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Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1

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Abstract

Objective—Preterm parturition is a syndrome caused by multiple etiologies. Although intra-amniotic infection is causally linked with intrauterine inflammation and the onset of preterm labor, other patients have preterm labor in the absence of demonstrable infection. It is now clear that inflammation may be elicited by activation of the Damage-Associated Molecular Patterns (DAMPs), which include pathogen-associated molecular patterns (PAMPs) as well as “alarmins” (endogenous molecules that signal tissue and cellular damage). A prototypic alarmin is high-mobility group box-1 (HMGB1) protein, capable of inducing inflammation and tissue repair when it reaches the extracellular environment. HMGB1 is a late-mediator of sepsis, and blockade of HMGB1 activity reduces mortality in an animal model of endotoxemia, even if administered late during the course of the disorder. The objectives of this study were to: 1) determine whether intra-amniotic infection/inflammation (IAI) is associated with changes in amniotic fluid concentrations of HMGB1; and 2) localize immunoreactivity of HMGB1 in the fetal membranes and umbilical cord of patients with chorioamnionitis.

Methods—Amniotic fluid samples were collected from the following groups: 1) preterm labor with intact membranes (PTL) with (n=42) and without IAI (n=84); and 2) preterm prelabor rupture of membranes (PROM) with (n=38) and without IAI (n=35). IAI was defined as either a positive amniotic fluid culture or amniotic fluid concentration of interleukin-6 (IL-6) ≥2.6 ng/mL. HMGB1 concentrations in amniotic fluid were determined by ELISA. Immunofluorescence staining for HMGB1 was performed in the fetal membranes and umbilical cord of pregnancies with acute chorioamnionitis.

1. Amniotic fluid HMGB1 concentrations were higher in patients with IAI than in those without IAI in both the PTL and preterm PROM groups (PTL IAI: median 3.1 ng/mL vs. without IAI; median 0.98 ng/mL; $p<0.001$; and preterm PROM with IAI median 7.3 ng/mL vs. without IAI median 2.6 ng/mL; $p=0.002$);

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2. patients with preterm PROM without IAI had a higher median amniotic fluid HMGB1 concentration than those with PTL and intact membranes without IAI ($p<0.001$); and
3. HMGB1 was immunolocalized to amnion epithelial cells and stromal cells in the Wharton's jelly (prominent in the nuclei and cytoplasm). Myofibroblasts and macrophages of the chorioamniotic connective tissue layer and infiltrating neutrophils showed diffuse cytoplasmic HMGB1 immunoreactivity.

1. Intra-amniotic infection/inflammation is associated with elevated amniotic fluid HMGB1 concentrations regardless of membrane status;
2. preterm PROM was associated with a higher amniotic fluid HMGB1 concentration than PTL with intact membranes, suggesting that rupture of membranes is associated with an elevation of alarmins;
3. immunoreactive HMGB1 was localized to amnion epithelial cells, Wharton's jelly and cells involved in the innate immune response; and
4. we propose that HMGB1 released from stress or injured cells into amniotic fluid may be responsible, in part, for intra-amniotic inflammation due to non-microbial insults.

Keywords

preterm prelabor rupture of membranes; intra-amniotic infection; intra-amniotic inflammation; chorioamnionitis; alarmin; RAGE; amniotic fluid; danger signal

INTRODUCTION

Preterm parturition is a syndrome [1–3] clinically characterized by activation of the uterine components of the common pathway of parturition, including myometrial contractility [4–13], cervical ripening [14–19] and/or decidual membrane activation [20–22] with multiple etiologies [1–3,23–33]. Of the mechanisms of disease associated with the preterm parturition syndrome, only intra-amniotic infection/inflammation has been causally linked to preterm delivery [34–40]. Accumulating evidence indicates that not all patients with intra-amniotic inflammation have demonstrable infection using cultivation and molecular techniques [40–52]. This may be attributed either to infections which cannot be detected with standard microbiologic techniques (cultivation or molecular), because they are caused by microorganisms that are non-culturable (bacteria [41,42], viruses [53–57], etc.) or by microorganisms that have eluded detection with the currently available molecular microbiologic techniques. Another possibility to consider is that inflammation is of non-infection-related etiology. For example, we have proposed that "danger signals" can lead to the initiation of intra-amniotic inflammation and labor in the absence of an infection. Recently, we have generated experimental evidence that allergy or hypersensitivity type I can cause preterm labor in animals [58,59], and case reports suggest that this may be the case in humans [26,60–62]. Similarly, we have recently demonstrated that "maternal anti-fetal rejection" is an important and frequent mechanism of disease in preterm labor [29]. Such mechanism of disease is expressed by a unique pattern of chronic inflammation in the chorioamniotic membranes (chronic chorioamnionitis) [63,64] or the placental mass (villitis of unknown etiology) [65,66].

Inflammation is an ancient mechanism of host defense to control endogenous or exogenous damage and restore homeostasis in response to bacteria or tissue injury [67]. To initiate an appropriate inflammatory response, organisms develop several means of cellular communication to sense "danger signals"[68]. Exogenous signals are specific molecular patterns of pathogens including peptidoglycans, lipopolysaccharides, bacterial DNA, viral RNA, etc. and are collectively termed "Pathogen Associated Molecular Patterns (PAMPs)"

[69–71]. In contrast, endogenous danger signals are normal constituents of cells, which, when the cells sustain damage, leak outside the cells and are capable of eliciting an inflammatory response. Joost Oppenheim proposed the term “alarmins” to differentiate the endogenous molecules that signal tissue and cellular damage from PAMPs [72–74]. Examples of alarmins include heat shock proteins [75,76], S100 proteins [77,78], uric acid [79], hepatoma-derived growth factor [80], interleukin-1a [81], adenosine triphosphate [74], and high-mobility group box-1 (HMGB1)[72].

HMGB1 has been considered a prototypic alarmin [72]. The characteristics of alarmins are: 1) molecules are rapidly released after non-program cell death (i.e. necrosis), and also, by apoptotic cells; 2) immunocytes can produce and release alarmin without dying, using a specialized mechanism for secretion or the endoplasmic reticulum Golgi secretion pathway; 3) they can recruit and activate receptor-expressing cells of the innate immune system, including dendritic cells, and therefore, can activate the adaptive immune system; and 4) these molecules should restore homeostasis by promoting healing of the tissue that was destroyed, either because of the direct insult or the secondary effects of the inflammatory process [72].

Originally discovered as a nuclear protein [82,83], HMGB1 is the most widely studied member of the alarmin family. This protein can be released either passively by necrotic cells [84,85] or actively by stressed cells in response to injury [86,87]; it is capable of inducing inflammation [88–97] and tissue repair [98–105] when it reaches the extracellular environment. HMGB1 has been implicated as a late mediator of sepsis [106–109]. Furthermore, blockade of HMGB1 activity reduces mortality in animal models of endotoxemia [107] and sepsis [108,109], even if administered after the onset of the disease. These findings have generated considerable interest in HMGB1 as a potential therapeutic target for several infectious and inflammatory disorders [106,110].

HMGB1 mediates its biological activities through multiple receptors including Toll-Like receptors (TLRs) [89,93,94,111] and receptors for advanced glycation end products (RAGE) [112–117]. Binding of HMGB1 to RAGE can induce the production of pro-inflammatory cytokines, and chemokines, as well as neutrophil chemotaxis, in a manner that may be suppressed or stimulated by soluble, truncated forms of RAGE including the soluble form of RAGE (sRAGE) and endogenous secretory RAGE (esRAGE) [112]. We have examined the changes of amniotic fluid concentrations of pro-inflammatory cytokines [118–126], anti-inflammatory cytokines [127], chemokines [128–134], proteases/anti-proteases [135], matrix-metalloproteinase [136–143], pro- and anti-angiogenic factors [144–146], coagulation factors [147,148], adipocytokines [149–151], anti-microbial peptides [152] and prostaglandins [153–156] in patients intra-amniotic infection and /or inflammation (IAI) both at term and preterm gestations. Amniotic fluid concentrations of sRAGE and esRAGE have been reported to be elevated in patients with IAI in preterm gestations and decreased in labor at term [157], whereas the amniotic fluid concentration of HMGB1 in patients with IAI has not yet been examined.

The objectives of this study were to: 1) determine if IAI in preterm gestation is associated with changes in the amniotic fluid concentration of HMGB1; and 2) immunolocalize HMGB1 in the fetal membranes and umbilical cord of patients with histologic chorioamnionitis.

MATERIALS AND METHODS

Study design and population

A retrospective cross-sectional study was conducted by searching our clinical database and bank of biological samples. Patients with spontaneous preterm labor (PTL) with intact membranes and those with preterm prelabor rupture of membranes (PROM) who had amniotic fluid samples retrieved by trans-abdominal amniocentesis were included. Patients were subdivided into the following groups: 1) PTL and intact membranes with (n=42) and without intra-amniotic infection/inflammation (IAI) (n=84); and 2) preterm PROM with (n=38) and without IAI (n=35).

All women provided written, informed consent before the collection of amniotic fluid samples. The collection and utilization of the samples were approved by the Human Investigation Committee of the participating institutions and the IRB of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS). Many of these samples have been used in previous studies of the biology of sRAGE, esRAGE and other cytokines and inflammatory mediators in IAI, pregnancy, preterm labor and preterm PROM.

Clinical definition

The diagnosis of preterm labor was made in the presence of regular uterine contractions (at least 3 in 30 minutes) and documented cervical change in patients with a gestational age of 20 to 36 6/7 weeks. Preterm PROM was diagnosed with a sterile speculum examination with documentation of vaginal pooling and positive nitrazine and ferning tests. Intra-amniotic infection was defined as a positive culture for bacteria in amniotic fluid, and intra-amniotic inflammation as an amniotic fluid interleukin (IL)-6 concentration of 2.6ng/mL or more [43]. Intra-amniotic inflammation could be present in the absence of a positive amniotic fluid culture for microorganisms.

Sample collection

Amniotic fluid samples were obtained by trans-abdominal amniocentesis performed for evaluation of the microbial status of the amniotic cavity and/or assessment of fetal lung maturity. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe and cultured for aerobic/anaerobic bacteria and genital mycoplasmas. White blood cell (WBC) count [158], glucose concentration [159] and Gram stain [160] were also performed shortly after collection as previously described [158,159]. The results of these tests were used for clinical management. Results of amniotic fluid IL-6 concentration were used only for research purposes. Amniotic fluid not required for clinical assessment was centrifuged at 1300*g* for 10 min at 4°C and the supernatant was stored at −70°C.

Determination of HMGB1 in amniotic fluid

Concentrations of HMGB1 and IL-6 in amniotic fluid were determined by sensitive and specific enzyme immunoassays obtained from IBL International (Toronto, Canada) and from R&D Systems (Minneapolis, MN, USA), respectively. The initial assay validation was performed in our laboratory prior to the conduction of this study. Briefly, the immunoassay utilized the quantitative sandwich enzyme immunoassay technique and the concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for HMGB1 were 3.1% and 4.4%, respectively, and for IL-6; 8.7% and 4.6%, respectively. The sensitivity of the assay for HMGB1 was 0.2 ng/mL and for IL-6; 0.09 pg/mL. The results of amniotic fluid sRAGE and esRAGE concentrations in several samples have been previously reported [157], but were included in this manuscript in

order to provide a comprehensive picture of the relationship between HMGB1 concentrations and that of its soluble receptors.

Immunofluorescence staining of HMGB1 in the fetal membranes, and umbilical cord

Immunofluorescence staining was performed to determine if HMGB1 protein expression could be detected and the localization of the protein in the chorioamniotic membranes and umbilical cord. The placentas of women at term not in labor, at term in labor, and patients who delivered preterm with histologic chorioamnionitis were studied (n=6). The staining was performed using a mouse monoclonal anti-HMGB1 antibody (1:50; AbCam plc, Cambridge, UK). Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissues were placed on silanized slides and stained using a Ventana Discovery automatic staining system (Ventana Medical Systems, Tucson, AZ). Sections were incubated with primary antibodies in 1% (w/v) BSA in PBS for 1 h, followed by incubation with Alexa 488 goat anti-mouse IgG and Alexa 594 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA) in 1% (w/v) BSA for 30 min. Sections were mounted in ProLong® Gold antifade reagent with 4′-6-Diamidino-2-phenylindole (DAPI) (Invitrogen).

Statistical analysis

The Kolmogorov-Smirnov test and Shapiro-Wilk test were used to determine if the data was normally distributed. A two-tailed Mann-Whitney U test was used to compare continuous non-parametric variables. Comparisons between proportions were performed using Chi-square or Fisher's exact tests. Spearman rank correlation was utilized to assess correlations between two continuous variables. A p-value <0.05 was considered statistically significant. Analysis was performed with SPSS, version 15 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographics and clinical characteristics of the study population

Demographics and clinical characteristics of patients with spontaneous PTL with intact membranes and those with preterm PROM are displayed in Tables I and II respectively. There was no significant difference in the median gestational age at amniocentesis between patients with and without IAI (p=0.3 and p=0.6 respectively).

Immunoreactive HMGB1 in amniotic fluid was detected in 91% (186/205) of the samples. Nineteen samples had HMGB1 concentrations below the detection limit of the immunoassay. Those samples were from patients in the following groups: sixteen from the PTL with intact membranes group (12 without IAI, 4 with IAI), and 3 from the preterm PROM group (1 without IAI, 2 with IAI).

Intra-amniotic infection/inflammation was associated with an elevation of amniotic fluid HMGB1 concentration

Patients with PTL and intact membranes with IAI had a significantly higher median amniotic fluid concentration of HMGB1 than those without IAI (PTL with IAI: median 3.1 ng/mL; range: 0–31.2 ng/mL vs. PTL without IAI: median 0.98 ng/mL; range: 0–7.3 ng/mL; p<0.001; Figure 1). Similarly, the median amniotic fluid concentration of HMGB1 in patients with preterm PROM with IAI was significantly higher than those without IAI (preterm PROM with IAI: median 7.3 ng/mL; range: 0–65.4 ng/mL vs. preterm PROM without IAI: median 2.6 ng/mL; range: 0–9.7 ng/mL; p=0.002; Figure 2). As previously reported, IAI was associated with a higher median amniotic fluid concentration of sRAGE and esRAGE in both patients with PTL and those with preterm PROM (Tables I and II).

There was no significant difference in the median amniotic fluid concentration of HMGB1 in patients with PTL without IAI between those who delivered at term and preterm (term delivery, n=56, median 1.2 ng/mL; range: 0–6.7 ng/mL vs. preterm delivery, n=28, median 0.7 ng/mL; range: 0–7.3 ng/mL; p= 0.2). Among patients with PTL without IAI who delivered at term, there was a significant relationship between amniotic fluid concentrations of HMGB1 and IL-6 (Spearman Rho 0.4; p=0.007). In contrast, among patients with PTL with IAI, there was no significant relationship between amniotic fluid concentrations of HMGB1 and markers of inflammation (IL-6, WBC, glucose) or its soluble receptors (sRAGE and esRAGE) (each p>0.05).

There were 28 patients with PTL who delivered preterm without IAI, six of which delivered within 3 days after amniocentesis. The median amniotic fluid HMGB1 concentrations of patients without IAI who delivered within 3 days were not significantly different from patients with PTL who delivered at term without IAI (median 1.3 ng/mL range 0.8–3.1 ng/mL vs. median 1.2 ng/mL range 0–6.7 ng/mL; p=0.6).

Amniotic fluid HMGB1 concentrations changed with membrane status

Patients with preterm PROM without IAI had a higher median amniotic fluid HMGB1 concentration than patients with PTL and intact membranes without IAI (p<0.001; Figure 3). Although the median gestational age at the time of amniocentesis was slightly lower in patients with preterm PROM without IAI than those with PTL and intact membranes without IAI (preterm PROM without IAI: median 29.6 weeks, range: 23–34.3 weeks vs. PTL without IAI: median 31.3 weeks, range: 23.1–34.5 weeks; p=0.003), amniotic fluid concentration of HMGB1 did not change as a function of gestational age in patients with PTL without IAI who delivered at term (Spearman Rho = − 0.1; p = 0.3).

Among all patients with intra-amniotic infection/inflammation (n=80), those who had a positive culture for microorganisms (n=43) had a significantly higher (6-fold) median amniotic IL-6 concentration than those with sterile amniotic fluid (n=37) (median 49 ng/mL range 0.03–388 ng/mL vs. median 7.8 ng/mL range 2.6–923; p<0.001), whereas there was no significant difference in HMGB1 concentration between those with and without a positive amniotic fluid culture (median 5.8 ng/mL range 0–65 ng/mL vs. median 3.1 range (0–31.2; p=0.09).

Three women had intra-amniotic infection without a high amniotic fluid IL-6 concentration (<2.6 ng/mL). The first patient was diagnosed with PTL and underwent amniocentesis at 33.5 weeks. She delivered spontaneously 4 days later. This patient had a positive amniotic fluid culture for *Mycoplasma hominis* with an IL-6 concentration of 0.64 ng/mL and HMGB1 of 2.6 ng/mL. The other two patients presented with preterm PROM and underwent amniocentesis at 28.9 and 34.4 weeks, respectively. In both cases, amniotic fluid culture revealed *Ureaplasma urealyticum*. These patients had IL-6 concentrations of 0.02 and 0.53 ng/mL with HMGB1 concentrations of 10.9 and 3.3 ng/mL, respectively.

Immunofluorescence staining for HMGB1 in the fetal membranes and umbilical cord

In the chorioamniotic membranes, immunoreactivity of HMGB1 was prominent in the nuclei or cytoplasm of amniotic epithelial cells. Myofibroblasts and macrophages of the chorioamniotic connective tissue layer and infiltrating neutrophils showed diffuse cytoplasmic HMGB1 immunoreactivity (Figure 4). In the umbilical cord, stromal cells in the Wharton's jelly were strongly immunoreactive for HMGB1 both in the nuclei and cytoplasm (Figure 5a). In contrast, immunostaining of HMGB1 on umbilical vascular endothelial cells was negative (Figure 5b).

DISCUSSION

Principal findings of the study

1) amniotic fluid concentrations of HMGB1 were higher in patients with intra-amniotic infection/inflammation regardless of their membrane status (intact or ruptured); 2) in the absence of intra-amniotic inflammation, patients with preterm PROM had higher amniotic fluid concentrations of HMGB1 than patients with preterm labor with intact membranes; and 3) HMGB1 protein was immunolocalized to amnion epithelial cells, myofibroblasts and infiltrating macrophages of chorioamniotic membranes as well as stromal cells in Wharton's jelly of the umbilical cord.

Biology of HMGB1

HMGB1 is an evolutionarily conserved DNA-binding protein that stabilizes nucleosomes and facilitates gene transcription [88]. This protein is expressed in almost every cell type (except those without a nucleus such as erythrocytes and skin epithelial cells)[161] and can be actively released from cells in response to bacterial products (eg: peptidoglycan, lipopolysaccharide (LPS), CpG-DNA)[162–165], cytokines [eg: tumor necrosis factor (TNF)-α, interferon-gamma and transforming growth factor-β] [165–167]or tissue injury (i.e. ischemic/reperfusion, nerve crush injury)[86,87,168]. Due to the lack of a leader signal sequence, HMGB1 can not be actively secreted through the classic endoplasmic reticulum-Golgi, but requires a specialized pathway to translocate the protein from the nucleus to the cytoplasm and load it into secretory lysosomes [72,169]. Eventually, this protein can be secreted into the extracellular environment by either exocytosis [169] or released as exosomes [170,170]. The release of HMGB1 from the nucleus of activated macrophages and dendritic cells is controlled by a crucial acetylation step. Alternatively, HMGB1 can be passively released by necrotic cells [84,85] or cells infected with viruses (e.g. West Nile, influenza, etc.)[171,172] or mycobacteria [173,174]. It is noteworthy that, in granulocytes, HMGB1 is tightly sequestered in a detergent-insoluble form, which limits its release even under conditions of necrotic cell death [84,161].

Several types of receptors have been implicated in HMGB1 signaling, including RAGE [112–117] and members of the Toll-Like Receptors (TLR -2, -4 and -9) [89,93,94,111]. Upon binding to its receptors, HMGB1 activates immune cells or endothelial cells to produce pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ), chemokines and adhesion molecules [88–97] as well as maturation and migration of immune cells [92,97,114,115]. Moreover, HMGB1 can stimulate the migration of stem cells, which is important for tissue repair and regeneration after inflammation [98–105].

Since HMGB1 can elicit sustained inflammatory response in the extracellular environment, a few physiologic regulators are present to control biological activity of HMGB1. These molecules include thrombomodulin [175–177], CD24 and Siglec-10 [178,179], as well as heat shock protein-72 [180]. Vagal nerve stimulation has also been shown to inhibit TNF-α release and reduce systemic HMGB1 concentrations, as well as improve survival in animal models of sepsis through an alpha7 nicotinic acetylycholine receptor [181,182].

Recently, Rouhiainen et al. demonstrated that ultra-purified HMGB1 has weak pro-inflammatory activity in mononuclear cells [183]. Since HMGB1 has a natural propensity to bind negatively-charged molecules such as DNA, RNA, nucleosomes, PAMP or immune activating cytokines such as IL-1, it is possible that HMGB1 may form a complex with TLR agonists (i.e. LPS, IL-1) that triggers the respective TLR or cytokine receptors and sends an "alarm signal" [161].

HMGB1, a late mediator of sepsis

HMGB1 was proposed to be a late mediator of sepsis [107]. Wang et al identified this 30-kDa protein in the conditioned media of macrophage culture after stimulation with endotoxin. In an animal model of endotoxemia [107] and sepsis [184], HMGB1 was first detected in the circulation late during the course of the disorder. This profile of HMGB1 parallels the occurrence of death induced by endotoxemia [107] and sepsis [184], and distinguishes itself from TNF-α and other pro-inflammatory cytokines which are increased early after the administration of endotoxin (IL-1β) [108,109]. The administration of HMGB1-neutralizing antibodies conferred a dose-dependent protective effect even when administered 24 hours after the onset of sepsis [184]. These observations indicate that HMGB1 is a potential therapeutic target for sepsis and other inflammatory disorders [106,109,161].

Intra-amniotic infection/inflammation was associated with an elevation of HMGB1

The finding that intra-amniotic infection/inflammation in preterm gestations was associated with an approximate 3-fold increase in the amniotic fluid concentration of HMGB1 suggests that this alarmin may participate in the host response to intra-amniotic infection or cellular injury in spontaneous preterm delivery. We have proposed that intra-amniotic inflammation is induced by microorganisms which may be detectable by standard cultivation or molecular techniques [41,43,45]. However, the possibility that inflammation caused by endogenous alarmins released from stressed or injured cells due to non-microbial insults should also be considered. It is now clear that inflammation can be caused by multiple insults unrelated to microorganisms [72,74,185].

Microbial products such as LPS and endogenous mediators (alarmins) such as HMGB1 can stimulate IL-6 and other pro-inflammatory cytokines [186]. Yet, in the current study, there was no relationship between IL-6 and HMGB1 in patients with intra-amniotic inflammation. It is noteworthy that, among patients with intra-amniotic inflammation, the median HMGB1 concentration in amniotic fluid was not significantly different between patients with and without a positive amniotic fluid culture for microorganisms, while the median amniotic fluid IL-6 concentration was 6-fold higher in those with a microbiologically proven infection than in those with sterile amniotic fluid. One interpretation of these findings is that microorganisms and microbial products are a much stronger inducer of IL-6 than of HMGB1 in cases of intra-amniotic infection. Alternatively, it can be argued that the magnitude of the HMGB1 elevation in amniotic fluid is similar in patients with intra-amniotic inflammation, regardless of whether the stimuli is microbial. Evidence in support of this interpretation includes: 1) LPS and HMGB1 induce a distinct pattern of cytokine release from neutrophils based on microarray experiments [92,187,188]; and 2) the administration of an HMGB1-neutralizing antibody to mice with LPS-induced lung injury does not significantly reduce the concentration of several pro-inflammatory cytokines (TNF-α, IL-1α, etc.) [108], suggesting that HMGB1 acts at a more distal point in the endotoxin-induced pro-inflammatory cascade, which is different from the effects of LPS (specifically, some effects of LPS may be due to early mediators).

Of note, we also observed that, under certain circumstances, patients with a microbiologically-proven intra-amniotic infection had an elevation of HMGB1 concentration, despite no elevation of IL-6 (<2.6 ng/mL). The reasons for this dissociation between IL-6 and HMGB1 are not known at this time, but we believe that this is an important observation. It suggests that the action of microorganisms may be mediated through the conventional pathway of chemokines and cytokines, such as IL-1β, TNFα and IL-6 in some cases, but in others, it may be mediated by the action of what we now call alarmins. If this interpretation is correct, there may be an overlap in the mechanisms of host

response induced by bacteria. In some cases, the biological effects are mediated to the classical PAMPs, while in others; the effect of bacteria on cells may lead to the release of alarmins such as HMGB1. These alarmins could then trigger the inflammatory process. It is understandable why the term "DAMPs" [189] has been somewhat confusing in the literature – some authors use this term to refer to endogenous molecules released in response to cell injury not caused by bacteria, while others use the term DAMPs as an umbrella to include PAMPs as well as endogenous mediators, or alarmins.

Clearly it is possible that infection can elicit different cytokine responses. For example, the duration of microbial invasion of the amniotic cavity, the gestational age at which infection occurs (very early pregnancy or close to term), the clinical presentation in preterm labor or PROM and the timing of the retrieval of amniotic fluid can all contribute to explaining the differences in the profile of chemokines, cytokines, inflammatory mediators and alarmins at a given point in the natural history of the inflammatory process. Some patients present in preterm labor in the early phase of the inflammatory response in which HMGB1 is low – this is likely to represent the majority of patients (IL-6 will be elevated in such patients). In contrast, in patients in whom intra-amniotic infection is characterized by a prolonged exposure to microbial products and microorganisms, the host response may have evolved so that HMGB1 is elevated. This is the equivalent of what happens in some patients with sepsis, in which HMGB1 is typically a late mediator of septic shock and death [108].

Amniotic fluid HMGB1 concentrations did not correlate with sRAGE or esRAGE

RAGE, a member of the immunoglobulin receptor superfamily, is one of the main receptors for HMGB1 [117] and is expressed in several cell types including amnion epithelium, extravillous trophoblasts, decidua, endothelial cells, macrophages, smooth muscle cells and neurons. The soluble truncated form of RAGE has been proposed to act as a regulatory negative feedback mechanism to modulate the activity of RAGE or HMGB1 [112]. However, this soluble form (sRAGE) has also been shown to induce the production of pro-inflammatory cytokines and chemokines by monocytes [190] and spleen cells [191]. Both the soluble and endogenous secretory forms of RAGE (sRAGE and esRAGE) were elevated in patients with IAI at preterm gestations regardless of the membrane status [157]. However, in the current study, neither sRAGE nor esRAGE concentrations correlated with HMGB1. These findings could be explained by the nature of RAGE which can be induced by several pro-inflammatory molecules (other than HMGB1), including S100/calgranulins, amyloid and beta sheet fibrils [112]. Indeed, intra-amniotic infection has been associated with a significant increase in amniotic fluid concentrations of S-100B [192], calprotectin (heterocomplex of S100A8 or calgranulin-A and S100A9 or calgranulin-B)[152].

Amniotic fluid HMGB1 in patients without intra-amniotic infection/inflammation

In the absence of intra-amniotic infection/inflammation, there was a significant correlation between the amniotic fluid concentrations of HMGB1 and IL-6. In such instances, mild cellular injury or stress may be responsible for the presence of extracellular HMGB1 and IL-6 in the amniotic fluid, and these proteins may be beneficial for tissue repair and resolution of a minor cellular injury (e.g. preterm labor without infection that eventually results in delivery at term because the episode of preterm labor resolves without delivery).

The finding that there was no significant difference in HMGB1 concentrations in the amniotic fluid of patients without intra-amniotic inflammation who delivered preterm compared to those who delivered at term suggests that HMGB1 does not appear to be involved in the mechanism of preterm labor/delivery in the absence of an elevation of IL-6.

HMGB1 may be involved in the mechanisms of rupture of membranes

A major finding of the current study is that patients with preterm PROM had higher concentrations of HMGB1 in amniotic fluid than those with intact membranes. This observation is consistent with the findings from a previous study that HMGB1 is capable of stimulating matrix metalloproteinase (MMP)-9 in neuronal and glial cell cultures [193]. This group of proteins has been implicated in the mechanisms of membrane rupture [120,121,136–143,194,195]. In contrast, our findings for HMGB1 are different than the behavior of a large number of inflammatory mediators including prostaglandins [153–156], cytokines [118–121] and chemokines [128,130,196] in women with preterm labor and preterm PROM. In the overwhelming majority of cases, the concentrations of inflammatory mediators are higher in patients with preterm labor and intact membranes than in those with preterm PROM. The exception to this rule is the concentration and/or activity of matrix-degrading enzymes, which are implicated in the mechanisms of membrane rupture [120,121,136–143,194,195]. In such cases, the concentrations of MMP1, MMP8 and MMP9 are significantly higher in both the amniotic fluid [136,140,141] and fetal blood [121] in patients with preterm PROM than in patients with preterm labor and intact membranes, regardless of microbial status.

It remains to be determined if HMGB1 in amniotic fluid can stimulate MMP expression in the fetal membranes or by immune cells. It is also possible that higher concentrations of extracellular HMGB1 in amniotic fluid observed in patients with membrane rupture may reflect more cellular damage in this condition than in patients with preterm labor and intact membranes. Furthermore, a recent study also observed a lower HMGB1 mRNA expression in cervical biopsy specimens of patients with preterm PROM compared to those of patients with preterm labor and intact membranes [197].

Immunolocalization of HMGB1 in fetal membranes and umbilical cord

The sources of HMGB1 in the amniotic fluid of patients with intra-amniotic inflammation were unknown. In the current study, however, HMGB1 was immunolocalized to the cytoplasm of amnion epithelium cells, myofibroblasts and macrophages of chorioamniotic membranes, and infiltrating neutrophils in patients with histologic chorioamnionitis, indicating that HMGB1 may be translocated from the nucleus to the cytoplasm in these cells and eventually released into amniotic fluid. These findings are consistent with those reported in normal pregnancy at term, which showed strong nuclear and cytoplasmic expression of HMGB1 in amnion epithelial cells using immunohistochemistry and *in situ* hybridization [198].

Strengths and limitations of this study

This is the first report of HMGB1 concentration in the amniotic fluid of patients with preterm labor and preterm PROM. It is tempting to propose that this protein may reflect a “danger signal” responsible for the mechanisms of membrane rupture. However, due to the cross-sectional nature of the study, a temporal relationship between an elevation of HMGB1 in amniotic fluid and membrane rupture could not be established.

Conclusion

The observation that amniotic fluid concentrations of HMGB1 were increased in intra-amniotic infection/inflammation suggests that this alarmin participates in the host response to intra-amniotic infection or cellular injury during intra-amniotic inflammation in preterm gestation. The possibility that intra-amniotic inflammation caused by endogenous alarmins released from stress or injured cells due to non-microbial insults should be considered.

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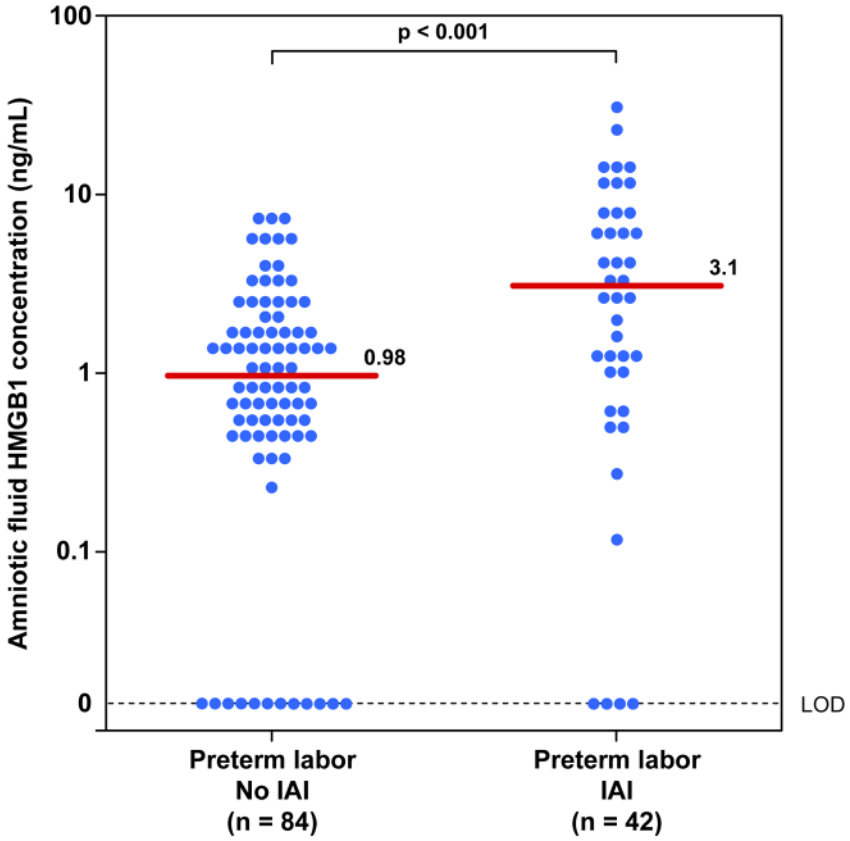

Figure 1.

Amniotic fluid concentrations of HMGB1 in patients with preterm labor and intact membranes (PTL) with and without intra-amniotic infection/inflammation (IAI). Patients with PTL and intact membranes with IAI had a significantly higher median amniotic fluid concentration of HMGB1 than those without IAI (PTL with IAI: median 3.1 ng/mL; range: 0–31.2 ng/mL vs. PTL without IAI: median 0.98 ng/mL; range: 0–7.3 ng/mL; p<0.001).

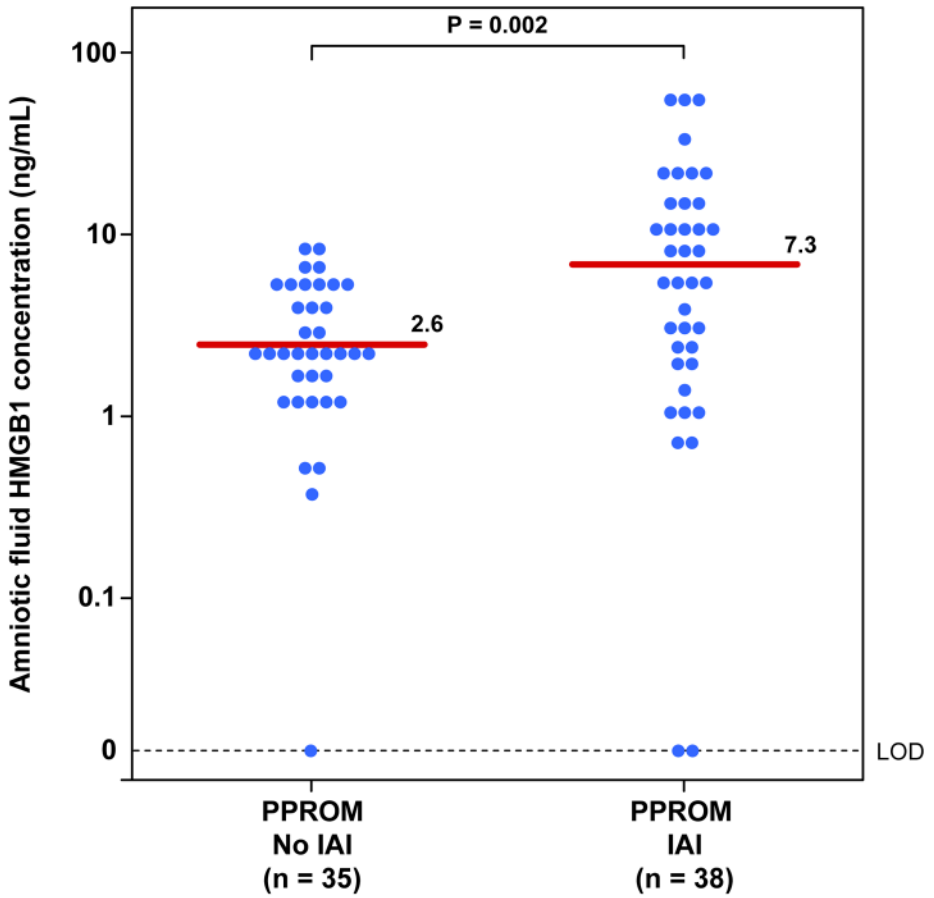

Figure 2.

Amniotic fluid concentrations of HMGB1 in patients with preterm prelabor rupture of membranes (PROM) with and without intra-amniotic infection/inflammation (IAI). The median amniotic fluid concentration of HMGB1 in patients with preterm PROM with IAI was significantly higher than those without IAI (preterm PROM with IAI: median 7.3 ng/mL; range: 0–65.4 ng/mL vs. preterm PROM without IAI: median 2.6 ng/mL; range: 0–9.7 ng/mL; p=0.002).

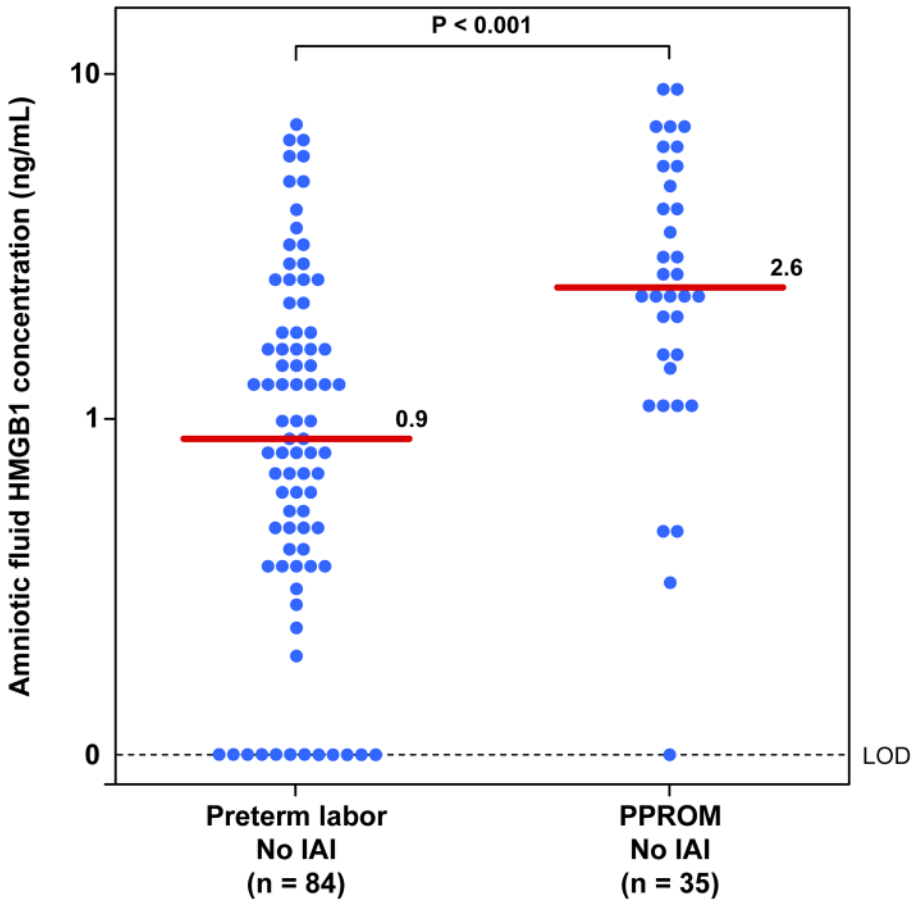

Figure 3.

Amniotic fluid concentrations of HMGB1 in patients with preterm labor and intact membranes (PTL) and preterm prelabor rupture of membranes (PROM) without intra-amniotic infection/inflammation (IAI). Patients with preterm PROM without IAI had a higher median amniotic fluid HMGB1 concentration than patients with PTL and intact membranes without IAI (preterm PROM without IAI: median 2.6 ng/mL; range: 0–9.7 ng/mL vs. PTL without IAI: median 0.98 ng/mL; range: 0–7.3 ng/ml; $p<0.001$).

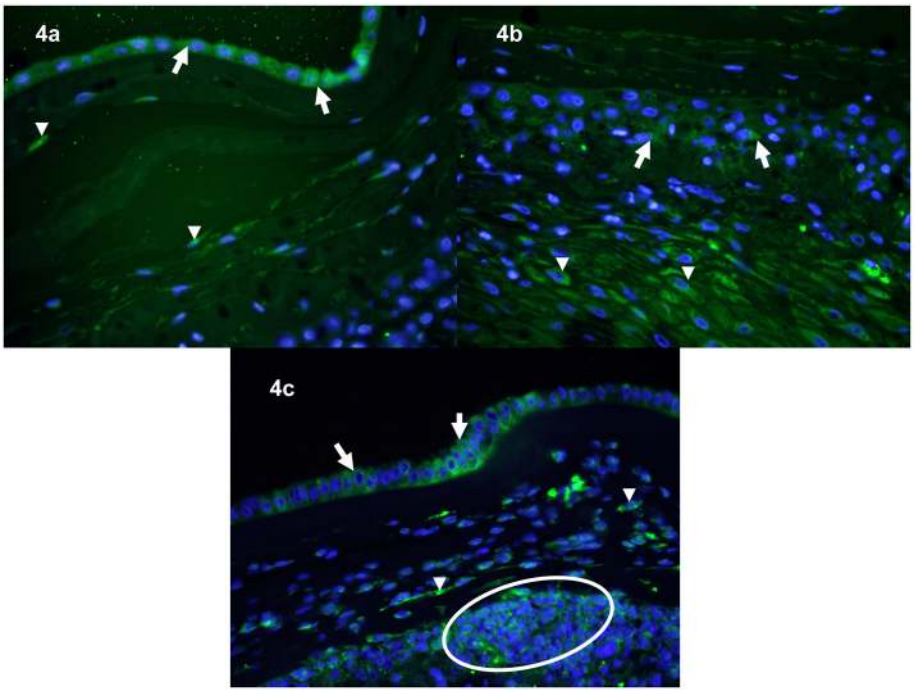

Figure 4.

Immunofluorescence images of HMGB1 immunoreactivity in the chorioamniotic membranes. a: Chorioamniotic membranes obtained from an uncomplicated pregnant woman who delivered at term. HMGB1 immunoreactivity (green) is evident in the amnion epithelial cells (arrows) and mesenchymal cells of the chorioamniotic connective tissue (arrowheads). X400. b: Chorioamniotic membranes obtained from a different case of normal term delivery. Scattered HMGB immunoreactivity (green) is observed in the chorionic trophoblasts (arrows) and decidual stromal cells (arrowheads). X400. c: Chorioamniotic membranes obtained from a patient who delivered preterm with acute chorioamnionitis and funisitis. Distinct immunoreactivity (green) is detected in the amnion epithelial cells (arrows), myofibroblasts of chorioamniotic connenctive tissue (arrowheads), and infiltrating neutrophils (circle). X400. DAPI (blue) was used to stain nuclei.

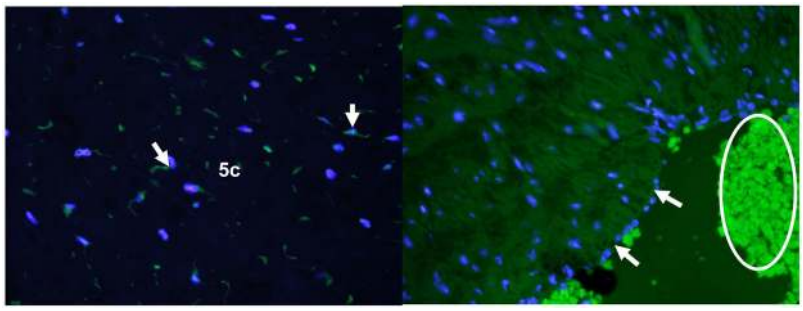

Figure 5.

Immunofluorescence images of HMGB1 immunoreactivity in the umbilical cord Wharton's jelly and umbilical vein. a: Umbilical cord Wharton's jelly obtained from a patient who delivered preterm with acute chorioamnionitis and funisitis. Distinct immunoreactivity of HMGB1 (green) is detected in the stromal cells of the Wharton's jelly. X400. b. Umbilical vein obtained from an uncomplicated pregnant woman who delivered at term. HMGB immunoreactivity (green) is absent in umbilical vein endothelial cells (arrows). Autofluorescence from RBCs are prominent (circle). X400. DAPI (blue) was used to stain nuclei.

Table 1

Demographics and clinical characteristics of patients with preterm labor with intact membranes (PTL)

	PTL no IAI n=84	PTL with IAI n=42	p
Maternal age (years)	23 (14–42)	24 (15–44)	0.7
Previous preterm delivery	15 (18%)	5 (11.9%)	0.4
Gestational age at amniocentesis (weeks)	31.3 (23.1–34.5)	30.7 (23.2–34.5)	0.3
Intraamniotic infection	0	20 (47.6%)	<0.001 *
Amniotic fluid IL-6 (ng/mL)	0.45 (0.03–2.07)	25.23 (0.64–923.1)	<0.001 *
Intra-amniotic inflammation	0	41 (97.6%)	<0.001 *
Interval to delivery (days)	38.9 (0–121.8)	1.1 (0–58.8)	<0.001 *
Delivery within 3 days	6 (7.1%)	25 (59.5%)	<0.001 *
Gestational age at delivery (weeks)	38.2 (29–41.4)	31.8 (23.3–39.0)	<0.001 *
Birthweight (grams)	3110 (1230–4470)	1940 (610–3520)	<0.001 *
Histologic chorioamnionitis	6 (12.5%)	14 (58.3%)	<0.001 *
HMGB1 detectable	72 (85.7%)	38 (90.5%)	0.6
sRAGE (ng/mL)	16.04 (0.49–44.77)	22.19 (0–83.65)	0.03 *
esRAGE (ng/mL)	7.22 (0–19.16)	10.86 (0–35.96)	0.001 *

Values are expressed as number (percent) or median (range)

IAI: Intraamniotic infection and/or inflammation

IL-6: Interleukin-6

* $p<0.05$

Table 2

Demographics and clinical characteristics of patients with preterm prelabor rupture of membranes (PROM)

	Preterm PROM no IAI n=35	Preterm PROM with IAI n=38	p
Maternal age (years)	26 (15–40)	29 (19–42)	0.2
Previous preterm delivery	10 (28.6%)	4 (10.5%)	0.07
Gestational age at amniocentesis (weeks)	29.6 (23–34.3)	28.9 (22.9–34.4)	0.6
Intra-amniotic infection	0	23 (60.5%)	<0.001 *
Amniotic fluid IL-6 (ng/mL)	0.77 (0.11–2.57)	21.13 (0.03–269.1)	<0.001 *
Intra-amniotic inflammation	0	36 (94.7%)	<0.001 *
Interval to delivery (days)	15.9 (0–79.2)	2.5 (0–41.9)	0.003 *
Delivery within 3 days	7 (20%)	20 (52.6%)	0.004 *
Gestational age at delivery (weeks)	31.7 (27–35.6)	30.4 (24.9–34.7)	0.01 *
Birthweight (grams)	1,770 (1,060–2380)	1,550 (740–2,600)	0.04 *
Histologic chorioamnionitis	10 (35.7%)	23 (69.7%)	0.008 *
HMGB1 detectable	34 (97.1%)	36 (94.7%)	1.0
sRAGE (ng/mL)	17.51 (1.7–37.1)	23.73 (1.5–52.8)	0.03 *
esRAGE (ng/mL)	6.43 (0.79–14.99)	9.39 (0–28.34)	0.01 *

Values are expressed as number (percent) or median (range)

IAI: Intraamniotic infection and/or inflammation

IL-6: Interleukin-6

* $p<0.05$