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Cigarette smoke condensate affects the retinoid pathway in human amnion

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ABSTRACT

Introduction: The preterm premature rupture of membranes (PPROM) is a frequent pathology responsible of more than 30% of preterm births. Tobacco smoking is one of the most frequently described risk factors identified and contributes to the pre term weakening of fetal membranes. As previously demonstrated, *all-trans* retinoic acid (atRA) regulates several genes involved in the extracellular matrix dynamics, an essential actor in fetal membrane ruptures. We hypothesized that cigarette smoke may affect this pathway in human amnion.

Methods: Amnion was obtained from full-term fetal membranes collected from non-smoking women after cesarean births and used either as explants or for the isolation of derived epithelial cells. The pro-healing and transcriptomic effects of atRA were studied by a scratch assay experiment and quantitative RT-PCR, respectively, after treatment with dimethyl sulfoxyde (DMSO), atRA, DMSO + cigarette smoke condensate (CSC), or atRA + CSC.

Results: Our results show a strong alteration of the retinoid pathway after CSC treatment on amnion-derived epithelial cells and explants. We first demonstrated that CSC inhibits the activity of the RARE reporter gene in amnion-derived epithelial cells. Then, atRA's effects on both the transcription of its target genes and wound healing were demonstrated to be inhibited or at least decreased by the CSC in human amnion epithelial cells.

Discussion: Here, we demonstrated that CSC altered the retinoid signal, already known to have roles in fetal membrane physiopathology. These results highlight a potential negative action of maternal smoking on the retinoid pathway in human amnion and more generally on pregnancy.

1. Introduction

Despite the well-established dangerous effects of tobacco during pregnancy, maternal smoking still occurs among 10–20% of women in high-income countries [1,2] and is responsible of numerous pathologies affecting all the actors of the *in-utero* life. Cigarette smoke is composed of a complex mixture of more than 4000 compounds [3], most of which pass easily through the placental barrier [4]. Many are thereby present in the amniotic fluid, such as cadmium [5] and cotinine [6], the main metabolite of nicotine. In this way, cigarette smoke compounds can reach the fetus and the embryo's annexes and lead to an increase of 33% of perinatal and neonatal mortality among smokers [7,8]. Moreover, maternal smoking has long been associated with several adverse pregnancy outcomes, such as spontaneous abortion, placental abruption, placenta previa, fetal growth restriction, childhood cancer, or, more recently, with a preterm premature rupture of membranes (PPROM) [9–12].

Fetal membranes are composed of two distinct layers: the internal layer, called the amnion, is in contact with the amniotic fluid, and the underlying chorion is in contact with the maternal decidua [13,14]. They both play a crucial role in human pregnancy, especially by protecting the fetus against mechanical pressure or external pathogens and by regulating the amniotic fluid homeostasis [15–17]. A PPROM, which is defined as their rupture before 37

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weeks of gestation, is thus considered a serious pathology, which can lead to preterm births (1/3 are due to a PPROM) and perinatal mortality in the most severe cases [18–20]. Several factors have been identified to predispose mothers to a PPROM, including choriodecidua infection, pollutant exposure, and smoking [21,22]. In addition to increasing the risk of a PPROM [11,23,24], cigarette smoke seems to have a dose-dependent effect, thus stepping up the risk of developing a PPROM at earlier gestational stages among heavy smokers (considered at more than 10 cigarettes per day) [25].

Physiologically, the rupture of membranes occurs at the end of pregnancy just after the onset of labor. This phenomenon is highly regulated and involves a step-by-step mechanism ending with the amnion fracture [26]. Several elements contribute to this rupture. First, internal pressures greatly increase during labor due to uterine contractions as well as fetal movements. Besides these mechanical factors, fetal membranes undergo drastic histological and biochemical modifications leading to their weakening at the end of pregnancy, thus enabling the rupture [27]. Extracellular matrix (ECM) changes play a major role in this programmed weakening process. Indeed, an imbalance between the ECM production and degradation at the end of pregnancy has been widely demonstrated in the literature and appears as one of the main factor responsible for fetal membranes weakening, allowing the rupture [28,29].

Vitamin A and its active derivatives have been found to play roles in the regulation and dynamic of the ECM in many tissues both during development and adulthood [30–34]. Furthermore, the retinoid pathway has been described to be functional in human fetal membranes [35] and appears as a pivotal modulator of genes involved in physiologic processes, such as amniotic fluid homeostasis [36] and, interestingly, ECM dynamics [37,38]. The disruption of such a pathway in fetal membranes could thus lead to numerous disorders and, in particular, affect their integrity.

As cigarette smoke is a potential disruptor of the retinoid pathway—as already demonstrated in the lungs—we have investigated its impact on the retinoid pathway in human fetal membranes, especially in the amnion, often considered the strongest layer and in direct contact with toxic substances present in the amniotic fluid. The objective of our study was to address the effect of cigarette smoke on the retinoid pathway at molecular and functional levels. We demonstrated that cigarette smoke condensate (CSC) treatment affects the transcriptional response to all-trans retinoic acid, an active derivative of Vitamin A, both in amnion-derived epithelial cells and explants. In addition, we demonstrated that CSC is also able to decrease the effect of atRA on wound healing using a scratch assay strategy.

2. Methods

2.1. Chemicals

atRA and DMSO were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Cell culture media were obtained from Gibco® Life Technologies (Carlsbad, United States). Fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) were purchased from GE healthcare (Buckinghamshire, United Kingdom). Collagen I was obtained from Stemcell (Grenoble, France). Superscript IV First-Strand-Synthesis System for RT-PCR, Taq polymerase and lipofectamine3000 were obtained from Life Technologies. CSC (40 mg/ml in DMSO) was obtained from Murty Pharmaceuticals (Lexington, KY, USA). It was prepared from Kentucky standard cigarettes 3R4F (University of Kentucky, KY), which were smoked using a Phipps-Bird 20-channel smoking machine designed for Federal Trade Commission testing. The particulate matter was then trapped onto filters and dissolved in DMSO.

2.2. Sample collection

Full-term fetal membranes were collected from non-smoking women with healthy pregnancies prior to labor from scheduled cesarean deliveries (Centre Hospitalier Universitaire Estaing, Clermont-Ferrand, France) and informed consent of the mothers. The research protocol was approved by the institutional regional ethics committee. Amnion was dissociated from the chorion and used, on the one hand, as tissue explants, and on the other hand, for the culture of primary amnion-derived epithelial cells.

2.3. Cell and tissue culture

Amnion explants and primary amnion-derived epithelial cells were cultivated under standard conditions (5% CO2, 95% humidified air, 37 °C) in Dulbecco's Modified Eagle Medium F-12 nutrient mixture (DMEM-F12 + GlutaMAX™-I) supplemented with 10% FBS, 50 mg/ml of streptomycin and 50 IU/ml of penicillin.

As previously validated [17], amnion explants were prepared by cutting squares of 1 cm^2 after three washes in PBS. The isolation of primary amniotic epithelial cells was conducted in three trypsinization steps (10, 20, and 30 min) followed by the scraping of the amnion. Cells were filtered to remove the collagen, centrifuged for 5 min at 1000 rpm and grown on culture dishes coated with collagen I (BioCoat™, Spectralab, Markham, Canada) in complete media. The absence of stromal cells was checked using vimentin immuno-labeling (data not shown).

The absence of CSC cytotoxicity (100 and 250 μg/ml) was checked using the measurement of lactate-dehydrogenase (LDH) concentrations in the media (data not shown).

2.4. CAT assay

After one passage, amnion-derived epithelial cells were grown at 70% confluence in 6-well plates and transfected with 1 μg of a plasmid expressing the chloramphenicol acetyltransferase (CAT) reporter gene under control of three retinoic acid responsive elements (RARE-DR5 type). Retinoic acid receptor expression plasmids (0.5 μg of human RARα and RXRα) were co-transfected to enhance the RAREs' activation ability, as well as 0.05 μg of pCH110 β-galactosidase expression vector, which allowed the normalization to the transfection efficiency. These plasmids were generously gift by Chambon's laboratory (IGBMC, Illkirch-Grafenstaden, France). Transfections were performed using 3.75 μl of Lipofectamine 3000 and 5 μl of Regeant 3000 per well. Cells were treated either with DMSO, atRA (1 μM), DMSO + CSC (100 μg/ml) or atRA + CSC 6 h after the transfection during 48 h (treatment was repeated after 24 h). Then, the expression of CAT and β-galactosidase were measured in the cellular extracts using the colorimetric immunoassay from Roche (Lyon, France) and a β-galactosidase assay kit (Agilent, Santa Clara, United States) respectively, according to the manufacturer's protocol. CAT inductions were calculated relative to DMSO or DMSO + CSC defined as 1 for atRA or atRA + CSC treatments, respectively. This experiment was repeated three times (each condition in duplicate).

2.5. RT-PCR and quantitative RT-PCR

Human amnion and primary amnion-derived epithelial cells were treated with either DMSO (vehicle of atRA) or atRA (1 μM) or with DMSO or atRA (1 μM) in combination with CSC (100 μg/ml for amnion-derived epithelial cells, 250 μg/ml for amnion explants). Total RNA was extracted from human amnion and epithelial cells using an RNeasy Lipid Tissue Mini Kit and an RNeasy Mini Kit, respectively (QIAGEN, Courtaboeuf, France). The reverse