Published in final edited form as:

J Pediatr Surg. 2008 April ; 43(4): 675–682.

FETAL WOUND HEALING USING A GENETICALLY MODIFIED MURINE MODEL: THE CONTRIBUTION OF P-SELECTIN

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Abstract

BACKGROUND/PURPOSE—During early gestation, fetal wounds heal with a paucity of inflammation and absent scar formation. P-selectin is an adhesion molecule that is important for leukocyte recruitment to injury sites. We used a murine fetal wound healing model to study the specific contribution of P-selectin to scarless wound repair.

METHODS—Linear excisional wounds were created on the dorsa of E15.5 and E17.5 gestation fetuses in wild-type and P-selectin (−/−) mice (term = 19 days). Wounds were harvested at various timepoints after wounding and analyzed using histology and immunohistochemistry.

RESULTS—The E15.5 wounds in both wild-type and P-selectin (−/−) fetuses healed scarlessly and with minimal inflammation, whereas E17.5 wounds healed with fibrosis and inflammation. However, the scars of the P-selectin (−/−) wounds appeared slightly different than wild-type. There were significantly more inflammatory cells in E17.5 wild-type wounds 6 hours after injury ($p < 0.001$), but the difference was no longer significant by 24 hours. Finally, re-epithelialization was slower in the E15.5 knockout wounds compared to their wild-type counterparts.

CONCLUSIONS—Absence of P-selectin delays inflammatory cell recruitment and re-epithelialization of fetal wounds; however, scar formation still occurs in late gestation wounds. The contribution of specific molecules to fetal wound healing can be elucidated using murine knockout or transgenic models.

Keywords

Fetal wound healing; Scarless wound healing; P-selectin; murine fetal wound healing model; knockout fetal wound healing model

INTRODUCTION

It is now widely accepted that early to mid-gestation fetal dermal wounds across many species from rodents (1) to rabbits (2), sheep (3) and monkeys (4) have the remarkable capacity to heal without fibrosis and scar formation and with rapid regeneration of dermal and epithelial tissues. In contrast, adult wound healing is considered imperfect, as it results in fibrosis, scarring, and

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poor epidermal and dermal tissue restoration. Although the sterile amniotic fluid was once thought to play a role in scarless fetal dermal wound healing, it quickly became clear that the ontogeny of fetal wound healing transitions, so that by late-gestation, wounds heal with scarring (5). The mechanism of scarless fetal wound healing is yet to be clearly elucidated. Many researchers have found differences among various fetal and adult tissue components, cytokine profiles, and molecules that are differentially expressed in the fetus and in the adult (6,7). Since wound healing is such a complex process with intricate interplay between many tissues, cell types and molecules, it is difficult to elucidate the magnitude of the contribution that a specific molecule makes to the scarless wound healing process.

Traditionally, large animal models have been used to study fetal wound healing due to the ease of working with large fetuses; however, smaller rodent models have recently been reported that require the use of microsurgical techniques but are easily available and cheaper to maintain than large animals (8–11). Additional advantages of a murine model include a well-characterized genome and the availability of genetically modified knockout or transgenic mice with targeted loss or activation of gene function (9). A syngeneic murine model in which 15-day gestation skin from wild-type or IL-10 knockout mice was grafted onto the back of same strain adult mice has been used to demonstrate the necessity of this cytokine to scarless wound repair (12). Since this model includes grafting onto adult mice, which potentially alters the healing environment, it can only be assumed that fetal healing occurs as it would in its own environment. In contrast, we have used an in-utero murine fetal wounding model using P-selectin knockout mice to study the specific contribution that this molecule makes to scarless fetal wound healing as it naturally occurs.

One of the most dramatic differences between fetal and adult dermal wound healing is the paucity of inflammation in fetal wounds (13). P-selectin is a leukocyte adhesion molecule involved in leukocyte transmigration to injury sites from the circulation. Thus, this molecule may potentially contribute to the lack of inflammatory cells noted in fetal wounds. We previously showed that P-selectin is expressed less by the fetus than the adult, which may explain the diminished inflammatory response in fetal wounds (14). Therefore, we hypothesized that the absence of P-selectin plays a key role in scarless wound healing and that all wounds (even those created in late-gestation) created in mice lacking P-selectin will heal scarlessly.

MATERIALS AND METHODS

Approval for conducting this study was obtained from the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). Six to eight week-old wild-type and P-selectin (–/–) C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME.) were mated and vaginal plug dates were noted. Since the mice had theoretically mated the night before, the plug date was considered gestational day 0.5. The pregnant mice were separated from the male mice and were provided with food and water *ad libitum*. Surgery was performed on either gestational day 15.5 (E15.5) or day 17.5 (E17.5); term = approximately 19 days. Prior to surgery, a pregnant mouse was placed into an anesthesia induction chamber and exposed to isoflurane (3%, 1L/min flow rate, Butler Animal Health Supply, Dublin, OH). The anesthetized mouse was then placed supine on the operating board and anesthesia was continued via a nose cone. The abdomen was then shaved and prepped with alcohol and povidone-iodine. Buprenorphine (0.1mg/kg) was administered subcutaneously for post-operative pain control, and penicillin (10⁶ U/kg) was administered intramuscularly for surgical infection prophylaxis. A midline laparotomy was performed and the bicornate uterus was examined. An operating microscope (original magnification × 9) was used to perform the rest of the procedure. The uterus was returned to the abdomen in order to prevent dessication. The fetus to be wounded was gently brought into visualization using a sterile cotton swab, with the uterus maintained

mostly within the warm abdominal cavity. The fetal dorsum was identified through the semi-transparent uterine wall and a 7-0 prolene purse-string (Harrell Medical, Inc, Canby, OR.) was made on the uterine wall. Within the purse-string, a small hysterotomy was made using fine forceps and sharp microscissors. The fetal dorsal skin overlying the lower spine was grasped using fine forceps and a 2 mm, full-thickness, linear incisional wound was made using microscissors. Placing a sterile 2mm punch biopsy tool next to the wound validated wound length. The wound was marked with India ink (Fischer Scientific, Pittsburgh, PA), warm sterile saline was injected into the uterine cavity, and tightening and tying the purse-string suture closed the hysterotomy. The incisional wounds always tended to gape open (0.5 – 1mm) after creation, and were not sutured closed. One milliliter of warm sterile saline was injected into the maternal peritoneal cavity prior to closure. The fascia and skin were closed in a single-layer, running 4-0 nylon suture (Harrell Medical, Inc. Canby, OR.). The pregnant mouse was then placed into a cage, covered in a towel, warmed under a warming lamp, and observed until she fully awakened from anesthesia. Four fetuses per dam were wounded in both E15.5 and E17.5 pregnant mice.

Fetal mice were harvested at various time points: 6, 24, 36, 48 hours and 7 days post-wounding. Five wild-type and five P-selectin (–/–) mice were harvested at each time point. Notably, there were no differences among the survival rate of wild-type and P-selectin (–/–) fetal mice. There were also no obvious signs of wound infections among either the wild-type or P-selectin (–/–) maternal mice. Wounds (located by the India ink mark) were harvested with a wide surrounding border of unwounded tissue and with an inferior border of muscle in order to preserve the wound configuration. The harvested tissues were placed in 1% formaldehyde for 24 hours, then dehydrated, bisected through the mid-wound, and embedded in paraffin. Serial 4µm sections of the paraffin-embedded wounds were made and placed onto Superfrost Plus slides (Fischer Scientific, Pittsburgh, PA). Standard hematoxylin and eosin staining was performed for sections at all time points. Masson's trichrome staining for collagen visualization was additionally performed for the 7-day wounds.

Immunohistochemistry using anti-CD45 (a pan-leukocyte marker) was performed on the 6-hour and 24-hour wounds. The 36-hour and 48-hour wounds were stained with anti-Integrin β4 in order to better visualize the epithelial basement membrane. Serial 4µm paraffin wound sections were rehydrated, immersed in target retrieval solution (Dako North America, Inc., Carpinteria, CA) and placed in a steamer for 20 minutes. The tissue sections were allowed to cool for a minimum of 1 hour after steaming until they were at room temperature. They were then blocked with non-immune rabbit serum (ABC kit, Vector Laboratory, Burlingame, CA) for 30 minutes, then the primary antibody (either rat anti-mouse CD45 antibody (BD Biosciences, Franklin Lakes, NJ) or rat anti-mouse Integrin β4 (Abcam Inc, Cambridge, MA)) or a non-specific rat immunoglobulin control antibody was applied, and the slides were incubated overnight at 4°C. The slides were then washed in phosphate buffered saline (PBS) and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. The slides were rinsed in PBS again, then incubated with biotinylated rabbit anti-rat antibody (ABC kit). Following a PBS wash, an avidin-biotin complex (ABC kit) was added to the tissues. After another PBS wash, the labeled tissues were developed with chromagen 3.3' – diaminobenzidine (Sigma, St. Louis, MO) and lightly counterstained with hematoxylin. Stained protein expression was examined using a Leitz Diaplan microscope and representative sections were photographed at various magnifications. In order to quantify the number of stained leukocytes, the wounds were photographed at 5 specific points (each wound margin, lateral to each wound margin and the wound base) at 60X magnification, and the number of stained cells per field were counted and averaged over 5 fields.

RESULTS

In order to validate the model, wild-type fetal wounds were first examined. Wounds created at E15.5 healed with minimal inflammation, regeneration of dermal and matrix components and absent fibrosis and scar formation. In contrast, wild-type fetal wounds created at late-gestation (E17.5) healed with a robust inflammatory response, dense collagen deposition and scar formation. Therefore, as expected, the wild-type E15.5 wounds represented scarless healing, whereas the E17.5 wounds represented healing with scar (Figure 1).

Comparison between Wild-type and P-selectin (–/–) fetal wound healing

1. Fibrosis and Scar Formation—Comparison was made between wild-type and P-selectin (–/–) fetal wounds harvested 7 days post-wounding. Similarly to wild type fetuses, P-selectin (–/–) fetuses wounded at E15.5 had healed in scarless fashion, whereas fetuses wounded at E17.5 had healed with fibrosis and scarring (Figure 2). Although the P-selectin (–/–) E17.5 wounds healed with scarring despite the absence of this leukocyte adhesion molecule, there were some differences in the scar character between the null and wild-type wounds. At 7 days post-wounding, the scars of the P-selectin (–/–) wounds were concave, contracted, and had less epithelial height (WT: 2.3 ± 0.9 vs. PS (–/–): 0.6 ± 0.1 , $p < 0.01$, $n = 4$ per group) compared to the protruberant wild-type wounds (Figure 3).

2. Inflammation—Since only a few neutrophils were noted in the wounds, we decided to stain for all leukocytes using a pan-leukocyte marker in the mouse, anti-CD45. Both the wild-type and P-selectin null fetuses wounded at E15.5 exhibited a minimal inflammatory response at 6 and 24 hours post-wounding. In fetuses wounded at E17.5, a robust inflammatory response was noted in both wild-type and P-selectin (–/–) wounds (Figure 4). In fact, there was no significant difference between the number of leukocytes within the injury site at 24 hours (WT: 17.8 ± 12.8 vs. PS (–/–): 12.2 ± 7.2). However, when examined 6 hours post-wounding, significantly less leukocytes had transmigrated to the injury site in the P-selectin (–/–) wounds than the wild-type wounds (WT: 50 ± 20 cells/hpf vs. PS (–/–): 25 ± 20 cells/hpf, $p < 0.001$, $n = 5$ per group). These results suggest that there is a delay in leukocyte recruitment in fetuses deficient in P-selectin, but the response accelerates to match that of wild-type fetuses by 24 hours post-wounding (Figure 5).

3. Re-epithelialization—By 48 hours post-wounding, re-epithelialization had been completed in the E15.5 wounds in both wild-type and P-selectin (–/–) fetuses. In order to clearly visualize the basement membrane, immunostaining was performed using a $\beta 4$ integrin-specific antibody that specifically stains the basal keratinocytes. Up-regulated expression was noted in response to wounding. An interesting difference was observed between the wild-type and P-selectin (–/–) wounds: at 48 hours after wounding, the neoepithelial layer in the wild-type wounds was almost indistinguishable from the surrounding unwounded epithelium, whereas keratinocyte proliferation and hair follicle regeneration was still observed in the P-selectin (–/–) wounds at the same time point. When harvested at 36 hours, the wild-type wounds showed a similar hyperplastic epithelium, which suggests that this phenomenon represents a progression of epithelial maturation that includes keratinocyte proliferation in order to reconstitute the basement membrane after keratinocyte migration across the wound bed (15, 16). Therefore, the fetuses deficient in P-selectin appeared to have delayed epithelial maturation compared to their wild-type counterparts (Figure 6).

DISCUSSION

One of the hallmarks of scarless fetal wound healing is the paucity of an inflammatory response. Adult wound repair involves an extensive inflammatory response, which is thought to be

instrumental in supplying cytokine and growth factor signals that orchestrate wound repair (17). However, fetal wounds across many species have consistently shown an absent inflammatory response, which suggests that tissue repair is not dependent on inflammatory cells (7,18). In fact, when fetal wounds are maximally stimulated, such as with a large injury, bacteria or pro-inflammatory cytokines, they are able to mount a robust inflammatory response which is followed by fibrosis (19–22). Therefore, the presence of inflammation potentially plays a key role in fibrosis, and ultimately in scar formation. Martin et al. corroborated this notion by wounding adult mice that are genetically incapable of mounting a standard inflammatory response and demonstrating that although the wounds healed as rapidly as wild-type adult mice, the repair appeared scarless, as in the fetus (23).

In order to mimic fetal dermal wound repair in adults, it seems feasible to minimize the inflammatory response to wounding. This could involve targeting a molecule known to be important in wound inflammation. P-selectin, also known as CD62P or GMP-140, is a member of the selectin family of transmembrane adhesion receptors that are involved in early adhesive interactions between leukocytes, platelets and endothelial cells. It is stored in intracellular granules in platelets and endothelial cells and expressed on their surface upon stimulation, making the endothelium “sticky” to facilitate leukocyte capture (24). *In vitro*, isolated human granulocytes have been shown to roll on purified P-selectin using flow chamber systems (25). The critical role of P-selectin in the rolling phase of the adhesion cascade is supported in experiments performed on gene-targeted mice. In mice lacking P-selectin, leukocytes do not roll on the venular endothelium after surgical trauma (26). Also, when compared with wild-type mice, the number of circulating granulocytes is greater in P-selectin deficient mice. This suggests that P-selectin is necessary to remove the leukocytes from the bloodstream and into areas of inflammation (27,28). Delayed neutrophil infiltration into chemically inflamed mouse peritoneum has been shown following blocking antibodies against P-selectin in wild-type mice and in P-selectin deficient mice (29,30). Notably, L-selectin and E-selectin also participate in the rolling process. In the absence of P-selectin, trauma-induced rolling becomes L-selectin dependent, but the average leukocyte rolling velocity is three to five times faster in this case. This suggests that L-selectin is much less efficient than P-selectin in mediating the rolling process (31,32). An apparent redundancy exists between P- and E-selectin in mediating leukocyte rolling on cytokine-activated endothelium (33).

In our laboratory, we have demonstrated that P-selectin mRNA expression by porcine endothelial cells is reduced 3-fold in the mid-gestation fetus compared to the adult (14). Therefore, we hypothesized that a P-selectin deficiency is important for scarless fetal dermal repair. In order to minimize discrepancies between wound creation in fetal and adult mice, we used an in-utero model to compare the healing between fetal wounds created at E15.5 (that heal scarlessly) and wounds created at E17.5, which is late in gestation when healing generally occurs with fibrosis and scarring. If a lack of P-selectin is vital for scarless fetal wound healing, then even late-gestation fetal wounds in P-selectin deficient mice should heal in a scarless fashion. Our hypothesis was rejected, as we demonstrated that late-gestation fetal wounds in P-selectin (–/–) mice healed with fibrosis and scarring. However, we did find some interesting differences in various aspects of wound healing between P-selectin (–/–) and wild-type fetal mice.

As seen in the peritoneal inflammation model, we noted a diminished inflammatory response in the E17.5 P-selectin (–/–) wounds at an early time point (6 hours after wounding), which was less apparent by 24 hours. Subramaniam et al. noticed a similar delay in early neutrophil extravasation in wounds of adult P-selectin deficient mice (34). The early discrepancy can be explained by a decreased number of rolling leukocytes. With time, it seems feasible that the expression of other selectins is upregulated, so that the leukocyte influx “catches up” to that of wild-type wounds. Therefore, although P-selectin is important in leukocyte capture, it is not

vital due to the inherent redundancy of the adhesion molecules. When both P- and E-selectin are lacking, adult mice have been shown to display markedly diminished inflammation and delayed keratinocyte migration compared to wild-type mice (34). Unfortunately, our in-utero model could not be used to study fetal wound healing in P/E-selectin double deficient mice as these immunocompromised mice were prone to abortions and often could not tolerate surgery. P-selectin deficient fetal wounds were also noted to have delayed epithelial maturation compared to wild-type counterparts. Forty-eight hours after wounding, wild-type wounds had re-established an epithelial layer and hair follicles so that the wound site was indistinguishable from unwounded skin. However, P-selectin ($-/-$) wounds still showed keratinocyte proliferation and hair follicle regeneration. The delay in epithelial maturation may be due to a delay in growth factor and cytokine signals provided by the slower entry of the inflammatory cells to the wound. Finally, at 7 days post-wounding, the scars of E17.5 P-selectin ($-/-$) wounds had a different character than wild-type scars, appearing less protruberant and more contracted. When Subramaniam et al. examined adult excisional wounds, scar appearance among wild-type and P-selectin ($-/-$) mice was similar (34). We did not examine scar formation past 7 days of wounding, but based on the above study, it can be assumed that the scars eventually appear similar. Therefore, the early discrepancy in scar formation seen in our model may be explained by the delay in inflammation accompanied by a delay in growth factors and cytokines responsible for fibrosis and scar formation. Despite the early differences, the wound repair process in the P-selectin deficient mice ultimately draws alongside to that of the wild-type mice, likely due to the redundancy within the system.

In conclusion, although there are a few minor differences in wound healing in mice deficient in P-selectin, a lack of P-selectin does not prevent scarring in fetuses wounded during late-gestation. Therefore, P-selectin would not provide an adequate therapeutic target to limit scarring in adult wound repair. However, the over- or under-expression of certain molecules may have more of an impact on scarless healing than others. Because of the inherent redundancy in the system, it has thus far been difficult to elucidate the contribution of a specific molecule. With the increasing availability of genetically modified mice, this murine, in-utero wounding model facilitates the study of the impact that the loss or gain of a targeted molecule has on the still elusive arena of scarless fetal dermal repair.

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Figure 1.

Representative wound histologic sections 7 days after injury illustrate the transition from scarless to fibrotic healing in wild-type fetal skin. Skin wounded at E15.5 (A) shows hair follicle regeneration and re-epithelization identical to the surrounding unwounded skin. Skin wounded at E17.5 (B) has healed with a scar lacking in normal skin components. Wound area is marked by the vertical bars. The black arrows indicate India Ink injected at the time of injury. (Hematoxylin and eosin stains, original magnification $\times 10$).

Figure 2.

Representative E17.5 wound histologic sections 7 days after injury illustrate a similar transition from scarless to fibrotic healing in P-selectin ($-/-$) fetal skin. Skin wounded at E15.5 (A) shows hair follicle regeneration and re-epithelization identical to the surrounding unwounded skin. Skin wounded at E17.5 (B) has healed with a scar lacking in normal skin components. Wound area is marked by the vertical bars. (Hematoxylin and eosin stains, original magnification $\times 10$).

Figure 3.

Representative E17.5 Masson's trichrome-stained tissue sections 7 days post-wounding demonstrate differences in scar character among P-selectin (-/-) (A) and wild-type (B) fetal wounds. Wound area is marked by the vertical bars. Note the differences in epithelial height (black arrows) and the protruberant wild-type wound compared to the contracted, concave P-selectin (-/-) wound. (Original magnification $\times 10$).

Figure 4. Immunohistochemical staining for leukocytes in 24-hour E17.5 fetal wounds in wild-type (A) and P-selectin (-/-) (B) wounds using an anti-CD45 antibody. Black arrows highlight stained inflammatory cells. The arrow head marks an inflammatory cell that has recently transmigrated out of the neighboring vessel. (Original magnification $\times 60$).

Figure 5.

Bar graphs depicting the average number of CD-45 + cells in 5 fields at 60 × original magnification. At 6 hours post-wounding, there was a significant difference among wild-type and P-selectin (–/–) wounds (*p<0.001, n=5 per group), whereas there was no difference among the groups at 24 hours after wounding.

Figure 6.

Immunohistochemical staining for basal keratinocytes in E15.5 wounds using anti-Integrin $\beta 4$ antibody demonstrates completed re-epithelization in a wild-type wound at the 48-hour time point (A) and hyperplastic epithelium (black oval) demonstrating keratinocyte proliferation (B) and hair follicle regeneration (arrowheads) (C) still evident in a P-selectin (-/-) wound, indicating that re-epithelialization is still in progress. Similar hyperplastic epithelium (black oval) is noted in a wild-type wound at an earlier time point (36 hours) (D). India Ink (black arrows) marks the wounded area. (Original magnification $\times 10$).