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In an *in-vitro* model using human fetal membranes, α-lipoic acid inhibits inflammation induced fetal membrane weakening

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ABSTRACT

Introduction: We established an *in-vitro* model for the study of human fetal membrane (FM) weakening leading to pPROM. In this model, granulocyte-macrophage colony-stimulating factor (GM-CSF) is a critical intermediate for both tumor necrosis factor-α (TNF; modeling infection/inflammation) and thrombin (modeling decidual bleeding/abruption)-induced weakening. Thus, inhibitors of FM weakening can be categorized as targeting GM-CSF production, GM-CSF downstream action, or both. Most progestogens inhibit both, except 17-α hydroxyprogesterone caproate which inhibits FM weakening at only one point, GM-CSF production. α-lipoic acid (LA), an over-the-counter dietary supplement, has also been previously shown to inhibit TNF and thrombin induced FM weakening.

Objective: To determine the point of action of LA inhibition of FM weakening.

Methods: FM fragments were mounted in Transwell inserts and preincubated with/without LA/24 h, then with/without addition of TNF, thrombin or GM-CSF. After 48 h, medium was assayed for GM-CSF, and FM fragments were rupture-strength tested.

Results: TNF and thrombin both weakened FM and increased GM-CSF levels. GM-CSF also weakened FM. LA inhibited both TNF and thrombin induced FM weakening and concomitantly inhibited the increase in GM-CSF in a concentration-dependent manner. In addition, LA inhibited GM-CSF induced FM weakening in a concentration dependent manner.

Conclusions: LA blocks TNF and thrombin induced FM weakening at two points, inhibiting both GM-CSF production and downstream action. Thus, we speculate that LA may be a potential standalone therapeutic agent, or supplement to current therapy for prevention of pPROM related spontaneous preterm birth, if preclinical studies to examine feasibility and safety during pregnancy are successfully accomplished.

1. Introduction

Preterm premature rupture of the fetal membranes (pPROM), a cause of about one-third of preventable spontaneous preterm births, is frequently associated with high levels of inflammatory cytokines [Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), tumor necrosis factor-α (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and also the generation of thrombin in amniotic fluid and fetal membranes [1–6]. In our *in-vitro* model of human fetal membrane (FM) weakening, both TNF (modeling infection/inflammation) and thrombin (modeling decidual bleeding/abruption) individually cause weakening of full thickness FM with concomitant biochemical and histological tissue changes similar to those reported in the supra-cervical FM zone of high morphological change or the physiological weak zone where the spontaneous FM rupture initiates [7–15]. There are no animal models to study *in-vivo* FM weakening. In our *in-vitro* model, described in detail in our recent four reports, GM-CSF has been identified as a critical intermediate in both the TNF and thrombin induced FM weakening pathways [9,16–18]. The following criteria demonstrate that GM-CSF is both necessary and sufficient for FM weakening: (1) GM-CSF is induced concomitantly in the choriodecidua with both TNF and thrombin induced FM weakening [16]; (2) Both TNF and thrombin induced membrane weakening is inhibited by preincubation with neutralizing antibody to GM-CSF [16]; (3) GM-CSF on its own also causes FM weakening [16]. Studies by others also support the importance of GM-CSF in clinically relevant inflammation

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and bleeding associated pPROM. In clinical specimens, membranes from pregnancies with chorioamnionitis stain more densely for GM-CSF than those from uncomplicated pregnancies [19]. FM from PROM at term before labor compared with term membranes after labor demonstrate greater GM-CSF chemotactic activity and granulocytic attraction [6].

FM weakening, a precondition for pPROM requires first, the generation of GM-CSF, and second, downstream weakening due to GM-CSF action. Similarly, inhibitors of the FM weakening process may prevent GM-CSF generation, inhibit downstream GM-CSF action, or act on both steps. This characterization facilitates understanding of the mechanisms by which specific inhibitors of FM weakening may work to prevent pPROM. Applying this strategy with our *in-vitro* model, we have recently demonstrated that three progestogens - Progesterone, 17-α hydroxyprogesterone (17-OHP; natural analog – not caproate) and medroxyprogesterone acetate (MPA) inhibit TNF and separately thrombin induced FM weakening by acting at two points, at the level of GM-CSF production and at the level of downstream GM-CSF action [17]. In contrast, the clinically used progestogen 17-OHPC inhibits the weakening pathway at only one point, GM-CSF production [18]. The limited proven efficacy of current clinical therapies to prevent pPROM or preterm labor and the continued high preterm birth rate in the US clearly support a search for additional therapeutic agents. Another approach may be to consider a supplement to the current therapy.

Previous *in-vitro* laboratory studies and studies in non-pregnant humans suggest that α-lipoic acid (lipoic acid) may be such a potential supplement. Lipoic acid is a natural dietary supplement that displays metal chelation, anti-oxidant and NFkB inhibitory properties and is commonly used for prophylaxis against diabetic neuropathy [20,21]. It is being trialed as a therapeutic agent for a variety of disorders (clinicaltrials.gov) [22]. Toxicity studies in rodent models have also been promising [23–27]. We have previously shown that in our *in-vitro* model system, lipoic acid can block both cytokine and thrombin induced FM weakening, and concomitant collagen remodeling as evidenced by inhibition of MMP9, MMP3 and PGE2 [28,29]. We hypothesized that lipoic acid inhibits TNF and thrombin induced FM weakening by inhibiting both GM-CSF and its downstream action. The purpose of this study was to further characterize the mechanism by which lipoic acid inhibits TNF and thrombin induced FM weakening in our model.

2. Materials and methods

2.1. Model system for study of human fetal membrane (FM) weakening

We have described our model system in detail in four recent reports [9,16–18]. Briefly, intact FM fragments were mounted in modified Transwell (Costar, Corning) inserts with amnion (fetal side) facing up and the choriodecidua (maternal side) facing down forming two separate compartments. Tissues were preincubated with/without lipoic acid for 24 h and then with/without addition of TNF, thrombin or GM-CSF. After 48 h, medium was assayed for GM-CSF in the choriodecidua compartment, and FM fragments were rupture-strength tested within the Transwell without any manipulation.

2.2. FM collection and preparation

Full thickness FM fragments from term uncomplicated repeat cesarean deliveries were collected after patient consent and approval by MetroHealth Medical Center's Institutional Review Board (# IRB10-00861; approval date 09/18/2010). None of the patients had a previous history of spontaneous preterm birth. FM tissue was obtained from areas remote from the weak zone region, washed in 2 × 250-ml changes of phosphate-buffered saline (pH 7.2–7.4), and mounted, choriodecidua side down, in 24 mm Transwell inserts secured with an O-ring to separate the choriodecidua and amnion chambers. The inserts were placed in six-well culture plates and 2 ml MEM+ [Minimum Essential Medium alpha with Earle's salts, supplemented with 1 mM L-glutamine, 2.24 g/L sodium bicarbonate (Mediatech, Manassas, VA), 10 ml/L Antibiotic-Antimycotic (Sigma Chemical Co., St. Louis, MO) and 50 mg/L gentamicin sulfate] was added to the upper (amnion) and the lower (choriodecidua) compartment [9,16–18]. Lipoic acid (at various concentrations) or vehicle (0.1% ethanol final concentration) was added to the choriodecidua compartment 24 h prior to the addition of weakening agents. Weakening agents [TNF (50 ng/ml), thrombin (10 U/ml), GM-CSF (200 ng/ml)] were then added to the choriodecidua compartment and cultures were incubated at 37 °C in 5% CO_2 and 100% relative humidity for an additional 48 h. After culture, medium from each compartment was collected, centrifuged for 15 min at 12,000 × g/10 °C and the supernatant was stored at −70 °C.

Concentrations of TNF, thrombin, GM-CSF and lipoic acid used in these experiments were determined from dose response studies in our previous publications [16,17,28,29]. Each of these were added to the choriodecidua side only, as our previous studies have shown that the choriodecidua (rather than the amnion) is the initial target tissue or the tissue of origin of each [16,17,30,31]. Lipoic acid in concentrations of 10^{-5} to 10^{-3} M, was added to the choriodecidua chamber to ensure that it reached the target tissue concomitant with the weakening agents.

2.3. Strength testing of FM

As reported previously, the FM fragments were strength-tested within the Transwell inserts using our rupture testing apparatus [9–12,16–18]. Briefly, Transwell-mounted FM were secured in a 2.5 cm diameter fixture between the aligned horizontal plates of the rupture testing equipment. A motor-driven 1 cm diameter spherical-head plunger aligned perpendicular to the FM was then forced against the amnion side. FM displacement and concomitant plunger force were recorded continuously until rupture. From these data, force (rupture strength in newtons) and maximum displacement (cm) needed to cause FM rupture were determined.

2.4. Granulocyte-macrophage colony-stimulating factor (GM-CSF) determination

GM-CSF levels in thawed supernatant from the fluid compartment adjacent to the choriodecidua of the FM were determined using the Human GM-CSF Quantikine ELISA Kit following the Manufacturer's protocol (R&D Systems, Minneapolis, MN). Intra- and inter-assay precision of the assay was 2.7% and 5.3 CV% respectively for cell culture supernatants at a sensitivity level of 3 pg/ml (*Escherichia coli* expressed), recombinant human GM-CSF as standard.

2.5. Materials

Recombinant human TNF and GM-CSF (both produced in HEK 293 cells), LA, and other miscellaneous reagents, unless otherwise stated, were from Sigma-Aldrich, St Louis, MO. Thrombin (from bovine plasma, 1500 NIH U/mg protein) was from Thermo Fisher Scientific, Pittsburgh, PA.

2.6. Statistical analysis

All experiments were performed at least in triplicate. Data were analyzed by ANOVA followed by post-hoc pair-wise comparisons (Holm-Sidak method) using Sigmaplot (Systat Software, Inc, Chicago, IL). Differences were considered significant when $p < 0.05$.