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The alarmin HMGB-1 influences healing outcomes in fetal skin wounds

Adrienne D. Dardenne, DVM, MS¹, Brian C. Wulff, PhD², and Traci A. Wilgus, PhD^{2,*}

¹Department of Veterinary Preventive Medicine/University Laboratory Animal Resources, Columbus, OH

²Department of Pathology, The Ohio State University, Columbus, OH

Abstract

In mice, cutaneous wounds generated early in development (embryonic day 15, E15) heal scarlessly, while wounds generated late in gestation (embryonic day 18, E18) heal with scar formation. Even though both types of wounds are generated in the same sterile uterine environment, scarless fetal wounds heal without inflammation but a strong inflammatory response is observed in scar-forming fetal wounds. We hypothesized that altered release of alarmins, endogenous molecules that trigger inflammation in response to damage, may be responsible for the age-related changes in inflammation and healing outcomes in fetal skin. The purpose of this study was to determine whether the alarmin high-mobility group box -1 (HMGB-1) is involved in fetal wound repair. Immunohistochemical analysis showed that in unwounded skin, E18 keratinocytes expressed higher levels of HMGB-1 compared to E15 keratinocytes. After injury, HMGB-1 was released to a greater extent from keratinocytes at the margin of scar-forming E18 wounds compared to scarless E15 wounds. Furthermore, instead of healing scarlessly, E15 wounds healed with scars when treated with HMGB-1. HMGB-1-injected wounds also had more fibroblasts, blood vessels, and macrophages compared to control wounds. Together, these data suggest that extracellular HMGB-1 levels influence the quality of healing in cutaneous wounds.

Keywords

alarmins; Damage associated molecular patterns (DAMPs); danger signals; fetal wound healing; high-mobility group box-1 (HMGB-1); scarless repair; skin

INTRODUCTION

Cutaneous wound healing is a highly coordinated, complex process that is not yet completely understood. The wound repair process has been extensively studied in mature skin revealing the three overlapping phases of inflammation, proliferation, and remodeling/scar formation (1). Detriments of scar tissue include restricted joint mobility, impaired growth and loss of organ function, as well as psychological and aesthetic impairments (2).

Many studies have shown that a unique type of healing occurs in embryonic skin during the first two trimesters of mammalian development (3, 4). In mice, skin wounds generated early in development (before embryonic day 16 or E16) exhibit a unique pattern of healing which leads to regeneration of skin with normal tissue architecture with an absence of scar tissue. In contrast, fetal wounds generated at late stages of development (beyond E16) heal with a

Correspondence and reprints: Traci A. Wilgus, PhD, The Ohio State University, Department of Pathology, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, Office: (614) 366-8526, Fax: (614) 366-9389, Traci.Wilgus@osumc.edu.

strong inflammatory response and scar formation (fibrotic wounds) (5–7). These developmental differences in inflammation, in addition to studies demonstrating that inducing inflammation leads to scarring in early fetal wounds that would otherwise heal scarlessly (6, 8), has led to the belief that inflammation is a key determinant of scar formation. Despite numerous studies indicating that a vigorous inflammatory response after wounding is restricted to more developed fetal skin, the reasons for developmental differences in wound inflammation are not entirely understood. Interestingly, inflammation is triggered only in late-stage fetal wounds despite the fact that regardless of age, fetal wounds are all created in the same sterile, amniotic fluid environment. This observation lead us to hypothesize that alterations in the expression or release of endogenous danger signals during skin development might be one reason why injury-induced inflammation is restricted to late gestation fetal wounds.

Alarmins are a recently described class of endogenous danger signals that stimulate inflammation in the absence of an external pathogen. Alarmins, which are sometimes referred to as damage-associated molecular patterns (DAMPs), are normal cell constituents that are released into the extracellular space following sterile injury or necrosis (9). Alarmins can also be secreted by activated immune cells (10, 11). Once these endogenous molecules are released into the extracellular space, they recruit and activate innate immune cells, leading to inflammation (12). Endogenous alarmins differ from exogenous danger signals such as pathogen-associated molecular patterns (PAMPs), which alert the immune system to the presence of microbial molecules and external threats. Several molecules have been implicated as alarmins, such as HMGB-1 (high-mobility group box-1), S100 proteins, and heat shock proteins (13). This study focuses on HMGB-1, which is a well-studied alarmin that displays the classic features of these endogenous danger signals (12).

HMGB-1 was initially described as a non-histone intranuclear architectural DNA binding protein which allows interactions between transcription factors and chromatin (9). HMGB-1 is normally localized in the nucleus; however, when cell damage occurs, HMGB-1 translocates to the cytoplasm and is released by the cell. Once in the extracellular space, HMGB-1 is able to stimulate inflammation by binding to RAGE (receptor of advanced glycation end products) or toll-like receptors (TLR4 and TLR2) present on inflammatory cells (14). Despite the importance of inflammation in wound repair, very few studies have examined the role of HMGB-1 in cutaneous wound healing (15–17), and none have evaluated its ability to regulate the initial stages of wound inflammation. Here, HMGB-1 was examined as a potential trigger for inflammation in fibrotic fetal wounds by comparing HMGB-1 expression and localization throughout the repair process in early-mid (scarless) and late (scar-forming) gestation wounds. In addition, the effects of exogenous HMGB-1 on scarless healing were assessed. The results implicate HMGB-1 as an important modulator of the healing response in fetal skin wounds.

MATERIALS AND METHODS

Animal experiments

A mouse model of wound healing was used for the present studies. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and the research was conducted in AAALAC-accredited facilities. Wounds were generated in fetuses of time-mated female FVB/NTac mice (Taconic, Germantown, NY) as described (18). Full-thickness dorsal skin wounds, approximately 2 mm in length, were created *in utero* on E15 or E18 under isoflurane (Abbott Laboratories, Abbott Park, IL) anesthesia. These ages represent times at which murine fetuses undergo scarless or fibrotic healing, respectively (6). One microliter of India ink (Fisher Scientific, Pittsburgh, PA) diluted to 10% in sterile PBS (phosphate buffered saline; Invitrogen Corporation, Carlsbad, CA) was

injected subcutaneously to demarcate the wound site. For some experiments, E15 wounds were injected subcutaneously with 200 or 400 ng of recombinant murine HMGB-1 (eBioscience, San Diego, CA) diluted in 10% India ink solution or an equal volume of PBS in India ink to act as a control at the time of wounding. These doses were chosen based on published studies which used 200–800 ng HMGB-1 for the treatment of adult mouse wounds (16). At various time points ranging from 2 hours to 7 days post-wounding, mice were euthanized by methods approved by The Ohio State University Institutional Animal Care and Use Committee. Wounds and skin samples from uninjured animals (control tissue) were harvested for analysis. Samples were fixed in 10% buffered formalin overnight and embedded in paraffin or frozen in TBS tissue freezing media (Triangle Biomedical Sciences, Durham, NC) for histology and immunohistochemistry. HMGB-1 expression and collagen deposition were analyzed, and the response of various cell types was assessed. Sample numbers vary for each parameter examined and are reported in the figure legends. Each sample represents wound tissue isolated from a separate animal.

HMGB-1 localization and cell identification

To assess the expression and localization of HMGB-1, 10 μ m cryosections of wounded and unwounded fetal skin were air dried and fixed in acetone for 15 minutes. After sections were washed in PBS, slides were treated with 0.3% hydrogen peroxide in methanol for 30 minutes then washed in PBS. Sections were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes, then incubated in anti-HMGB-1 antibody (Abcam Inc., Cambridge, MA; 1:120 dilution) in serum overnight at 4°C. To confirm the specificity of the staining, control slides were incubated with serum alone (primary antibody omitted) or with rabbit IgG (Vector Laboratories) at the same final concentration as the HMGB-1 antibody (data not shown). After slides were washed in PBS, the sections were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:200 dilution) in serum for 30 minutes and washed in PBS. Slides were then incubated in avidin-biotin-horseradish peroxidase complex (Vectastain ABC-HRP Kit, Vector Laboratories) for 30 minutes. Sections were again washed and incubated in DAB Solution (KPL, Gaithersburg, MD) in the dark for 8 minutes. Sections were counterstained with Hematoxylin-2 (Richard-Allan Scientific, Kalamazoo, MI) and rinsed in tap water. Slides were dehydrated and placed in Clear Rite 3 (Richard-Allan Scientific), and cover slips were mounted with Permount (Fisher Scientific).

Samples were also stained for various cell types at time points appropriate for the analysis of each cell type. Similar techniques to those described above for HMGB-1 staining were used for the detection of macrophages (anti-MOMA-2 antibody, Abcam), endothelial cells (anti-PECAM/CD-31 Antibody, B-D Pharmingen, San Diego, CA), and fibroblasts (anti-vimentin antibody, Abcam) in cryosections, except that the type of serum, primary antibodies and secondary antibodies were changed. Paraffin sections were used for the detection of mast cells, neutrophils and myofibroblasts. After deparaffinization and rehydration, sections were stained with 0.2% toluidine blue (Fisher Scientific) in 0.7 N HCl to detect mast cells as described (7) or immunohistochemistry was performed to detect neutrophils (anti-Ly-6G antibodies, BD Pharmingen) or myofibroblasts (anti-alpha smooth muscle actin, Sigma, St. Louis, MO). For myofibroblast detection, a mouse-on-mouse staining kit was used as specified by the manufacturer (Vector Laboratories). Immunohistochemistry (anti-cleaved caspase-3, Abcam) or TUNEL staining (Frag-EL Kit, EMD Millipore, Billerica, MA) was also performed in paraffin sections as recommended by the manufacturers to identify apoptotic cells.

Assessment of collagen

Masson's trichrome staining, which differentially stains collagen blue, was used to evaluate the wound bed for collagen deposition and to assess healing. Staining was performed as described previously (18). Picosirius red-staining was also used to characterize collagen deposition (19, 20). This stain takes advantage of the birefringent properties of collagen and allows for visualization of collagen fiber orientation under polarized light. Compared to adult skin, fetal skin is known to have a greater proportion of type III collagen, which is thin and appears green in color (20). Adult skin contains predominately type I collagen, which is thicker and appears orange-red in color (20). Sections were deparaffinized, rehydrated, and incubated in 0.1% sirius red in saturated picric acid (Electron Microscopy Sciences, Hatfield, PA) for 90 minutes at room temperature, then washed in 0.01 N HCl (Electron Microscopy Sciences) for 2 minutes. Sections were then dehydrated, cleared, and cover slips were mounted prior to viewing on a microscope equipped with polarizing filter attachments.

Quantification of staining

Images were generated from slides using an Axioskop 40 microscope with AxioCam MRc5 and Axiovision40 version 4.6.3.0 software (Carl Zeiss Imaging Solutions, Thornwood, NY). Dermal mast cells, MOMA-2-positive macrophages and vimentin-positive fibroblasts were counted in captured digital images. Mast cells and macrophages were analyzed at early stages in the repair process, so the number of cells was counted in one high-power field immediately adjacent to either side of the wound. Fibroblasts were analyzed at later time points and were counted in the wound bed/scar. To control for possible differences in dermal thickness or wound bed size, dermal area was determined for each field using Axiovision software. Cell densities (cell number per mm²) were then calculated. For mast cells and macrophages, cell densities for the right and left sides of each wound were averaged to obtain the mean cell density per wound. Blood vessel density (percentage of PECAM-positive area) was calculated as described previously, except that Image J software (NIH, Bethesda, MD) software was used.

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Statistical differences were determined by unpaired student's t-tests, with p-values < 0.05 considered statistically significant.

RESULTS

HMGB-1 expression in unwounded fetal skin

Immunohistochemical staining for HMGB-1 was performed to assess baseline levels of expression in early and late gestation fetal skin. Both E15 (Figure 1A) and E18 (Figure 1B) unwounded skin showed positive staining in the nuclei of basal keratinocytes. However, more intense staining was observed in E18 skin compared to E15 skin, indicating that basal keratinocytes in E18 skin have a higher baseline expression of HMGB-1.

HMGB-1 expression in E15 and E18 wounds

In addition to unwounded skin, HMGB-1 expression patterns were also compared in E15 and E18 wounds. Figure 2 provides a side-by-side comparison of HMGB-1 staining in E15 (Figure 2A, C, E, and G) and E18 wounds (Figure 2B, D, F, and H). HMGB-1 was confined to the nuclei of basal keratinocytes for the first 12 hours after injury in both E15 and E18 wounds (Figure 2A and B, respectively). At these early time points, the nuclear staining pattern of HMGB-1 resembled that of unwounded skin (Figure 1). However, beginning at 24 hours post-wounding, a reduction in nuclear staining in keratinocytes near the wound margin

can be seen in both age groups (Figure 2C and D), which was also evident at 48 hours post-wounding (Figure 2E and F). During the course of healing, E18 wounds displayed a loss of nuclear HMGB-1 staining in a large number of keratinocytes extending away from the wound margin, whereas in E15 wounds only a small number of keratinocytes at the wound margin lacked nuclear staining. Loss of nuclear HMGB-1 staining has been established as an indication of extracellular HMGB-1 release (15, 16, 21). At 72 hours post-wounding, the HMGB-1 staining pattern seen in E15 wounds (Figure 2G) was similar to adjacent unwounded skin, indicating that HMGB-1 expression and localization had returned to normal. In contrast, the majority of keratinocytes in E18 wounds (Figure 2H) continued to lack nuclear staining. Interestingly, cytoplasmic staining was observed in E18 wounds at later time points after injury which was not evident in E15 wounds. Cytoplasmic staining has been described just prior to HMGB-1 release from the cell (22, 23). Overall, these results suggest that HMGB-1 is released by a greater number of keratinocytes at the wound edge for a longer period of time in scar-forming E18 wounds.

Effects of HMGB-1 treatment on scar formation

The enhanced and prolonged HMGB-1 release in E18 wounds suggested by immunohistochemistry alluded to a relationship between HMGB-1 release and scar formation. To determine whether HMGB-1 levels affect scar formation, E15 wounds were treated with HMGB-1 at the time of injury. Normally, E15 wounds heal with minimal HMGB-1 release (Figure 2), no inflammation, and no scar. Masson's trichrome and picrosirius red staining were used to evaluate collagen deposition in E15 wounds treated with PBS or recombinant HMGB-1. As expected, PBS-injected E15 wounds healed without scar formation; however, an increasing proportion of the wounds healed with scarring when injected with 200 or 400 ng of recombinant HMGB-1 (Figure 3A). Furthermore, a dose-dependent increase in scar size was seen with increasing amounts of HMGB-1 (Figure 3B). Representative photomicrographs of trichrome- and picrosirius red-stained sections are also shown. Scarless repair was seen in trichrome-stained PBS-injected E15 wounds (Figure 3C). The majority of collagen fibers within the wound bed were primarily weak green to yellow in color and appeared similar in structural organization to collagen in areas of skin distant to the wound in picrosirius red-stained sections (Figure 3D). Larger scars, with the characteristic loss of hair follicles, were seen with increasing doses of HMGB-1 (Figure 3E and G), and the majority of the collagen fibers within the wound bed were oriented in one direction (Figure 3 F and H). The fibers also appeared thicker compared to control wounds and produced a strong orange-red color. These data suggest that HMGB-1 is capable of stimulating scar formation and influencing the quality of the collagen produced in E15 wounds. The scars induced by HMGB-1 did not appear to result from differences in wound closure or apoptotic cell load, as HMGB-1-injected wounds healed at a similar rate (Supplemental Figure 1A) and contained comparable numbers of cleaved caspase-3-positive cells (Supplemental Figure 1 B–E) and TUNEL-positive cells (data not shown) as control wounds.

Characterization of dermal fibroblasts and angiogenesis

To explore potential mechanisms for the stimulatory effects of HMGB-1 on scar formation, various cell populations were characterized. Due to the difficulty in obtaining large numbers of samples with this animal model the high dose of HMGB-1 (400 ng), which caused the largest scars to form, was used for further analysis. First, fibroblasts were examined, as HMGB-1 could enhance scar tissue deposition by increasing the number of collagen-producing fibroblasts or the number of myofibroblasts, which generally correlate to increased scar formation in fetal wounds (18, 24). At 7 days post-wounding, HMGB-1-treated wounds contained higher numbers of vimentin-positive fibroblasts compared to PBS-treated wounds (Figure 4 A–C); however, no myofibroblasts were detected even after

HMGB-1 treatment (Figure 4 D–E). The effects of HMGB-1 on were also examined, as some studies have shown a relationship between increased angiogenesis and more scar tissue production (18). HMGB-1 caused a significant increase in the density of PECAM-positive blood vessels in the wound bed/scar compared to control wounds (Figure 5), suggesting that elevated levels of angiogenesis were occurring in the presence of HMGB-1.

Assessment of inflammation

The response of inflammatory cells was also assessed, since HMGB-1 could also enhance scarring indirectly by promoting inflammation. Mast cells, neutrophils, and macrophages were examined. Toluidine blue, which stains mast cell granules blue, was used to assess mast cell density at 12 and 24 hours post-wounding (Supplemental Figure 2). No significant differences in mast cell numbers were found between groups. In addition, no observable mast cell degranulation, a marker of mast cell activation, was seen in either treatment group. Scarce Ly-6G-positive neutrophils were observed in either PBS- or HMGB-1-injected wounds at 12 or 24 hours after injury (data not shown). Neutrophils that were detected were clearly within blood vessels and had not migrated into the tissue. The results suggest that HMGB-1 does not induce mast cell activation or neutrophil infiltration in fetal wounds at the dose used here. HMGB-1 did, however, significantly increase the number of MOMA-2-positive wound macrophages (Figure 6A). Because macrophage numbers usually peak later than mast cell activation and neutrophil infiltration in a wound, macrophages were assessed 48 hours after injury. Many positively stained macrophages can be seen in wounds after HMGB-1 treatment (Figure 6C) compared to only a few positive cells in control wounds (Figure 6B).

DISCUSSION

Fetal skin has the unique ability to heal by regenerating normal skin after injury. During this type of healing process, there is very little, if any, inflammation at the site of injury and no scar tissue is produced; however, fetal skin is only capable of undergoing scarless healing at early stages of development. Late in gestation, fetal skin begins to heal more like fully developed skin, with a strong inflammatory response and the formation of scar tissue. Despite the fact that differences in injury-induced inflammation are believed to dictate what type of healing will take place in fetal wounds (scarless or fibrotic), the exact mechanisms that limit inflammation in early gestation wounds while allowing for a vigorous inflammatory response in late gestation wounds are not known.

Many different ‘danger signals’ could trigger inflammation in a skin wound, including PAMPs, which alert the immune system to external threats, and alarmins, which are endogenous molecules released by damaged cells that stimulate inflammation in the absence of infection. Since both early and late gestation fetal wounds are generated in the same sterile environment but only late gestation wounds exhibit inflammation after injury, we hypothesized that an increase in the expression or release of alarmins after injury may explain why late gestation wounds heal with inflammation and subsequent scarring. For the studies described here, we focused on HMGB-1, which demonstrates the classic features of alarmins (12), has been demonstrated to have pro-inflammatory effects (25), and has been implicated as a biomarker of systemic inflammation (26).

While the current study is the first to examine HMGB-1 during the process of fetal wound healing, there have been a limited number of studies published in adult injury models suggesting that HMGB-1 is involved in the repair process. Straino and colleagues showed that HMGB-1 promotes wound closure, granulation tissue formation, and angiogenesis in a diabetic wound model, and HMGB-1 expression and release has been described in animal models of tail injury/lymphedema and burn injury (15, 16, 27). Additionally, studies have

shown that HMGB-1 stimulates migration and regulates collagen synthesis in cultured fibroblasts (16, 17, 28).

In the present study, developmental differences in HMGB-1 expression were found in the epidermal layer of the skin. Basal keratinocytes in uninjured E18 skin stained more intensely for HMGB-1 than E15 skin. In addition, spatial and temporal differences in HMGB-1 localization were observed after injury. Compared to E15 wounds, E18 wounds exhibited a stronger reduction of nuclear HMGB-1 staining and enhanced cytoplasmic staining in keratinocytes, a staining pattern that also extended a longer distance from the wound margin and persisted for a longer period of time in E18 wounds. Similar spatial and temporal alterations in HMGB-1 staining have been described other types of injury models (15, 27). Reduced nuclear and/or elevated cytoplasmic HMGB-1 staining generally indicates that the cell has recently or is in the process of releasing HMGB-1 (15, 16, 21–23). The timing of HMGB-1 release during an inflammatory response varies depending on the model used (26, 29), and in the case of injury likely depends on the severity of the damage (15, 27, 30). In E18 fetal wounds, strong release of HMGB-1 occurred at 48–72 hours, suggesting that HMGB-1 may be important for maintaining or extending the inflammatory response. Overall, the HMGB-1 staining patterns observed here suggest that extracellular HMGB-1 release in response to cutaneous wounding is heightened in fibrotic wounds that heal with a strong inflammatory response (E18) compared to wounds that heal scarlessly and with minimal inflammation (E15).

The enhanced and prolonged release of HMGB-1 observed in scar-forming E18 wounds prompted additional studies to evaluate HMGB-1 as a potential pro-fibrotic molecule. Recombinant HMGB-1 was introduced into E15 wounds, which normally heal with limited HMGB-1 release and without scarring, and the effects on collagen deposition were assessed. While control wounds injected with PBS healed scarlessly, wounds were induced to heal by fibrosis in the presence of HMGB-1. A higher incidence of scar formation, larger scar sizes with more disorganized collagen, and a higher number of fibroblasts were seen with the addition of HMGB-1. These results are in line with studies correlating HMGB-1 release with fibrosis in other organs (31–33). HMGB-1 has also been shown to stimulate fibroblast migration (16, 28). Because HMGB-1 can signal in fibroblasts (28) and more fibroblasts were present in HMGB-1-treated wounds, α -smooth muscle actin staining was used to determine whether myofibroblasts were present in HMGB-1-treated wounds. Although myofibroblasts are often observed in fetal wounds that heal with scarring (18, 24), it is unlikely that HMGB-1 was stimulating the differentiation of fibroblasts to myofibroblasts in fetal wounds, since these cells could not be detected even after HMGB-1 injection. The increase in collagen deposition in the presence of HMGB-1 seen here and the suggestion by others that HMGB-1 is involved in fibrosis are somewhat contradictory to a recent study concluding that HMGB-1 reduces collagen production by cultured dermal fibroblasts (17). It could be that fibroblasts respond differently to HMGB-1 depending on the conditions (*in vitro* versus *in vivo*) or that HMGB-1 affects the way that fibroblasts organize or remodel collagen which, in addition to the level of collagen production, is also important for scar formation. Alternatively, it is possible that the changes in collagen deposition observed in our model were not due to direct effects of HMGB-1 on fibroblasts, but rather scar formation was being induced indirectly.

Several potential indirect mechanisms for the stimulatory effects of HMGB-1 on scar formation were assessed in the current study. First, the vascularity of the wounds was examined. The density of PECAM-positive vessels was increased in wounds injected with HMGB-1. Previous studies have suggested that HMGB-1 can stimulate angiogenesis (16, 34–36), and higher levels of angiogenesis have been associated with scar formation both in adult and fetal wounds (18, 37–39). Interestingly, HMGB-1 can undergo oxidation in the

presence of reactive oxygen species (40), and oxidized extracellular HMGB-1 has been shown to induce apoptosis in cancer cells *in vitro* through a mechanism partially dependent on the activation of caspase-3 (41). The likelihood of HMGB-1 becoming oxidized in fetal wounds does not seem high based on the relatively low numbers of reactive oxygen species-generating inflammatory cells in early gestation wounds. Nonetheless, an increase in cellular apoptosis in response to exogenous HMGB-1 was explored as a possible explanation for the fibrotic healing response in HMGB-1-injected wounds, but no differences in the number of cleaved caspase-3-positive or TUNEL-positive cells were found. In addition, wound closure occurred at the same rate in wounds injected with PBS or HMGB-1, suggesting that scar formation did not occur as a result of delayed healing in the presence of HMGB-1.

Based on the strong association of inflammation with scar formation/healing outcomes in fetal wounds (5, 6, 8) and the link between HMGB-1 and inflammation (9, 12), the effects of HMGB-1 on inflammation were assessed as another potential indirect mechanism for the increased scar tissue production. The numbers of several types of inflammatory cells were compared in control and HMGB-1-injected E15 wounds, including mast cells, neutrophils, and macrophages. Very few neutrophils were observed, even in the presence of HMGB-1, and no differences in the overall number or activation of mast cells were detected. We have recently shown that E15 mast cells degranulate less in response to certain stimuli compared to E18 mast cells (7), so it is possible that mast cells in more mature skin would respond differently to HMGB-1. It should also be noted that while the numbers of mast cells were not different and no overt signs of mast cell degranulation were visible in histological sections, mast cells are capable of secreting cytokines and other pro-inflammatory mediators even in the absence of classical degranulation (42). The possibility that mast cells were selectively releasing cytokines in HMGB-1-treated wounds without degranulating, which cannot be easily assessed *in vivo*, cannot be ruled out. In addition to mast cells and neutrophils, macrophage numbers were assessed using immunohistochemistry for the macrophage marker MOMA-2. Significantly more macrophages were found in HMGB-1-treated wounds, which could explain in part why these wounds healed with scars. Elevated macrophage recruitment to wounds has been tied to more severe scarring (43, 44), and lower numbers of macrophages have been described in scarless fetal wounds (5, 45).

In conclusion, the studies presented here showing an increase in the release of HMGB-1 in E18 fibrotic fetal wounds and the stimulation of scar tissue production by HMGB-1 in E15 wounds suggest that this alarmin may drive scar formation in fetal wounds. There are likely multiple mechanisms involved in the promotion of scarring by HMGB-1. The possibilities include direct stimulation of fibroblasts, as well as indirect pathways linked to increases in angiogenesis and/or macrophage recruitment in the wound. Future studies will have to be performed to determine whether HMGB-1 could be used as a target to control wound inflammation and minimize scarring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HMGB-1 immunostaining in unwounded fetal skin

Immunostaining was used to evaluate HMGB-1 expression in unwounded fetal skin. Representative photomicrographs of E15 (A) and E18 (B) skin are shown (scale bars = 50 μm ; $n = 4-6$ per group). HMGB-1 staining was localized to the nuclei of basal keratinocytes, with more intense nuclear staining in E18 skin.

Figure 2. HMGB-1 localization in fetal wounds

Immunostaining was used to examine HMGB-1 expression patterns in healing fetal wounds. Representative photomicrographs of E15 (left panels; A, C, E, G) and E18 (right panels; B, D, F, H) wounds harvested at 12 (A, B), 24 (C, D), 48 (E, F) or 72 (G, H) hours post-wounding are shown (scale bars = 50 μ m; n = 4–6 per group at each time point). Wound margins are marked with arrows. Reduced nuclear staining and increased cytoplasmic staining in basal keratinocytes (indicated by brackets) were observed beginning at 24 hours post-wounding and is more pronounced in E18 wounds. India ink (black), which was used for wound identification, can be seen in the dermis in G and H.

Figure 3. Evaluation of healing outcomes in wounds treated with HMGB-1

The presence and size of scars after healing was assessed in Masson's trichrome-stained sections. The percentage of wounds that healed with a scar is represented graphically (A). PBS-treated wounds healed scarlessly, as expected (nd, none detected). Average scar widths were determined for wounds that healed with scars (B). Bars represent mean scar width $\pm$ S.E.M. (* $p < 0.05$; $n = 6-7$ per group). Representative Masson's trichrome (left panels; C, E, G) and picrosirius red (right panels; D, F, H) staining of E15 wounds treated with PBS or HMGB-1 at 7 days post-injury. Wound beds/scars are marked with arrows; scale bars = 100 μ m. Wounds treated with PBS (C, D) have no scar. Wounds treated with 200 ng (E, F) and 400 ng (G, H) HMGB-1 healed with increasingly large scars.

Figure 4. Effects of HMGB-1 on wound fibroblasts

Immunostaining was used to identify vimentin-positive fibroblasts and α -smooth muscle actin-positive myofibroblasts at 7 days post-wounding. The density of vimentin-positive fibroblasts was determined in the wound beds/scars (A). Bars represent average fibroblast density (number of vimentin-positive cells/mm²) $\pm$ S.E.M. (* $p < 0.05$; $n = 4-7$ per group). Representative images of vimentin-stained E15 wounds injected with either PBS (B) or 400 ng HMGB-1 (C) are shown. No α -smooth muscle actin-positive myofibroblasts were detected in wounds treated with PBS (D) or 400 ng HMGB-1 (E). Wound margins/scars are marked with arrows; scale bars = 50 μ m. The inset in E shows a blood vessel from an adjacent field of view at high magnification with positive staining, which was used as an internal positive control (inset scale bar = 20 μ m).

Figure 5. Alterations in blood vessel density after HMGB-1 treatment

Immunohistochemical staining for PECAM was used to identify dermal blood vessels in E15 wounds at 7 days post-wounding. The percent area of PECAM-positive staining within the wound bed/scar was used to estimate blood vessel density (A). Bars represent average blood vessel density $\pm$ S.E.M. (* $p < 0.05$; $n = 5-7$ per group). Representative images of PECAM-stained E15 wounds injected with PBS (B) or 400 ng HMGB-1 (C) are shown. Wound margins/scars are marked with arrows; scale bars = 50 μ m.

Figure 6. Macrophage recruitment in HMGB-1-treated wounds

Immunostaining for MOMA-2 was used to determine macrophage density at the wound margin 48 hours after injury (A). Bars represent average macrophage density (number of MOMA-2-positive cells per mm²) $\pm$ S.E.M. (* $p < 0.05$; $n = 8$ per group). Representative photomicrographs are shown for E15 wounds treated with PBS (B) or 400 ng HMGB-1 (C). Wound margins are marked with large arrows and small arrows are used to highlight MOMA-2-positive macrophages; scale bars = 50 μ m.