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Adhering maternal platelets can contribute to the cytokine and chemokine cocktail released by human first trimester villous placenta

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Abstract

Placental villous explant culture has been increasingly recognized as suitable model to study secretion of inflammatory and immune modulating factors by human placenta. Most of these factors likely derive from the syncytiotrophoblast, whereas extraplacental sources such as maternal peripheral blood cells are rarely considered. Due to their small size and absence of a nucleus, platelets adhering to perivillous fibrinoid of normal placenta are frequently ignored in routine immunohistochemistry. Here we demonstrate adhering maternal platelets on first trimester placental villi after explant culture and point out that platelet-derived factors must be considered when analysing the inflammatory secretion profile of human placenta.

Keywords

placental explant culture; platelets; cytokines; chemokines

Introduction

Fetal-maternal cross-talk on the level of growth factors, cytokines and chemokines has been studied *in vitro* using different culture models, including primary trophoblasts and trophoblast derived cell lines, cultured either as monolayers or spheroids. In recent years, placental villous explant culture has been increasingly recognized as another suitable model probably best reflecting the *in vivo* situation. The major advantage of culturing intact villous tissue is based on the fact that cells remain in their natural microenvironment and preparatory stress is reduced to a minimum [1,2]. By the use of placental villous explant culture, release of placenta-derived angiogenic and immunomodulatory factors have been analyzed for pregnancy pathologies, such as gestational diabetes mellitus (GDM) [3], intrauterine growth restriction (IUGR) [4] and preeclampsia [5-7]. For most of these analyzed factors it seems likely and well-proven that the syncytiotrophoblast is the only source, whereas extraplacental sources, such as remaining maternal peripheral blood cells have not been considered. Amongst peripheral blood cells, platelets are suggested as

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important players modulating inflammatory and immune responses [8,9]. They are 2µm small, anucleated disc-shaped bodies, which derive from megakaryocytes in the bone marrow and contain different types of granules filled with coagulation and angiogenic factors, adhesion molecules, cytokines and chemokines. Though the surface of human syncytiotrophoblast in principle is not considered to induce aggregation of maternal platelets, adherence of the latter to perivillous fibrinoid is a regular finding in normal term placenta. However, owing to the absence of a nucleus and their small size, platelets are frequently ignored in routine immunohistochemistry. Here we show that maternal platelets can be detected on first trimester placental villi after explant culture and point out that platelet-derived factors such as chemokine (C-C motif) ligand 5 (CCL5, also known as regulated on activation, normal T cell expressed and secreted (RANTES)) and chemokine (C-X-C motif) ligand 4 (CXCL4, also known as platelet factor 4 (PF4)) can distort analysis of the intrinsic inflammatory and immune modulating secretion profile of human first trimester placenta.

Methods

Human placenta tissue samples

First trimester placenta tissues (n=6, mean gestational week: 9.5 ± 1.9) were obtained after approval by the ethical committee of the Medical University of Graz and with informed consent from healthy women undergoing elective pregnancy terminations.

Placental explant culture

Placental first trimester explants were cultured as described previously [10]. In brief, placental villous tissues were washed thoroughly in buffered saline and dissected into small pieces of approximately 5 mg moist mass. Placental explants were cultured in 12 well dishes (nunc, Thermo Scientific) and 2ml/well DMEM/F12 (1:1, Gibco) supplemented with 10% FCS, penicillin/streptomycin, amphotericin B and L-glutamine, in a hypoxic workstation (BioSpherix) under 2.5% oxygen at 37°C for 48h. After incubation, placental explants were homogenized in RIPA buffer (Sigma-Aldrich) including Protease Inhibitor Cocktail (Roche Diagnostics) and total protein determined by Lowry method. Placental explants cultured in parallel were formalin fixed and paraffin embedded (FFPE) for immunohistochemical analysis.

Measurement of CCL5 and CXCL4

CCL5 (RANTES) and CXCL4 (PF4) were measured in explant homogenates and corresponding conditioned culture media using quantitative sandwich enzyme immunoassays (Human CCL5/RANTES and Human CXCL4/PF4 Quantikine ELISA, R&D Systems). Placental tissue homogenates were centrifugation at $8.000 \times g$ and 4 °C for 10 min. Conditioned culture media from explant culture were centrifuged at $1.500 \times g$ and 4 °C for 5 min. Clear supernatants from conditioned culture media or placental explant homogenates were subjected to immunoassays according to the manufacturer's instruction. RIPA buffer and complete culture medium incubated without explants served as blank for measurements of tissue homogenates and conditioned supernatants, respectively. Samples were analyzed in duplicates and obtained concentrations normalized to total tissue protein.

Immunohistochemistry and Immunocytochemistry

FFPE placental explant sections (5 µm) were mounted on Superfrost Plus slides (Menzel/Thermo Fisher Scientific) and deparaffinised according to standard protocol. Slides were boiled in Epitope Retrieval Solution pH 9.0 (Novocostra, Leica) for 7 min at 120°C in a decloaking chamber (Biocare Medical). Immunostaining was performed using the UltraVision Detection System HRP Polymer Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. In brief, endogenous peroxidase was blocked using the hydrogen peroxidase block for 10 min. After three washing steps with TBS including 0.05% Tween 20 (TBS/T; Merck) background blocking was performed using Ultra Vision Protein Block for 5 min. Primary antibodies (see table 1) were diluted in Antibody Diluent (Dako) and incubated on slides for 45 min at RT. Thereafter slides were washed three times and detection achieved by incubation with anti-rabbit UltraVision HRP-labelled polymer (15 min) and 3-amino-9-ethylcarbazole (AEC, Thermo Scientific) for 10 min. Nuclei were stained with hemalaun and slides were mounted with aqueous mounting agent Aquatex (Merk Millipore). FFPE platelets were used as positive control. For this purpose platelets-rich plasma (500µl) obtained from healthy donors was fixed in formalin at RT overnight. Formalin fixed platelets were washed in buffered saline and incubated with 5% gelatin for 45 min at 37°C. Gelatin embedded platelets pellets jelled at 4°C and underwent another formalin fixation step. Fixed gelatin embedded platelets were subsequently embedded in paraffin by standard procedure and subjected to immunostaining as described above.

Results and Discussion

In order to test whether or not platelets can be detected in placental villous explant culture, placental explants were formalin fixed and analyzed immunohistochemically after culture. Immunohistochemistry for CD42b, a surface membrane protein of platelets, not only demonstrated platelets in fetal blood vessels, but also identified maternal platelets adhering to fibrin deposits on the villous surface (Fig. 1a). Staining of adjacent sections with anti-fibrin antibody confirmed that identified platelets adhered to perivillous fibrin-type fibrinoid (Fig. 1b). Notably, maternal platelets were detected on explants from all studied placentas, indicating that adherence of maternal platelets to villous surface is indeed a common process in first trimester of gestation. Whether aggregation of maternal platelets on the syncytial surface represents initial stages of perivillous fibrinoid deposition, or whether focal degeneration of the syncytiotrophoblast with exposure of basal lamina components to maternal blood induces blood clotting and closure of focal defects by fibrinoid plugs has been discussed over decades [11-13]. However, since placental explants were thoroughly rinsed before culture, adherence of maternal platelets may not be the result of culture conditions but may rather occur *in vivo* and persist throughout cultivation. Interestingly, maternal platelet aggregates were also described on villous surface of dually perfused human term placenta cotyledons [14].

Immunohistochemistry for well-described platelet-derived factors CCL5 (Fig. 1c) and CXCL4 (Fig. 1d) showed distinct staining of maternal platelets adhering to placental villi, while villous trophoblast, Hofbauer cells, fetal endothelium, and villous stroma were unstained. Formalin fixed and paraffin embedded platelets served as positive control and

showed considerable CCL5 (Fig. 1e) and CXCL4 (Fig. 1f) staining, whereas negative controls for rabbit (Fig. 1g) and mouse antibody (Fig. 1h) revealed no staining. ELISA analysis of placental explant homogenates and corresponding conditioned culture media revealed considerable levels of CCL5 and CXCL4 (Fig. 1i). Since neither CCL5 nor CXCL4 was detected in the villous trophoblast layer, adhering platelets seem to be the only source for both chemokines detected in conditioned culture media.

Thus, presence of adhering maternal platelets on the villous surface of placental explant culture or perfused placenta cotyledons must be considered when studying the secretion profile of human placenta. While some factors may derive exclusively from the syncytiotrophoblast, others may come from both the trophoblast and adhering platelets, and yet others such as CCL5 and CXCL4 may be released solely by adhering platelets. Beside coagulation and angiogenic factors, platelet granules contain adhesion molecules, cytokines and chemokines, which are exocytosed upon activation and enhance hemostasis and inflammation [9]. Release of platelet-derived factors by adhering platelets may for instance provide an explanation for previous studies showing high levels of CCL5 secretion in early and term placental villi, even though no production of this chemokine has been detected in isolated trophoblast cultures [15,16]. However, CCL5 and CXCL4 may only be one examples for platelet-derived chemokines in placental explant culture. Considering the diverse array of chemokines contained in platelets granule, including chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL5, CXCL7 and CCL3 (or macrophage inflammatory protein (MIP)-1 α), a precise verification of their origin seems to be mandatory when defining the placental role in fetal-maternal chemokine and cytokine cross-talk.

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Figure 1. Localization of adhering platelets and analysis of platelet-derived factors in human first trimester explant culture

Immunohistochemical staining for CD42b (a) identified maternal platelets (arrowhead) adhering to perivillous fibrin-type fibrinoid, which was confirmed by staining of adjacent sections for fibrin (b). CCL5 (c) and CXCL4 (d) were detected in platelets adhering to perivillous fibrin deposits, while villous trophoblast, Hofbauer cells, fetal endothelium, and villous stroma were unstained. Scale bar represents 50 μ m. Formalin fixed and paraffin embedded human platelets showed intense CCL5 (e) and CXCL4 (f) staining. Negative

control for rabbit IgG (**g**) and negative control mouse IgG (**h**) revealed no staining. Explant homogenates and corresponding culture supernatants were analyzed by ELISA (**i**) and values normalized to total tissue protein. Values are given as mean $\pm$ SEM.

Table 1

Applied antibodies

antigen	working concentration	species	clone	Source
CCL5/RANTES	2 µg/ml	rabbit	polyclonal	Acris, Germany
CD42b	4.5 µg/ml	rabbit	polyclonal	Proteintech, UK
CXCL4/PF4	0.9 µg/ml	rabbit	polyclonal	abcam, UK
Fibrin	2 µg/ml	mouse	clone E8	BeckmannCoulter, USA

negative controls				
negative control, mouse IgG1	2 µg/ml	mouse		Dako, Denmark
negative control for rabbit IgG	4.5 µg/ml	rabbit		Neomarkers, ThermoScientific, USA