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SHORT COMMUNICATION

Evaluation of the Placental Environment with a New in vitro Model of Histocultures of Early and Term Placentae: Determination of Cytokine and Chemokine Expression Profiles

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We aimed to set up and validate a new in vitro model of placental histocultures, for the evaluation of cytokine and chemokine profiles of the placental environment, over a long culture period.

Micro-explant cultures from 6 early and 6 term placentae were set up on collagen sponge gel supports at a liquid/air interface. At various times during culture, we analyzed tissue morphology and cell death by microscopy and quantified beta-hCG production and mRNA levels for beta-hCG and insulin-like 4 (INSL4). Levels of IL-6, LIF, TNF alpha, IL-10, IFN-gamma, IL-16 and RANTES in the medium were measured by ELISA on days 1, 4 and 7 of culture. SDF-1 mRNA expression was determined by real-time PCR at the same time points.

Histocultures from early and term placentae remained viable until day 10. High levels of IL-6 and LIF production, low levels of TNF alpha, IL-10 and IFN-gamma production and significant SDF-1 expression were observed. These data indicate that placental histoculture is a suitable and reliable in vitro model for studying the placental environment.
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INTRODUCTION

Attempts to establish models for studying the placental environment and particularly the expression of different cytokines have been made by several groups. In vitro models involving dispersed placental cells cannot be used to evaluate the interactions between the various cell subpopulations. Ex vivo studies have been performed, using cultured explants of human placentae [1,2]. However, such explants generally remain viable for only 24 to 48 h, limiting the study of potential modifications of the placental environment in response to various stimuli or experimental conditions. Other models of placental explant cultures have been established, but the cytokine/chemokine profiles were not evaluated in such systems [3,4]. Tissue culture on collagen sponge gel supports at a liquid/air interface has been validated for several organs [5–7]. No such in vitro model has been developed for placental fragments yet, despite its clear potential value for studying the placental environment. The aim of this study was to develop and validate a histoculture system using placental micro-explants. We monitored viability of the histoculture throughout the culture period, by morphological examination and cell death evaluation, quantification of beta-hCG production and mRNA expression for beta-hCG and insulin-like 4 (INSL4). We also investigated the expression profiles of selected pro- and anti-inflammatory cytokines and chemokines involved in the regulation of placental viral replication, to examine if this system could be used to further evaluate the

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impact of the placental environment on mother-to-child transmission of pathogens.

MATERIALS AND METHODS

Placental histocultures

Six term placentae were obtained immediately after elective cesarean section, and 6 first-trimester placentae (8–10 weeks amenorrhea) were obtained following elective abortions carried out by vacuum aspiration in accordance with French ethics guidelines.

During the 2–3 hours following collection, placental chorionic villi were isolated and washed extensively 3 times with PBS (Dulbecco, Invitrogen, Paysley, Scotland, UK). Collagen sponge gels (1.5 cm^2, Gelfoam®, Pharmacia Upjohn, Kalamazoo, MI, USA) were placed into the wells of 6-well plates containing 3 ml of medium per well (RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin, 0.1% gentamycin, 1% amphotericin B, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate (Gibco BRL Ltd, Paisley, Scotland, UK). Villi were cut into 2–3 mm blocks and placed on top of the collagen sponge gels at the interface between the medium and the air (9 blocks per collagen sponge and per well). Histocultures were maintained at 37 °C (5% CO_2/95% air). Each experiment was performed in triplicate. The culture medium was collected and fresh medium added to each well on days 1, 4, 7, 10, 14, 18 and 21. The 9 placental blocks on a given sponge were collected on day 1, 4, 7 or 10.

Morphological and cell death evaluation of the placental histocultures

Micro-explants from a representative histoculture of an early and a term placenta on days 0, 1, 4, 10, 14, 17 and 21 were fixed in 4% buffered formaldehyde and embedded into paraffin. Five-micrometer sections were cut, stained with hematoxylin and eosin and observed under a light microscope. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) technique was performed to detect apoptotic cells with an in situ cell death detection kit (Roche, Nutley, NJ) on tissue sections at the same time points.

Evaluation of beta-hCG and cytokine production

The concentration of total beta-hCG in each histoculture supernatant was evaluated by a non-isotopic immunometric method (sensitivity: 2 mIU/ml) performed on an automated Advia-Centaur multi-analyzer (Bayer Diagnostics, Tarrytown, NY, USA). Enzyme-linked immunosorbent assays (ELISAs) were performed using commercial kits according to the instructions of the manufacturer for interleukin 6 (IL-6) and RANTES (Quantikine R&D Systems Europe Ltd, Oxon, UK), interleukin 16 (IL-16) (Biosource International, Nivelles, Belgium), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF alpha), gamma interferon (IFN-γ) (Immunotech, Beckman Coulter, Marseille, France). Human LIF was quantified by ELISA as described previously [8].

Detection of beta-hCG, INSL4, stromal cell-derived factor-1 (SDF-1) mRNA

Tissues of 5 early and 5 term placental histocultures were homogenized. Total RNA was then extracted from the 9 placental blocks (30–50 mg) per sponge. cDNA was obtained using the Taqman® reverse transcription kit (Applied Biosystems, New Jersey, USA). cDNAs were amplified by quantitative real-time PCR, using the ABI-PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, California) with specific oligonucleotide primer sets and Taqman® probes (Table 1) [9,10]. RNA levels were normalized by quantifying 18S RNA (20×, Applied Biosystems, Foster City, California) in the same reaction. Each sample was analyzed in triplicate. Gene expression in each sample was quantified by means of the comparative threshold cycle (CT) method. Beta-hCG, INSL4 and SDF-1 mRNA levels are expressed with respect to those in a reference term placental tissue (calibrator considered as the 1× sample), similar for each experiment and run in parallel in each analysis.

Statistical analysis

Production of beta-hCG and SDF-1 expression in histocultures of early and term placentae were compared using the non-parametric Mann and Whitney test. Production of cytokines in placental histocultures was plotted as histograms with error bars indicating the standard deviation.

RESULTS

On days 1, 4, 7 and 10, the villi were morphologically similar to day 0 villi (Figure 1Aa, Ba). In the histoculture of the early placenta, we observed a well-defined inner cytotrophoblastic layer and outer layer of syncytiotrophoblasts with a differentiation of the cytotrophoblastic cells into syncytiotrophoblastic cells on day 7 (Figure 1Ad). A thin layer of syncytiotrophoblastic cells was observed in the histoculture of the term placenta (Figure 1B). Fetal vessels and Hofbauer cells were observed in the villous stroma. Necrotic zones appeared between days 14 and 21 (Figure 1Ag,h, Bg,h). On days 17 and 21, cell subpopulations were no longer visible within the villi (Figure 1Ag,h, Bg,h). For the early placenta, no apoptotic cells were detected before day 10 by TUNEL analysis. From day 10 to 21, 5 to 10 apoptotic cells per microscopic field (×400) were observed in the cyto and the syncytiotrophoblast. Apoptotic cells were only detected at day 21 for the term placenta (>10 apoptotic cells per microscopic field) (data not shown).

Beta-hCG levels peaked on days 1 to 4 in the supernatants of early (7620 ± 1858 mIU/ml) and term (13 ± 2 mIU/ml) placental histocultures. Beta-hCG levels decreased between day 4 and day 7 of culture and reached a minimum after day 14 (1150 ± 981 mIU/ml for early placentae, 4 ± 1 mIU/ml for term placentae). Beta-hCG mRNA levels peaked on day 4

Table 1. Primers and probes used to amplify beta-hCG, INSL4 and SDF-1 (α/β) mRNA from placental histocultures

	Forward primer	Reverse primer	Probe
Beta-hCG	5′-GCTACTGCCCCACCATGACC-3′	5′-ATGGACTCGAAGCGCACATC-3′	5′-FAM-CCTGCCTCAGGTGGTGTGCAACTACC-TAMRA-3′
INSL4	5′-CATATTGCCCCATGCCTGAG-3′	5′-TGTTGGAGGTTGACACCATTTCT-3′	5′-FAM-AGACATTCACCACCACCCCAGCAGG-TAMRA-3′
SDF-1 (α/β)	5′-CACTCCAACTGTGCCCTTCA-3′	5′-CTTGTTTAAGCTTTCTCCAGGTACTC-3′	5′-FAM-CAACAACAGACAAGTGTGCATTGACCCG-TAMRA-3′

(2.11 ± 0.29 and 0.021 ± 0.014, for early and term placentae, respectively, with respect to the calibrator) and then decreased from day 7. INSL4 mRNA levels peaked on day 1 for both early and term placentae (0.47 ± 0.27 and 0.036 ± 0.011, respectively, with respect to the calibrator) and then decreased from day 7 to day 10.

In histocultures of early placentae, IL-6 and LIF were produced in large amounts (mean peaks of 12,978 ± 8967 pg/ml and 2498 ± 1519 pg/ml, respectively). The levels of RANTES, IL-16, TNF alpha, IL-10 (on days 1 and 4) and IFN-gamma were low, either below or at the limit of detection (Figure 2). In histocultures of term placentae, RANTES, LIF, IL-6 and IL-16 were produced in detectable amounts (peak mean 13 ± 11, 2458 ± 1490, 27,366 ± 11,338 and 39 ± 14 pg/ml, respectively). In contrast, TNF alpha, IL-10 and IFN-gamma levels were below or at the limit of detection (Figure 2). SDF-1 mRNA was detected up to day 7 in histocultures. Levels peaked on day 1 (0.13 ± 0.11 with respect to the calibrator) and were maximal on days 1 and 4 (0.35 ± 0.12, 0.35 ± 0.24, respectively, with respect to the calibrator) for histocultures of early and term placentae, respectively.

DISCUSSION

We aimed to set up a new in vitro model of placental environment to evaluate cytokine/chemokine profiles in a long-term culture system. Collagen sponge cell supports were used to perform placental histocultures as previous culture systems of trophoblastic cells or placental tissue with collagen containing supports allowed proper structural preservation and synthesis of trophoblastic products [11]. Moreover, collagen is a major component of the chorionic villi mesenchyma and trophoblastic cells express collagen receptors [12,13]. In the histoculture system, micro-explants are much smaller than the usual explants which means that the contact of the tissue micro-explant with the air is much better which changes local gas exchange conditions. Micro-explant villi of early and term placentae were morphologically viable until at least day 10, with no significant alterations occurring until day 14 and no significant cell death before day 10 for early placenta and before day 21 for term placenta. Interestingly, differentiation from cytotrophoblastic cells to syncytiotrophoblastic cells, as occurs in vivo in the placenta during pregnancy, was observed in early histocultures [12]. This indicated that under our experimental conditions, mechanisms resulting in cell differentiation that takes place during particular stages of placental development are preserved, providing additional evidence that placental villi are intact. The higher production of beta-hCG in early placental histoculture in comparison to term placental histoculture ($P < 0.01$) is consistent with the in vivo situation [14]. Furthermore, the INSL4 gene, a newly discovered member of the insulin-related gene family preferentially expressed in trophoblastic cells [10] was also detected in the placental histocultures. Based on morphological features, apoptosis assessment, protein secretion profiles and mRNA synthesis

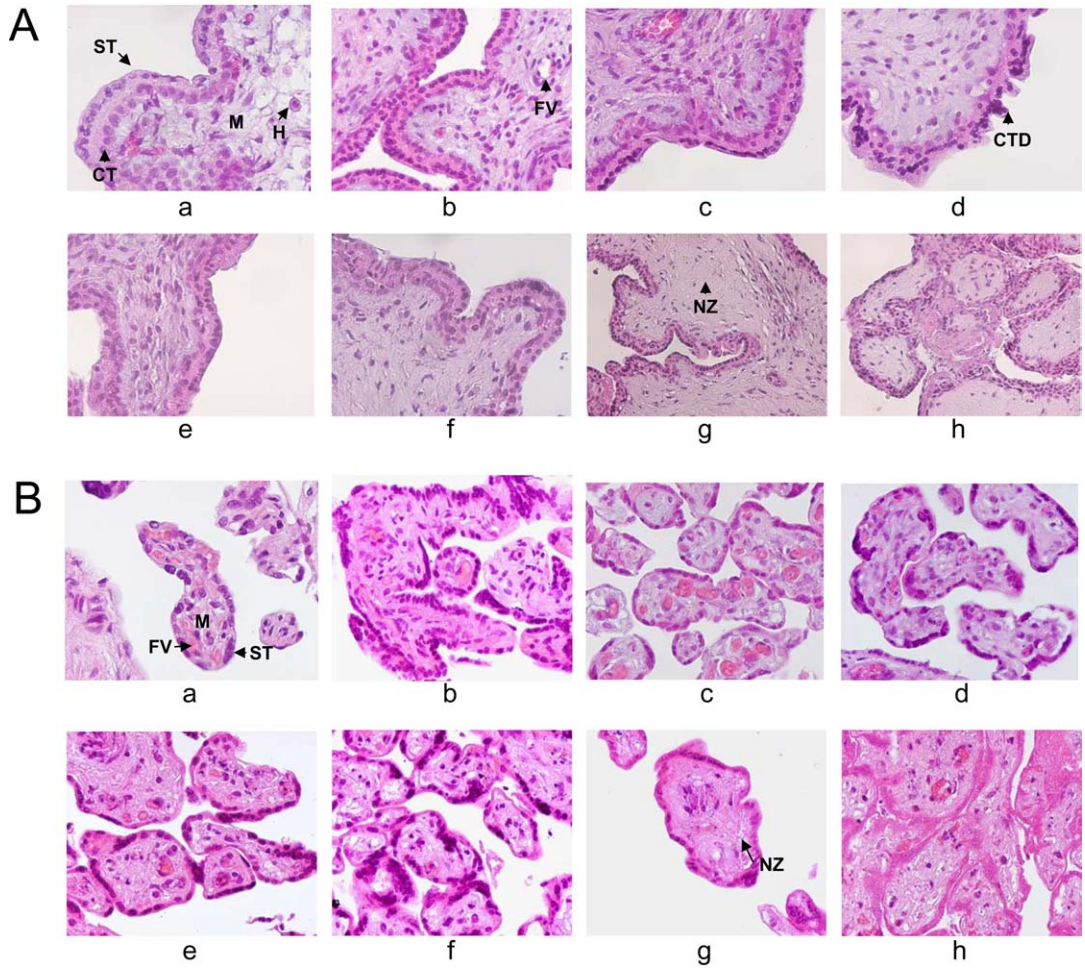

Figure 1. Cross section of placental villi from histocultures of an early (A) and a term (B) placenta on days 0 (a), 1 (b), 4 (c), 7 (d), 10 (e), 14 (f), 17 (g) and 21 (h) (×40 for Aa–f and ×20 for Ag,h and B). CT: cytotrophoblast, ST: syncytiotrophoblast, FV: fetal vessel, H: Hofbauer cell, M: mesenchyma, CTD: cytotrophoblast differentiation, NZ: necrotic zone.

patterns of beta-hCG and INSL4, placental histocultures are viable for a 7 to 10 day period. Several cytokines known to be involved in the regulation of viral replication were then studied. Histoculture cytokine profiles were compared with the cytokine profiles observed in 24-h culture supernatants of classical placental explants from 5 early and 5 term placentae. The placentae were obtained in the same conditions as the histocultures and processed as described elsewhere [1,2]. The histocultures of term placentae displayed the same cytokine/chemokine pattern as the 24-h term placental explants, with high levels of LIF, IL-6, RANTES and IL-16 (for explants: 186 ± 115, 1593 ± 437, 968 ± 524, and 498 ± 245 pg/ml, respectively) and low levels of TNF alpha, IFN-gamma and IL-10 (below or at the limit of detection). The cytokine/chemokine profile of histocultures of early placentae was similar to that for 24-h early placental explants, with high production of IL-6 and LIF (for explants: 7146 ± 5260 and 404 ± 301 pg/ml, respectively) and low production of TNF alpha, IL-10 (on days 1 and 4) and IFN-gamma (below or at the limit of detection). The production of RANTES and IL-16 was higher in 24-h explants (422 ± 218 and 734 ± 309 pg/ml, respectively). Differences in the preparation and culture of the placental tissue and differentiation of cytotrophoblasts into syncytiotrophoblasts during the histoculture of early placentae may explain these differences between histocultures and 24-h cultures of early placentae.

In summary, the expression profiles of IL-6, RANTES, TNF alpha, IFN-gamma and IL-10 were comparable to the one observed in 24-h placental explant cultures and to the one described in the literature [2,15–17]. Furthermore, a high expression of LIF was detected in accordance with data published on LIF mRNA expression [18]. For the first time, to our knowledge, we investigated IL-16 expression in human placenta and found a significant production of this cytokine in the supernatant of histocultures of term placenta in accordance with the findings at the murine fetomaternal interface [19]. Furthermore, our data confirmed the expression of SDF-1 in placental histocultures as already observed in a previous study in explants of term placentae by in situ hybridization [20]. We observed a higher mRNA expression in term versus early placentae ($P < 0.05$). Altogether, our study shows that histocultures can be used as a new long-term (up to 10 days)

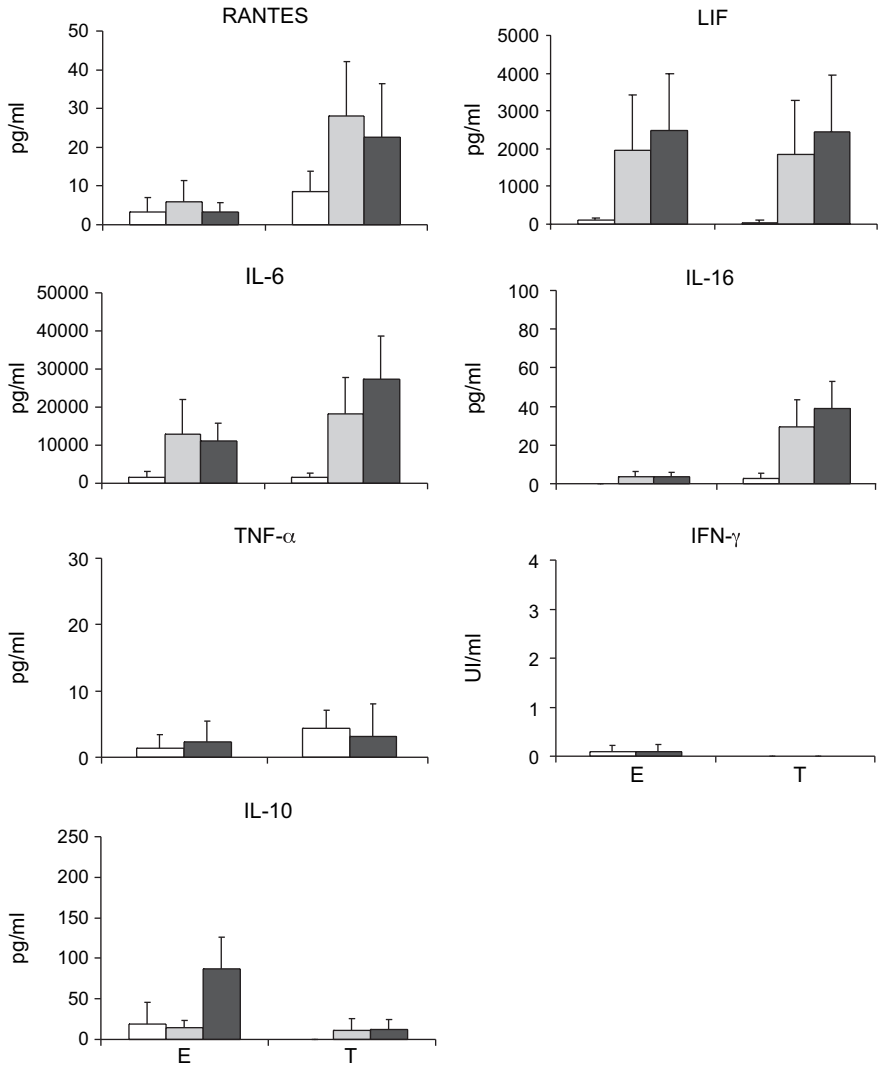

Figure 2. Cytokine levels in the medium of 5 early (E) and 5 term (T) placental histocultures at various time points (□ day 1, ■ day 4, ■ day 7) (for day 4 and day 7, histograms represent accumulation of cytokines during 3 days in culture).

culture system for first-trimester and term human placental tissue that may approximate the in vivo situation because of maintenance of the complex interactions between the various cell subpopulations at the chorionic villi level. Thus, placental histoculture may be useful for the evaluation of the placental environment such as the expression of cytokines or chemokines following viral infection or the influence of drugs, toxins or other exogenous agents.

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