Placenta 35 (2014) 1049–1056

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Decidual GM-CSF is a critical common intermediate necessary for thrombin and TNF induced *in-vitro* fetal membrane weakening

D. Kumar [a, *], R.M. Moore [a], A. Nash [a], E. Springel [b], B.M. Mercer [b], E. Philipson [c], J.M. Mansour [d], J.J. Moore [a, b]

[a] Department of Pediatrics, Case Western Reserve University, Cleveland, OH, USA
[b] Department of Reproductive Biology, Case Western Reserve University, Cleveland, OH, USA
[c] Women's Institute-Cleveland Clinic, Cleveland, OH, USA
[d] Mechanical and Aerospace Engineering, Case Western Reserve University, Cleveland, OH, USA

ARTICLE INFO

Article history:
Accepted 3 October 2014

Keywords:
Decidua
GM-CSF
TNF
Thrombin
Fetal membranes
Weakening
Biomechanics
PPROM

ABSTRACT

Introduction: Inflammation/infection and decidual bleeding/abruption are highly associated with pPROM. As no animal model for pPROM exists, we have developed an *in-vitro* model system for the study of human fetal membrane (FM) weakening/rupture. Using it we have demonstrated that both TNF/IL-1 (modeling inflammation) and thrombin (modeling bleeding) weaken full thickness FM in a dose dependent manner concomitant with inducing biochemical changes similar to those seen in the FM physiological weak zone.

Methods: As the physiological site of infection and bleeding is the choriodecidua (CD), we modified our model system with full thickness FM tissue mounted on modified Transwell culture inserts to permit directional TNF/thrombin exposure on the decidua only (rather than both sides of the FM). After incubation, medium was sampled separately from the CD facing (maternal side) or from the amnion facing (fetal side) compartments and probed for cytokine release and confirmed with western blots. The FM was strength tested within the insert.

Results: Full-thickness FM fragments exposed to TNF or thrombin on CD side only showed dose dependent weakening and biochemical changes consistent with previous reports. Concomitantly, GM-CSF increased markedly on the CD but not the amnion side. Numerous proteases including MMP1 and MMP3 also increased on the CD side. Pre-incubation with GM-CSF antibody blocked both thrombin and TNF induced weakening. Finally, GM-CSF weakened FM in a dose dependent manner.

Discussion: GM-CSF is a critical common intermediate in the thrombin and TNF FM weakening pathways.

1. Introduction

Preterm premature rupture of the fetal membranes (pPROM) and resultant premature birth is a major cause of infant morbidity and mortality [1,2]. Infection/inflammation with cytokine production, and decidual bleeding/abruption with thrombin production, respectively, increase the risk of pPROM but the actual mechanism(s) of pPROM remain obscure [2–7].

There are no appropriate animal models for the study of pPROM. Therefore, we developed an *in-vitro* model system to facilitate study of the mechanisms of human FM weakening and rupture. First we designed and built FM strength testing equipment and developed methodology that allowed us to systematically measure and map human FM biomechanical properties over the entire FM surface and to correlate these with local biochemical properties [8]. Using this technology, in conjunction with an adaptation of the FM explant culture system reported by Menon et al., we then developed an *in-vitro* model system to study the process of human FM weakening [8–10]. This model system is novel and unique in its ability to measure FM weakening, the major parameter of clinical interest, as well as concomitant and presumably causative biochemical changes within the FM.

Inflammation/infection and decidual bleeding/abruption with thrombin production are both highly associated with pPROM. Using our *in-vitro* model system we have previously shown that cytokines (TNF and IL1-beta) as well as thrombin, cause weakening of full thickness FM [4,9,11,12]. In parallel with weakening, these agents cause biochemical and histological changes in the tissue that mimic

* Corresponding author. CWRU, MetroHealth Medical Center, 2500 MetroHealth Drive, Cleveland, OH 44109, USA. Tel.: +1 216 778 5946; fax: +1 216 778 3252.
E-mail addresses: dkumar@metrohealth.org, dxk91@case.edu (D. Kumar).

http://dx.doi.org/10.1016/j.placenta.2014.10.001
0143-4004/

those seen in the physiological weak zone (FM rupture initiation site) described by ourselves and others in the FM region overlying the cervix [9,11–18].

Initial investigation using our model system suggested differences in the manner that agents associated with inflammation (TNF/IL1) and the agents associated with bleeding (thrombin) induced FM weakening [4]. At issue was the process by which the amnion, the strongest component of the FM, weakened. Thrombin was shown to be capable of directly weakening the isolated amnion (stripped free of choriodecidua) and was thus hypothesized to initially and primarily act there. The cytokines TNF and IL1 could only weaken amnion in the presence of choriodecidua (CD). TNF and IL1 were shown to induce as yet unidentified soluble factors from the CD that secondarily weakened amnion [4].

The mechanism of thrombin induced weakening of isolated amnion was further investigated. We found that although some of the weakening effect on isolated amnion caused by thrombin might be mediated through the cells of the amnion, potent amnion weakening occurred with no viable cells present [19,20]. Thrombin can weaken amnion ECM preparations in which all the cells have been stripped off or killed. Thus thrombin's direct weakening of isolated amnion does not require any viable cell-mediated processes. In experiments done to explain this phenomenon we demonstrated that thrombin can activate the large pool of pro-MMP2 in cell free amnion ECM to the active MMP2 form. Active MMP2 (and possibly other MMPs activated by thrombin) could clearly degrade/weaken the amnion. Thrombin, however, was found capable of directly weakening the cell-free amnion ECM even in the presence of MMP inhibitors. Thus activation of MMPs is not necessary for thrombin-induced amnion weakening. Ultimately, we found that thrombin itself can directly degrade the amnion ECM [19].

In spite of these findings, however, the conundrum remained that direct action of thrombin on the amnion matrix, on pro-MMP-2 in the amnion ECM, or even on amnion cells, requires thrombin to traverse the chorion in significant concentration from bleeding points in the decidua. This may happen with extensive hemorrhage, but with smaller bleeds, thought to be associated with pPROM, it probably does not. Thus we have hypothesized that thrombin's initial target of action in FM, like TNF's, is probably within the CD rather than directly on the amnion.

Accordingly, in order to more accurately mimic the directionality and the initial point of contact of both TNF and thrombin on the CD (rather than both sides of the FM), we redesigned our model system. Intact FM, mounted on modified Transwell (Costar, Corning, NY) culture inserts are exposed to TNF or thrombin only on the CD (maternal facing) side. After incubation, medium can be sampled separately from the CD facing (maternal side) compartment or from the amnion facing (fetal side) compartment. The FM can then be strength tested within the insert following which the tissue is available for biochemical and IHC analyses. Using this new upgraded model system we demonstrated that either TNF or thrombin applied to only the CD side causes FM weakening in the same manner as when both sides were exposed. Thrombin was not detected on the fetal side of the FM confirming our suspicion that it acts initially on the CD, not the amnion. We also identified a single cytokine, GM-CSF, produced by CD in large amounts in parallel with both TNF and thrombin induced FM weakening. Blockade of this induced GM-CSF prevented FM weakening by both TNF and thrombin. Finally, GM-CSF alone caused dose dependent FM weakening.

2. Methods

2.1. Fetal membrane collection and preparation

Full thickness fetal membrane (FM) fragments from term uncomplicated repeat Cesarean section placentas taken from FM areas distal to the paracervical weak zone region were rinsed in phosphate buffered saline (PBS, pH 7.2) and mounted, CD side down, in 24 mm Transwell (Costar, Corning, NY) inserts, secured with o-rings and placed in six well culture plates. These were incubated with 2 ml MEM+ [Minimum Essential Medium with Earle's salts, 1 mM L-glutamine and 2.24 g/L sodium bicarbonate (pH 7.4)], (Mediatech, Manassas, VA) containing 100:1 Antibiotic-Antimycotic Solution (Sigma Chemical, St. Louis, MO) added to the upper and lower chambers. After 24 h culture at 37 °C and 5% CO_2 and 100% relative humidity, medium was replaced with or without addition of thrombin or TNF added to the CD compartment ($N = 9$). Medium from each compartment was sampled after 48 h. Samples were immediately centrifuged for 15 min at 12,000 g and 10 °C and supernatants were removed and stored at −70 °C until analysis. This study was approved by the MetroHealth Medical Center's Institutional Review Board.

2.2. GM-CSF and GM-CSF neutralizing antibody

HumanKine™ Granulocyte Macrophage-Colony Stimulating Factor (human recombinant, expressed in HEK 293 cells; ED_{50} 0.4–2 ng/ml; purity ≥ 95%), obtained from Sigma–Aldrich, St Louis, MO, was used for the GM-CSF dose response study. HumanKine™ Granulocyte Macrophage-Colony Stimulating Factor is expressed as a 15–36 kD glycosylated monomer. GM-CSF neutralizing antibody AF-215-NA was obtained from R&D Systems, Minneapolis, MN.

2.3. Enhancement of the model system

The amnion provides the major strength component for FM and must be weakened if rupture is to occur. It has become apparent from our studies that the signal which initiates weakening of the FM due to either cytokines or thrombin moves directionally from the CD to the amnion. Cytokines do not weaken the isolated amnion but stimulate the CD to release soluble agents which secondarily weaken the amnion [4]. Thrombin is released from blood vessels in the maternal decidua and then either traverses the chorion to reach the amnion directly, or more likely also stimulates the CD to release agents that affect the amnion. In either case the pathway is directional from the maternal side of the FM (decidua) through the chorion to the maternal facing side of the amnion.

In the original version of our model system the tissue put in culture was bathed on all sides with medium containing the agents of interest. Thus thrombin, TNF, lipoic acid and other agents of interest reach both sides of the amnion tissue when an isolated amnion fragment is studied and only the fetal side when an intact FM fragment is studied [4,9,11,12].

Because of the clear directionality of the signals *in vivo*, we modified our culture system to be more authentic. We have developed a modified Transwell incubation system which allows us to apply biochemical agents of interest to a specific sublayer surface of the FM and follow the effect directionally through the tissue. Transwell culture systems were developed to allow a shelf of cells to grow on a porous synthetic substrate suspended above cells growing at the bottom of the plate. Thus two compartments are formed (above and below the suspended platform) and the products of the lower and upper cells can interact in a directional manner. Several groups have removed the substrate of the Transwell and attached a piece of either intact FM or a FM component (amnion or CD) in its place with a rubber ring. Agents can then be placed on a single side of the suspended tissue and products produced also assessed in a directional manner [21–24]. We have further modified this so that the Transwell with the tissue attached can be strength tested after incubations without any manipulation (Fig. 1A and B).

2.4. Strength testing of Transwell cultured FM (Fig. 1A and B)

After incubations, FM fragments were strength-tested within the Transwell insert using our published methodology and rupture testing equipment [8,13]. Briefly, post incubations, Transwell mounted FM were secured in a 2.5 cm diameter fixture in between the aligned horizontal plates of the rupture testing equipment. A motor-driven 1 cm diameter spherical-head plunger was forced into the fixed membrane, perpendicular to the Transwell mounted membrane surface. Displacement of the membrane and the resultant force were collected continuously. From these data, rupture forces and maximum displacements could be determined and used to generate force/displacement curves. From these curves, physical properties including rupture strength could be quantified.

2.5. Proteomic array analysis

Clarified thawed aliquots (1 ml) of medium from CD facing or amnion facing compartments of Control and thrombin or TNF treated cultures were subjected to Proteome Profiler Human Cytokine (36 cytokines/chemokines) or Protease (35 proteases) Array analysis and processed according to the Manufacturer's protocols (R&D Systems, Minneapolis, MN). Developed x-ray films (Fuji, Tokyo, JPN) were scanned and pixel density analysis determined using Image J Software (NIH, Bethesda, MD). Spot densities were normalized against respective reference array spots then against controls. Cytokine array results for GM-CSF and Plasminogen Activator Inhibitor-1 (PAI-1) were confirmed by Western Blot analysis.