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All-trans retinoic acid promotes wound healing of primary amniocytes through the induction of LOXL4, a member of the lysyl oxidase family

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ABSTRACT

Thirty percent of preterm births directly result from preterm premature rupture of fetal membranes (PPROM). Clinical management currently proposes using a collagen plug to mechanically stop loss of amniotic fluid. Vitamin A and its active metabolite (retinoic acid) have well-known pro-healing properties and could thus make good candidates as a proposable adjuvant to this mechanical approach. Here we investigate the molecular mechanisms involved in the pro-healing properties of all-trans retinoic acid (atRA) in fetal membranes via an approach using an in vitro primary amniocyte wound model and transcriptomics. The results demonstrate that atRA promotes migration in primary amniocytes, improving wound healing in vitro by up to 90%. This effect is mediated by the induction of LOXL4, which plays a crucial role in the dynamics of the extracellular matrix by regulating collagen reticulation. This new insight into how atRA exerts its pro-healing properties prompts us to propose using atRA as a candidate strategy to help prevent future PPROM.

1. Introduction

Human fetal membranes are composed of two distinct layers: the amnion (the internal layer), which lines the amniotic fluid, and the underlying chorion, which lines the maternal decidua (Parry and Strauss, 1998). Both are essential structures that specifically maintain healthy pregnancy through their numerous functions, including regulation of amniotic fluid homeostasis (Prat et al., 2012), paracrine actions (Denison et al., 1998; Riley et al., 1999), protection against pathogens, and mechanical resistance to internal and external pressure. Physiologically, at term, the rupture of the membranes occurs during labor via a step-by-step mechanism ending with fracture of the amnion, which is generally identified as the stronger layer (Méhats et al., 2011; Bryant-Greenwood, 1998; El Khwad et al., 2005; Moore et al., 2006). This phenomenon results from two main processes: a dramatic increase in internal pressures due to uterine contractions and fetal movements, and a weakening of fetal membrane composition and strength that enables the rupture. This programmed weakening process is mainly due to modifications in extracellular matrix (ECM) dynamics (Strauss, 2013). Indeed, the balance between ECM production and enzymatic degradation is altered at the end of pregnancy, resulting in a degradation of the ECM formed essentially by a strong collagen network. Human fetal membranes approaching full term show a characteristic increase in MMP8 and MMP 9 and decrease in TIMP1 and TIMP3 (Vadillo-Ortega et al., 1995; Arechavaleta-Velasco et al., 2004; Kumar et al., 2006).

The most common pathology involving fetal membranes is premature rupture (PROM), defined as a rupture taking place before the onset of labor. PROM can occur at term (called TPROM), with no risk to fetus, or preterm (called PPROM) when it occurs before 37 weeks of gestation. PPROM is responsible for 1/3 of preterm births and can lead to perinatal mortality in the most severe cases (Simhan and Canavan, 2005; Moutquin, 2003). Several factors have been associated with chronic PPROM (Hadley et al., 1990; Caughey et al., 2008), including choriodecidua infection (Bendon et al., 1999), pollutant exposure (Dadvand et al., 2013) and smoking (England et al., 2013). Besides these environmental causes, surgical procedures that challenge membrane integrity, such as amniocentesis and fetoscopy, are also reported as major risk factors for PPROM (Papanna et al., 2010a), potentially causing iatrogenic

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PPROM (iPPROM). Clinical management options for PPROM remain limited, mainly due to the fact that fetal membranes are very slow to heal (Gratacós et al., 2006; Devlieger et al., 2006). Current preventive treatment therefore revolves around prolonging pregnancy by preventing infection (Blanchon et al., 2013). Nevertheless, several therapeutic strategies to delay rupture have been studied, the most widely tested to date being attempting to seal iatrogenic wounds via a 'mechanical' approach using a collagen plug. However, despite promising results in animal models (Gratacós et al., 2000), this mechanical technique shows little efficacy in humans (Engels et al., 2014). Several studies have been led to improve plug-use by optimizing its healing capacities, mainly by focusing on its ability to promote amnion re-epithelialization, with various degrees of success. A long list of adjuvants to this inert matrix plug have already been tested, ranging from fibrinogen, plasma, platelets and amniotic fluid to mussel-mimetic glue, and also simply primary amniotic cells (Mallik et al., 2007; Engels et al., 2013; Liekens et al., 2008; Papadopulos et al., 2010; Kivelio et al., 2013).

Given the various complementation strategies already tested, vitamin A and its active metabolite (retinoic acid), which are already well described and used in clinical practice to promote wound healing (Thielitz and Gollnick, 2008; Johansen et al., 1998), could be promising candidates as adjuvants to help improve the efficacy of collagen plugs. Furthermore, their effects on wound healing (Abdelmalek and Spencer, 2006; Lee and Tong, 1970; Hattori et al., 2012) and ECM dynamics (Fisher et al., 1996; Barber et al., 2014) have been well documented, mainly in skin, eyes and developing organs (lung and heart). In fetal membranes, the presence and functionality of the retinoid pathway has already been demonstrated (Marceau et al., 2006) but its effect on gene regulation remains poorly studied (Blanchon et al., 2011). However, *all-trans* retinoic acid (atRA), the active metabolite of vitamin A, has been shown to regulate the expression of genes involved in both amniotic fluid homeostasis (Prat et al., 2015) and, more interestingly, the ECM dynamics of the membranes (Borel et al., 2010).

As part of this study to further investigate the global effects of atRA on human fetal membranes, we generated a large-scale transcriptome dataset and identified a list of genes regulated by the retinoid pathway in fetal membranes, more specifically in the amnion and primary amniocytes. These findings highlight the potential importance of members of the lysyl oxidase (LOX) family, which are key enzymes of ECM dynamics but also permit the oxidation of amine chemical functions localized on collagen lysines and elastin precursors, thus allowing the fibers to crosslink (Kagan et al., 1995; Smith-Mungo and Kagan, 1998). Lysyl oxidases thus emerge as the main actors involved in collagen reticulation and maintenance of the tensile strength of collagen fibrils in several tissues (Molnar et al., 2003; Mäki, 2009). The LOX family is composed of five members that share the same catalytic activity (C-terminal) but different N-terminal domains that confer new functions to the lysyl oxidase. Three of these LOX (LOX, LOXL1 and LOXL2) are already known to be expressed in placenta, maternal decidua and human fetal membranes (Hein et al., 2001). Note too that LOX activity has been shown to decrease in the amnion throughout pregnancy but more strongly at late gestational age, thus pointing to the potential importance of LOXs for ECM integrity in fetal membrane tissue (Casey and MacDonald, 1997).

Our atRA transcriptomics results were used here to investigate the wound healing properties of vitamin A by *in-vitro* scratch assay on primary amniocytes. We demonstrated ECM dynamics of one of the most important of all the various pathways regulated by atRA. Finally, and for the first time, we provide evidence of a direct link between a retinoids-induced LOX gene and the pro-healing properties of atRA on amniotic cells.

2. Materials and methods

2.1. Chemicals

All-trans retinoic acid (atRA), dimethyl sulfoxide (DMSO), trypsin, β-amino propionitrile (βAPN) and cycloheximide (CHX) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Cell culture media were obtained from Gibco® Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) were purchased from GE Healthcare (Buckinghamshire, UK). Collagen I was obtained from Stemcell (Grenoble, France). The Superscript III First-Strand-Synthesis System for RT-PCR, taq polymerase and lipofectamine 3000 reagent were obtained from Life Technologies.

2.2. Sample collection

Full-term fetal membranes were collected from healthy pregnancies after caesarean births (University Hospital–Estaing, Clermont-Ferrand, France) with informed consent from the mother. Membrane samples were always collected a certain distance from the placental border and in the whole zone of intact morphology (ZIM). The research protocol was approved by the hospital center's IRB. Amnion was dissociated from the chorion and used for both tissue explants and culture of primary amniocytes.

2.3. Cell and tissue culture

Cells and tissues were cultivated under standard conditions (5% CO_2, 95% humidified air, 37 °C). Human amnion-like Wistar Institute Susan Hayflick (WISH) cell line was grown in Modified Eagle's Medium (MEM) supplemented with 10% FBS, 200 nM glutamine, 50 mg/mL streptomycin and 50 IU/mL penicillin. Amnion explants, primary amniocytes and amnion-derived AV-3 cells were cultivated in Dulbecco's MEM F-12 nutrient mixture (DMEM-F12 + GlutaMAX™-I) supplemented with 10% FBS, 50 mg/mL streptomycin and 50 IU/mL penicillin. Amnion explants were prepared by cutting 1 cm^2 squares just before incubation. Primary amniotic epithelial cells were isolated as follows. After repeat washes in PBS, the amnion was cut into squares and incubated in 5 mL of a solution of 0.25% trypsin for 10 min at 37 °C. The trypsin was removed and replaced by 7 mL of fresh trypsin solution for 20 min. Then, the trypsinated cells were collected in complete media and centrifuged for 5 min at 1000 rpm. Finally, the remaining amnion was incubated for 30 min in a third bath of trypsin (7 mL) collected in complete media after scraping the amnion epithelia. This solution was filtered to remove the collagen, then centrifuged for 5 min at 1000 rpm. Cells from the second and third trypsinization steps were pooled and grown on culture dishes coated with collagen I (BioCoat™, Spectralab, Markham, Canada) in complete media.

2.4. Transcriptomics study

The transcriptomics study was performed on primary amniotic epithelial cells, amnion and chorion collected from 12 patients after caesarean births. These samples were treated with atRA or DMSO (as atRA vehicle) for 6, 12, 24 and 48 h, and transcriptomics analysis was conducted only at 24 h. These same samples (6, 12, 24 and 48 h) were also used for RT-qPCR experiments (see Section 2.8). Explants and cells were frozen at −80 °C after treatment. RNA was extracted and quality-controlled using the Agilent RNA 6000 Nano Kit. Samples with a RNA integrity number (RIN) higher than 6 were integrated into the transcriptomics study. cDNA was then obtained using the Superscript III First-Strand-Synthesis System for RT-PCR. Retinoids-induction of membranes was controlled by RARβ RT-