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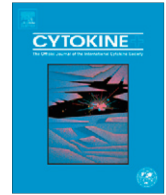

Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions

David Barrera [1], Nancy Noyola-Martínez [1], Euclides Avila, Ali Halhali, Fernando Larrea, Lorenza Díaz *

Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga, No. 15, Tlalpan, 14000 México D.F., Mexico

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ABSTRACT

Preeclampsia is associated with systemic inflammation and increased expression of placental Th1-cytokines. IL-10 and calcitriol inhibit proinflammatory cytokines expression in human placenta helping to fetal allograft toleration. Regulation of placental IL-10 by calcitriol and Th-1 cytokines has not yet been fully elucidated. Since it is believed that calcitriol promotes a shift from a Th1- to a Th2 profile, we hypothesized that it would stimulate IL-10 in a normal and an inflammatory scenario to conjointly restrain inflammation. Therefore, we investigated calcitriol effects upon IL-10 expression in cultured human trophoblasts obtained from normal (NT) and preeclamptic (PE) pregnancies. Similar studies in the presence of TNF-α (as an inflammatory stressor) were also performed. Calcitriol dose-dependently inhibited IL-10 expression in NT, PE and TNF-α-challenged trophoblasts ($P < 0.05$). This effect was prevented by a vitamin D receptor (VDR) antagonist. IL-10 expression was significantly stimulated by TNF-α and IL-1β, inhibited by IFN-γ and was not affected by IL-6. Finally, calcitriol inhibited TNF-α and IL-1β stimulation upon IL-10. In summary, in cultured human trophoblasts, calcitriol down-regulates IL-10 expression under normal as well as under natural and experimental inflammatory conditions. This effect is mediated by the VDR and might involve direct inhibition of TNF-α. In view of these and previous results it seems that in placenta calcitriol suppresses both Th1- and Th2 cytokines while undertakes the anti-inflammatory effects of IL-10 by itself, since both factors exert this task redundantly. The regulation of IL-10 by IFN-γ suggests that this cytokine could be a viable candidate to explain low IL-10 levels in preeclampsia.

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1. Introduction

Inflammation is central for reproductive success, since ovulation, menstruation, implantation, and parturition include some features of this complex biological process. However, exacerbated inflammatory responses may lead to pregnancy complications such as spontaneous abortion, preterm labor, preterm rupture of membranes and preeclampsia (PE) [1]. In normal pregnancy, tumor necrosis factor-α (TNF-α) modulates growth and invasion of trophoblasts into maternal spiral arteries. Nevertheless, when produced in excess, TNF-α acts as a master proinflammatory cytokine, playing a dominant role in the initiation and perpetuation of chronic inflammatory diseases such as PE [2]. Previous studies from our laboratory demonstrated that calcitriol is able to revert TNF-α-dependent induction of inflammatory cytokines in cultured human trophoblasts [3]. These studies showed calcitriol as a pregnancy-supporting factor, particularly during early fetal allograft acceptation and pathological inflammatory conditions. However; in human placenta, interleukin (IL)-10 regulation by calcitriol under normal and inflammatory settings has not yet been studied. The production of calcitriol results from 1α-hydroxylation of 25-hydroxyvitamin D_3 (25-OHD_3), which depends on the expression and activity of the cytochrome P450 25-OHD_3-1-α-hydroxylase (CYP27B1). Conversely, calcitriol levels are down-regulated by the calcitriol-degrading enzyme 25-hydroxyvitamin D_3-24-hydroxylase (CYP24A1). During pregnancy, vitamin D metabolism is boosted by increased renal and placental CYP27B1 expression, as well as placental-specific methylation of the CYP24A1 gene, which altogether result in increased production and decreased catabolism of calcitriol [4,5]. Adequate vitamin D intake and/or enough sun exposure are essential for maternal and fetal health, since pregnant women with sub-optimal vitamin D levels are at risk of developing PE, gestational diabetes mellitus, and bacterial vaginosis, among other pathologies affecting both the mother and the fetus [6–8]. Calcitriol stimulates innate immunity by

Abbreviations: NT, normal trophoblasts; PE, preeclampsia; VDR, vitamin D receptor; CYP27B1, 25-OHD_3-1-α-hydroxylase; CYP24A1, 25-hydroxyvitamin D_3-24-hydroxylase; 25-OHD_3, 25-hydroxyvitamin D_3; AMPs, antimicrobial peptides; TBP, TATA-binding protein.

* Corresponding author. Tel.: +52 5 555 73 11 60; fax: +52 5 556 55 98 59.
E-mail address: lorenzadiaz@gmail.com (L. Díaz).

[1] These authors contributed equally to this work.

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enhancing bacterial killing while helping to prevent exacerbated inflammation by regulating adaptive immunity. Therefore, calcitriol exhibits two key immunomodulatory properties with potential beneficial effects for successful maintenance of pregnancy: its ability to shift T cell responses from Th1 to Th2, and its capacity to orchestrate antimicrobial defenses [9,10]. During the past few years considerable research has been conducted in order to investigate the role of locally generated calcitriol as a modulator of fetal–placental development and function. In this regard, we have previously shown that calcitriol regulates the secretion of various placental hormones and inhibits proinflammatory cytokines such as interferon-γ(IFN-γ), IL-6 and TNF-α [3,11,12]. Yet, studies regarding calcitriol regulation of IL-10 are still lacking. Human placenta expresses a wide spectrum of cytokines, and specifically, trophoblasts produce IL-10 in a gestational age-dependent manner [13]. IL-10 was first recognized for its ability to inhibit activation of lymphocytes, monocytes and macrophages; but its main function is to restrain inflammatory responses, in a similar manner as calcitriol acts upon adaptive immunity. In the context of pregnancy, the role of IL-10 appears to be as a suppressor of active maternal immunity to allow immune-tolerance of the fetal allograft [14]. Nevertheless, previous findings regarding IL-10 interventions *in vivo* and *in vitro* suggest that this cytokine can be deleterious to situations in which a strong immune response is required, for example, microbial infections [15–17]. Indeed, IL-10 inhibits several macrophage functions, including microbicidal properties and presentation of antigens to Th1 cells. Moreover, IL-10 downregulates the expression of important antimicrobial peptides (AMPs) [18,19]. Conversely, calcitriol up-regulation of AMPs gene expression has been demonstrated in different cell types including trophoblasts [9,20].

Since both IL-10 and calcitriol redundantly suppress active maternal immunity while differentially regulate the innate immune response, we were interested in knowing how calcitriol modulates placental IL-10 in normal trophoblasts (NT) and under natural and experimentally-simulated inflammatory conditions. In addition and considering that TNF-α and IL-10 are two mutually regulated cytokines with opposite roles on systemic inflammatory responses, we also investigated the effects of TNF-α and other inflammatory cytokines upon placental IL-10 synthesis.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS) and Trizol were from Invitrogen (CA, USA). TaqMan Master reaction, TaqMan probes, capillaries and the reverse transcription (RT) system were from Roche (Roche Applied Science, IN, USA), calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd. (Basel, Switzerland). Enzymes used for cell cultures and recombinant cytokines were from Sigma–Aldrich (MO, USA). The concentration of IL-10 in the culture media was measured by ELISA (Duo Set ELISA; R&D systems, MN, USA). All oligonucleotides for real time polymerase chain reaction (PCR) were synthesized by Invitrogen. The vitamin D antagonist (23S)-25-dehydro-1-hydroxyvitamin D_3-26,23-lactone (TEI-9647) was kindly donated from Teijin Pharma Ltd. (Tokyo, Japan).

2.2. Placenta donors

This study was approved by the Institutional Human Ethical Committee. Placentas were acquired after obtaining written informed consent from each donor. Subjects were considered to be PE when their blood pressure was found to be at least 140/90 mm Hg in two different time intervals of 6 h apart. In addition, hypertension should have been associated with urinary excretion of >300 mg protein per 24 h. Patients with chronic hypertension (before pregnancy), diabetes mellitus, cervico-vaginal infections, renal, and other systemic illnesses were excluded from the study. All placentas in this study were obtained by cesarean section.

2.3. Trophoblast cell culture and experimental conditions

Trophoblasts were cultured as previously described [21]. Briefly: Villous tissue was enzymatically dispersed and cells were separated on density percoll gradients. An additional purification step after percoll-separation was performed, which consists in allowing cells to attach to the surface of the flasks for 18 h before aspirating the cell culture media in order to remove all non-adherent cells (which include T-lymphocytes and granulocytes). However, other contaminating cells such as macrophages and fibroblasts may remain in our cell preparation. Therefore, our cell cultures are further referred as enriched trophoblastic cell preparations. As determined by Tscherning-Casper et al. [22], by using this method at least 85% of cells correspond to trophoblasts. Before plating, the viability of percoll-enriched cells was estimated by dye exclusion (0.4% trypan-blue). Cells were plated at a density of 3×10^6 cells per 25 cm^2 flasks with 4 mL each of supplemented medium (DMEM-HG) [100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml Fungizone], containing 10% heat-inactivated-FBS. Incubations were performed in humidified 5% CO_2–95% air at 37 °C. Cells were allowed to attach overnight, culture media was aspirated and incubations continued in supplemented medium conditioned with charcoal-stripped 10% heat-inactivated-FBS in the presence of different calcitriol concentrations or its vehicle (0.1% ethanol), with or without 10 ng/mL TNF-α during 24 h. The effects of the VDR antagonist TEI-9647 (1×10^{-6} M) upon calcitriol actions were also tested. In order to study the effects of proinflammatory cytokines upon the gene expression profile of IL-10, we incubated the cells in the presence of different concentrations of TNF-α, IL-1β, IFN-γ and IL-6. Medium was collected and stored at −70 °C for ELISA analysis, and RNA was extracted from cells for gene expression studies. Expression of human chorionic gonadotropin (hCG-β5) was used as control of the cell culture system. The basal expression of CYP27B1 and soluble fms-like tyrosine kinase-1 (sFlt-1) was used as a marker of PE.

2.4. IL-10 immunoassay

Measurement of secreted IL-10 in the culture media was done using specific ELISA, following the manufacturer's directions. Briefly: ninety-six-well plates were coated with capture antibody and incubated overnight at room temperature. Plates were washed and subsequently blocked in PBS containing 1% bovine serum albumin for at least 1 h. After washing, standards and samples were added and incubated for 2 h at room temperature. Plates were washed and coated with detection antibody for 2 h. Developing was carried out with streptavidin-HRP and further substrate addition. Optical density of each well was determined by using a microplate reader (Multiskan MS photometer type 352, Labsystems, Helsinki, Finland) set to 450 nm. Assay sensitivity was 31.25 pg/mL.

2.5. PCR amplifications

Calcitriol effects upon gene expression were studied by extracting total RNA from treated cells using Trizol reagent. In all cases, the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm and a constant amount of RNA (1 µg) was reverse transcribed using a RT assay. Primers and probes for PCR

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