Placenta 31 (2010) 867–872

Contents lists available at ScienceDirect

Placenta

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

Heat-killed *Lactobacillus rhamnosus* GG Modulates Urocortin and Cytokine Release in Primary Trophoblast Cells

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ARTICLE INFO

Article history:
Accepted 9 April 2010

Keywords:
Placenta
Lactobacilli
Preterm delivery
Urocortin
Cytokines

ABSTRACT

A number of studies are showing that probiotic treatment induces an anti-inflammatory state. Intrauterine infection can lead to preterm delivery by modulating immune function and efforts to prevent this condition are ongoing nowadays. *Lactobacillus rhamnosus* GG (LGG) is a probiotic known to ameliorate inflammation by increasing local anti-inflammatory mediators in urinary and gastrointestinal tracts. The present study then analyzed the effect of heat-killed LGG over β-hCG, progesterone, interleukins (IL) 4 and 10, tumor necrosis factor-α (TNF-α), corticotropin releasing hormone (CRH) and urocortin (Ucn) release by primary trophoblast cells. Normal human term placentas (n = 6) were collected and purified trophoblast cells were incubated in the presence of LGG, lipopolysaccharide (LPS) or either LGG + LPS during 3 h, after which the target substances were quantified by ELISA and real-time PCR. LGG did not affect β-hCG, progesterone, or CRH secretion. Conversely, LGG increased IL-4 protein and mRNA expression ($P < 0.05$) while IL-10 and Ucn secretion were increased in a dose dependent manner and the highest dose of LGG increased significantly IL-10 mRNA ($P < 0.05$). LGG did not alter TNF-α, while LPS exposure increased TNF-α protein ($P < 0.001$) and mRNA expression ($P < 0.01$). Conversely, LGG treatment reversed LPS-induced TNF-α release at both protein ($P < 0.01$) and mRNA levels ($P < 0.05$) in a dose dependent fashion. In conclusion, LGG stimulates IL-4, IL-10 and Ucn expression and reverses LPS-induced TNF-α release from trophoblast cells, with no change in β-hCG or progesterone release, suggesting that this probiotic may play a role as an immunomodulatory agent in human placenta without altering basic trophoblast functions.

1. Introduction

Probiotics are live microorganisms that promote host immunomodulation by colonizing and protecting tissues against microbial infection [1]. They act on both innate and adaptive immunity by modifying cytokine production of different cell populations [2] and are effective in colonizing the vagina and curing women with bacterial vaginosis, or at least preventing its recurrence [3]. Low concentrations or even absence of vaginal lactobacilli are correlated to bacterial vaginosis, a condition that may lead to intrauterine infection and is associated with a 40% increased risk of preterm delivery [4]. Intrauterine infection activates the innate immune system, which prematurely initiates the parturition mechanisms through the production of cytokines and chemokines at the reproductive tissues [5,6]. Most of the cytokines are expressed in the placenta and associated membranes [7] and the anti-inflammatory cytokines, interleukins (IL) 4 and 10 are considered to have a protective role during pregnancy [8]. Tumor necrosis factor-α (TNF-α), conversely, synergizes with oxytocin by increasing prostaglandin E2 (PGE2) synthesis via cyclooxygenase-2 in the myometrium and thereby promotes myometrial contractility, leading to term or preterm labor [5–7].

The peptides of the corticotropin releasing hormone (CRH) family have also been implicated in inflammatory processes. CRH and Urocortin (Ucn) are expressed in the placenta and fetal membranes [9,10] and are involved in the mechanisms leading to preterm delivery. CRH displays pro-inflammatory effects in trophoblast cells by increasing lipopolysaccharide (LPS) induced TNF-α and IL-8 release [11]. Ucn, in turn, stimulates IL-4 and IL-10 secretion and reverses LPS-induced TNF-α release from trophoblast cells via CRH-R2 receptors [12], suggesting a possible role for Ucn as an anti-inflammatory agent in human trophoblasts [13,14].

Lactobacillus rhamnosus GG (LGG) is a lactobacilli strain frequently integrated in the elaboration of fermented milks. In rats,

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0143-4004/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.placenta.2010.04.007

LGG decreases LPS-induced systemic inflammation [15–17] and LGG effects may be mediated by two secreted proteins, p75 and p40; they promote epithelial cell growth, inhibit apoptosis and protect epithelial barriers from hydrogen peroxide damage [18]. The aim of the present study was to investigate whether LGG treatment would affect the production and release of hormones (β-hCG and progesterone) and immunomodulatory factors (IL-4, IL-10, CRH and Ucn) from cultured human trophoblast cells. In addition, we investigated if LGG would be able to attenuate or even reverse lipopolysaccharide-induced TNF-α expression in a primary trophoblast cell culture model.

2. Materials and methods

2.1. Collection of placenta

Normal term placentas (>37 weeks of gestation) were collected after uncomplicated elective caesarean delivery in absence of labor (n = 6), in the Division of Obstetrics and Gynecology of the University of Siena (Siena, Italy). Approval from the Local Human Investigation Committee was obtained and all participants signed an informed written consent before being included in the study.

2.2. Primary trophoblast cell culture

Syncytiotrophoblast was prepared using a modification of the method of Kliman et al [19]. After the removal of the decidual tissue and blood clots, approximately 60 g of placental tissue were digested with 0.125% trypsin (Sigma–Aldrich, Steinheim, Germany) and 0.02% deoxyribonuclease-I (Sigma–Aldrich) in phenol red free DMEM (Invitrogen, Paisley, UK), during three times for 30 min at 37 °C. The dispersed trophoblast cells were filtered through a 200-μm-pore-size nylon gauze and were loaded onto a discontinuous Percoll gradient of 5–75% (Sigma–Aldrich) followed by centrifugation at 2500g for 20 min. Cells between the density markers (Amersham Biosciences, Uppsala, Sweden) of 1.049 and 1.062 g/ml were collected. Cells (10^7 per well) were plated in six-well plates with DMEM culture medium containing 10% charcoal stripped fetal calf serum (CSFCS – Invitrogen) and 1% antibiotic/antimycotic solution (Invitrogen). Purified trophoblast cells were cultured for 3 days at 37 °C under 5% CO_2/95% O_2 for 72 h. After 72 h, trophoblast cells aggregated to form a syncytium and were washed twice with Hank's solution pre-warmed at 37 °C, and cultured for 16 h in DMEM free of CSFCS and antibiotics. Cell viability was assessed by trypan blue exclusion and biochemical viability was confirmed by the measurement of human chorionic gonadotropin (β-hCG) by a commercially available ELISA kit (Radim, Rome, Italy), while the purity of the trophoblast culture was confirmed by the immunostaining of more than 90% of the cells positive for cytokeratin and less than 1% of the cells positive for vimentin, using primary antibodies (Dako, UK) at a dilution 1:1000 (for both cytokeratin and vimentin).

2.3. L. rhamnosus strain GG (LGG)

LGG powder (LGG ID 1271), was kindly donated by Anidral S.R.L. (Novara, Italy) with a cellular viability of 350×10^9 UFC/g. Heat killed LGG was prepared as previously described [20,21]. Briefly, bacteria at a concentration of 10^{10} CFU/mL in cell culture medium were heated at 80 °C for 20 min and heat-killed bacteria were centrifuged at 8000 rpm for 10 min, so supernatants could be collected and then diluted at 3 different concentrations in culture medium. Syncytiotrophoblast cells were incubated at 37 °C with LGG at 0, 10^6, 10^8 and 10^{10} CFU/mL in duplicate for 3 h and then culture medium was collected and stored at −80 °C until use. Protein concentrations of samples were determined by the method of Bradford using a protein assay kit (Bio-Rad, Milano, Italy) with bovine serum albumin as a standard.

2.4. Lipopolysaccharide (LPS) treatment

With the purpose of investigating LGG effects on pro-inflammatory cytokine secretion in the presence of an inflammatory stimulus, LPS from *Escherichia coli* serotype 0111:B4 (Sigma–Aldrich) was used in a concentration (100 ng/mL) known to induce cytokine secretion by trophoblast cells in primary culture [11,12,22]. Thirty min after LGG treatment at 0, 10^6, 10^8 and 10^{10} CFU/mL, trophoblast cells were challenged with LPS for 3 h and the supernatants were collected and kept frozen at −80 °C until assayed for the content of the pro-inflammatory cytokine TNF-α.

2.5. Hormone and cytokine assays

Measurement of the hormones and cytokines in the cell culture supernatants was performed by ELISA using commercially available kits in accordance with the manufacturer's instructions. IL-4 (range: 1.1–58 pg/mL) and IL-10 (range: 12.5–400 pg/mL) kits were purchased from Abcam, UK. TNF-α (range: 39.0–250 pg/mL), CRH (range: 0–100 ng/mL) and Ucn (range: 0–100 ng/mL) kits were purchased from Phoenix peptides USA, while β-hCG (range: 0.0–2000 mIU/l) and progesterone (range: 0.05–40 ng/mL) assays were acquired from Radim-Italy.

2.6. RNA extraction and quantitative RT-PCR

Trophoblast primary cells were disrupted and homogenized by passing the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to a RNase-free syringe, and total RNA was digested with RNase-free DNase while the resulting RNA was cleaned up and concentrated according to the instructions of the manufacturer (RNase protect Micro Kit Qiagen, Hilden, Germany). We performed reverse transcription (RT) using the high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) with 100 ng RNA. Subsequently, TaqMan real-time PCR was carried out for all the genes analyzed and the size of amplification products was confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide.

We used the TaqMan gene expression assays (Applied Biosystems) reported in Table 1 and the following thermal cycle protocol was applied: initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °CC for 1 s and 60 °C for 20 s using 100 ng

Table 1
TaqMan gene expression assays (Applied Biosystems) used to perform the real time PCR.

Gene name	Alias	Gene symbol	Ref Seq	Assay ID	Amplicon length
Eukaryotic 18S rRNA		18S	NM_002192.2	Hs99999901_s1	187
Urocortin	MGC129974 MGC129975 UI UROC	UCN	NM_003353.2	Hs00175020_m1	67
Interleukin 4	BCGF-1 BCGF1 BSF1 IL-4 MGC79402	IL4	NM_000589.2	Hs00929862_m1	70
Interleukin 10	CSIF IL-10 IL10A MGC126450 MGC126451 TGIF	IL10	NM_000572.2	Hs00961622_m1	74
Tumor Necrosis Factor (TNF superfamily, member 2)	DADB-70P7.1 DIF TNF-α TNFA TNFSF2	TNF	NM_000594.2	Hs99999043_m1	85