of collagen by fibroblasts,[21-23] the inhibition of collagen degradation by the suppression of collagenase,[24,25] and the induction of tissue inhibitors of metalloproteinases (TIMPs)[25] that limit collagenase activity. The net result is an increase in production but a decrease in degradation of collagen. It is generally believed that the presence of these cytokines at the site of injury initiates the inflammatory response. Application of TGF-β and PDGF to fetal wounds was found to evoke an inflammatory response and induce adult-like healing.[5,6] The addition of neutralizing antibodies to TGF-β has been reported to result in a more organized matrix with reduced scarring.[26]

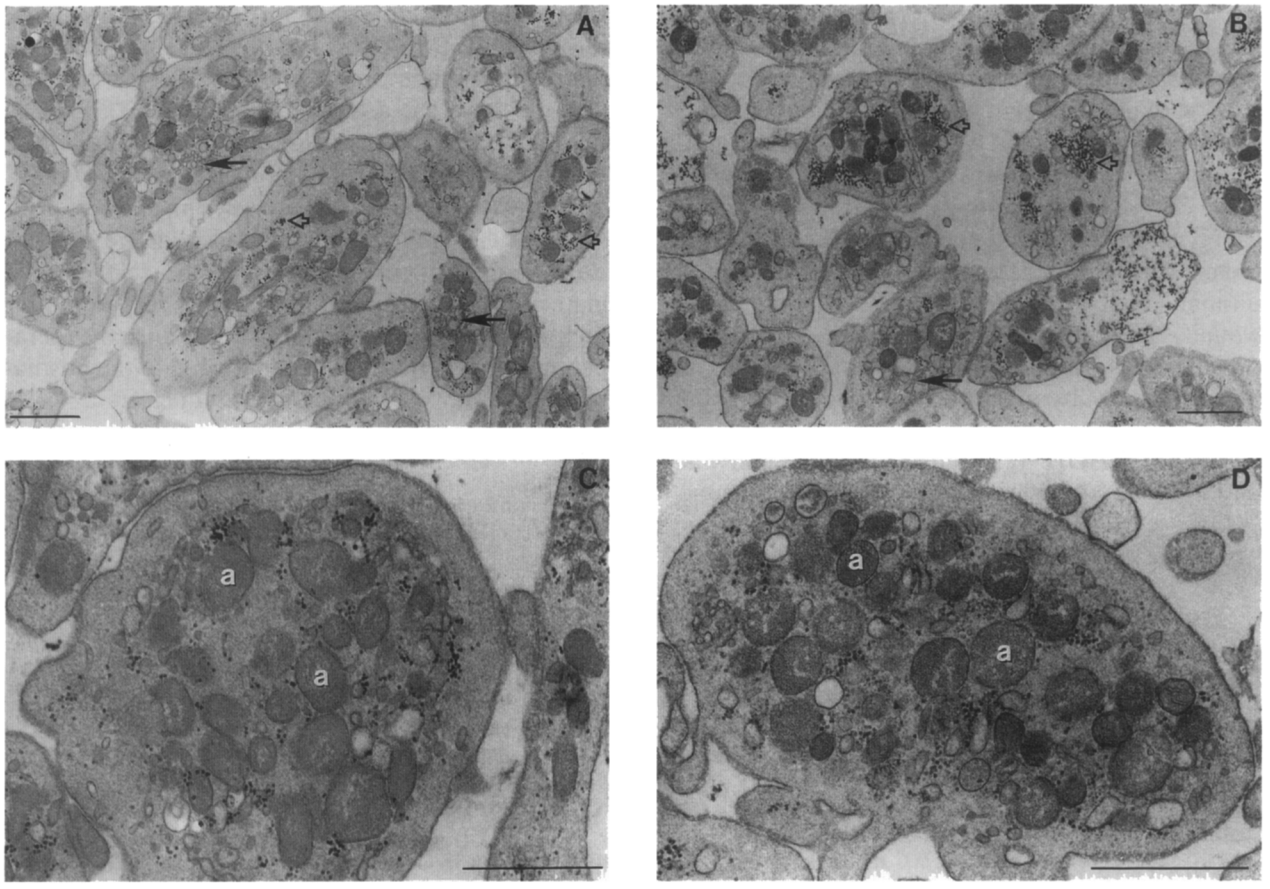

Fig 1. Transmission electron micrographs of adult (A,C) and fetal (B,D) platelets. Fetal platelets contain abundant glycogen granules (open arrows) characteristic of most fetal tissue. Mitochondria, golgi complex (solid arrows), and alpha granules (a) are similar in structure and quantity in the adult and fetus (horizontal line = 1 μm).

In the present study, TGF-β1, TGF-β2, and PDGF-AB were present in both adult and fetal sera, but the concentrations were significantly lower in fetal sera. Serum cytokine content more closely resembles the amount of cytokines released by platelets under physiological conditions. Because the response of fetal and adult platelets to various agonists differs,[11,27-29] adequate comparison of cytokine release after aggregation to a specific agonist is not possible. Essentially all the PDGF and about 90% of the TGF-β in serum is of platelet origin. Therefore these results reflect platelet release. Platelet counts are comparable for adults and fetuses[30-33]; hence, this cannot explain the differences in serum cytokine content. The present observations indicate that a lower amount of cytokines is released by fetal platelets. This may be related to a lower cytokine content within fetal platelets or to decreased secretory capabilities.

Electron microscopy showed the average fetal platelet size to be similar to that of the adult, but several large platelets were found in the fetus. Similar findings have been noted for the human fetus.[34] However, electron microscopy is not a reliable method of assessing platelet size. Bleyer et al also noted that human fetal platelets early in gestation tend to be larger, rounder, and contain fewer granules.[34] In contrast, in the present study it was noted that platelet alpha granules were similar in number, distribution, and heterogeneity in the fetus and adult.

Table 1. Cytokine Content of Adult and Fetal Sera

	PDGF-AB	TGF-β1 Active	TGF-β1 Total	TGF-β2 Active	TGF-β2 Total
Adult	972 ± 185	3,045 ± 810	29,864 ± 5,050	109 ± 11	13,407 ± 1,395
Fetus	383 ± 72*	1,020 ± 50*	13,895 ± 1,770*	66 ± 12*	6,758 ± 734*

NOTE. Data are expressed as mean ± SEM in picograms per microliter. Adult sera contained significantly greater quantities of PDGF-AB, TGF-β1, and TGF-β2 (*$P < .05$). Active TGF-β1 and active TGF-β2 also were significantly higher in the adult.

Alpha granules contain not only cytokines but other procoagulant factors, and thus their morphological appearance may not correlate with cytokine content.

Most of the TGF-β in serum was in latent form,[13,14] but the amount of latent TGF-β activated at the wound site is unknown. Longaker et al found that most TGF-β in the wound fluid of adult and fetal sheep also was in latent form.[15] Therefore, local factors that activate latent TGF-β will determine the ultimate biological effects of this cytokine at the wound site. The lower cytokine content of fetal serum provides less cytokine for activation and thus will influence the subsequent biological activity. Furthermore, the poor aggregation of fetal platelets to collagen and ADP[11] released at the site of injury also may influence the release of cytokines at the wound.

Both TGF-β1 and TGF-β2 have fibrotic effects when injected subcutaneously or added to wounds.[35-38] In addition to the total amount of TGF-β present, the ratio of TGF-β isoforms is believed to play a role in the regulation of scarring and fibrosis.[15] It was noted that the fetus has a lower ratio of total TGF-β1 to TGF-β2. The significance of this altered ratio in the modulation of inflammation remains unclear. TGF-β2 is not detectable in adult human serum and its role in scarless healing in the human fetus is, at present, not clear. A better understanding of the role of fetal platelet cytokines in the inflammatory response after fetal wounding would significantly enhance the therapeutic maneuvers employed to modulate the postnatal inflammatory response and its sequelae.

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Discussion

T.M. Crombleholme (Boston, MA): How platelets are harvested can cause activation that can artificially alter the concentrations observed when assayed. How were the platelets harvested, both in the adult and the fetus?

Have you looked at varying gestational age in the fetus and differences between fetal and adult platelets?

It's well recognized that pregnant women's platelets don't activate as well as platelets of nonpregnant women. They seem to be selectively unresponsive to many agents that cause platelet activation, and that goes away as soon as they deliver, seemingly an effect of some humoral factor. Did you look at fetal platelet activation in relation to that of adults?

O.O. Olutoye (response): With regard to harvesting, whole blood was obtained from the adults by peripheral venipuncture and from the fetal umbilical vein during fetal surgery. We harvested whole blood and separated the serum. The platelets actually had been activated and had released contents of their granules into the serum. We have only looked at cytokine release and not the total platelet content. Although the cytokine content of these platelets may differ, the amount of cytokine actually released is what is physiologically important, and that is what we have tried to study.

Although it would be interesting to look at platelets later on in gestation when there is a transition to an adult-like healing response, we chose only to look at platelets at midgestation (when the fetus does not scar) and compare them with the platelets of adults (who do scar) to provide a clear comparison.

With regard to the effect of pregnancy on platelet function, we have performed other studies that showed that fetal platelets do not aggregate as well as adult platelets. We also compared pregnant female platelets with adult male platelets, and in our model there were no significant differences in aggregation.

H.R. Ford (Pittsburgh, PA): As you know, TGF-β is a very potent down-regulator of the inflammatory response both in vitro and in vivo; therefore, how do you reconcile these factors with your findings? Is it possible that what you are observing is a mere epiphenomenon, and that other factors may be responsible for the observed changes in wound healing in the fetus?

O.O. Olutoye (response): It would be foolhardy on my part to assume that fetal platelets are the key to scarless healing. However, platelets definitely play a significant role in the minimal inflammation noted following fetal wounding. The reduced release of cytokines, which induce inflammation and fibrosis when applied to fetal wounds, helps to shed some light on this aspect of fetal wound healing.

N.S. Adzick (Philadelphia, PA): Do fetal pigs heal in a scar-free manner at 60 days' gestation? Do fetal pigs show a more adult-like repair with scar formation when wounded late in gestation?

O.O. Olutoye (response): Pigs have a gestational period of about 114 days. Previous work by Dr Caldwell's group in Chicago noted the absence of scarring in fetal pig wounds created at 60 days' gestation. We have confirmed these findings in our laboratory and noted that scarless healing occurs in the fetal pig up to day 80 of gestation. At about day 100 of gestation, there is a transition to adult-like healing, with fibrosis and scar formation. This is in keeping with observations noted with other animal models (sheep, mice).