CHARACTERIZATION OF TROPHOBLAST CELLS AND DECIDUAL LYMPHOCYTES ISOLATED FROM FETAL-MATERNAL INTERFACE

Daniela Constantinescu1, Carmen Cozmei1, Laurette Graziella Cozma1, Maria Țepeluș2, Andreea Chiriac3, Eugen Carasievici2,4

1. Institute of Public Health, Iași, 2. Immunology and Genetics Laboratory, ‘Sf. Spiridon’ Hospital, Iași, 3. ‘Cuza Vodă’ Maternity, Iași, 4. Immunology Department, ‘Gr. T. Popa’ University of Medicine and Pharmacy, Iași

Abstract. Aim. The aim of our study was to describe the leukocyte phenotype at the fetal-maternal interface after separation of the trophoblast cells and uterine leukocytes from endometrial tissue samples. Materials and methods. The tissues were processed and submitted to 3 cycles of enzymatic digestion then the cells were separated by centrifugation over a Percoll gradient. We investigated by flow-cytometry the lymphocyte subpopulations in peripheral blood or endometrial tissue samples obtained after legal abortion in 10 normal first trimester pregnancies. Leukocyte tissue distribution and trophoblast invasion in the endometrium were investigated by immunohistochemistry. Results. Our data show that NK cells are the predominant lymphocytes in pregnant uterus, that they express a higher level of CD56 compared to their circulatory equivalent, lack CD16 and partially coexpress CD3. One third of the uterine lymphocytes are T cells, with a CD4/CD8 ratio of 1. Immunohistochemistry investigation showed that leukocytes aggregate around spiral arteries and endometrial glands and also disseminate in the stroma. Conclusion. We devised a feasible method for isolation of trophoblast cells and uterine leukocytes from fetal-maternal interface that could be useful in further investigations in order to outline some useful immune tests for detecting and monitoring pregnancies with high obstetrical risk.

Key-words: fetal-maternal interface, trophoblast, lymphocytes, NK cells

Rezumat. Scop. Scopul acestei lucrări a fost acela de a descrie fenotipul populațiilor leucocitare de la nivelul interfeței materno-fetale după separarea trofoblastului și a leucocitelor din probe de țesut endometrial. Material și metodă. Fragmentele tisulare au fost prăjite și supuse la trei cicluri de digestie enzimatică apoi celulele au fost separate prin centrifugare pe gradient de Percoll. Am investigat flow-citometric subpopulațiile limfocitare din sânge periferic și țesut endometrial obținut prin chiuretaj solicitat de 10 gravide cu sarcină normală în primul trimestru. Distribuția tisulară a leucocitelor și invazia trofoblastului în endometru au fost investigate prin imunohistochimie. Rezultate. Rezultatele noastre arată faptul că celulele NK reprezintă populația limfocitară predominantă în uterul gravid, ele exprimând un nivel crescut de CD56 în comparație cu echivalentul lor circulant, un nivel redus de CD16 și coexprimă parțial CD3. O treime din limfocitele uterine sunt celule T cu un raport CD4/CD8 de 1. Imunohistochimia a relevat faptul că leucocitele sunt fi într-o agregare în jurul arterelor spiralate și a glandelor endometriale, fi disseminate în stroma. Concluzie. În această lucrare descriem optimizarea unei tehnici fuzabile de separare a trofoblastului și a leucocitelor uterine de la nivelul interfeței materno-fetale, metodă ce va servi investigațiilor ulterioare care...
INTRODUCTION
Leukocytes are an important component of the fetal-maternal interface during the first trimester of pregnancy, representing 40 – 45% of the decidual cells (1, 2).
Decidua, the pregnancy-modified endometrium, is infiltrated at the implantation site by NK cells and T lymphocytes being in close active contact with the invading fetal trophoblast cells (3). There are two populations of trophoblast cells: the villous trophoblast that covers the placental villi and mediates the nutritive changes between the mother and the fetus. The extra-villous trophoblast is responsible with the invasive behavior, it migrates in the maternal decidua, surrounds and replaces the endothelium of the maternal spiral arteries changing them to low-resistance vessels which are not any more responsive to maternal stimuli and offer larger amounts of blood to the fetus (4, 5).
The immune cells and their products at the maternal-fetal interface contribute to limiting the invasiveness of the trophoblast in a way that allows the fetus to develop and mature to term and the mother to survive to the increasing demands of the new being. From the immunological point of view pregnancy is a puzzling issue; it is still unclear how the immune cells of the mother tolerate the fetus which, due to the paternal genes, is half non-self. For a normal evolution of pregnancy to term, a well-balanced equilibrium between invasive trophoblast and maternal immune cells is required, in order to support the complex endocrine and cytokine network which regulate the activity at the fetal-maternal interface.

MATERIAL AND METHODS
Preparation of trophoblast cells and uterine leukocyte suspensions
We processed placental tissue obtained after legal abortions in 10 normal first trimester pregnancies (6-14 weeks). The tissues were transported to the laboratory in ice cold heparinised saline 0.9% NaCl, during the first 30 minutes after the curettage, due to the protease-rich nature of these tissues. Processing of the samples was performed in the laminar-flow cabinet, preserving the sterility of the products. After removing the blood clots and fragments of the fetal membranes, the tissue fragments were rinsed several times in phosphate buffered saline (PBS) (pH 7.4) to remove residual blood and then mechanically disaggregated with scissors, to fragments of approximately 3-10 mm³ volume. Minced tissue was transferred to the digestion bottle
containing 20 U/ml DNAse I type IV (Sigma), 0.125 % trypsin (Sigma), 50 µl CaCl₂ (Reactivul Bucureşti) 100mM, 50 µl MgSO₄ (Reactivul Bucureşti) 800 mM and RPMI 1640 (Sigma) up to 50 ml. The presence of divalent ions is necessary for trypsin activation. The enzymatic tissue digestion was performed at 37°C, in the shaking water bath. After incubation, tissue pieces were allowed to settle for 5 min then half of the supernatant containing the syncytiotrophoblast cells was discarded. Two more cycles of enzymatic digestion were performed, in similar conditions except that the second and third digestion mixtures did not contain DNAse. After the last incubation trypsin activity was stopped by adding heat inactivated fetal calf serum (FCS) up to 10% of the final volume (6). Undigested tissue was removed by filtration through a dense mesh and filtrate was washed by centrifugation (620xg, 20°C, 10 min). After discarding the supernatant, the cell pellet was resuspended in RPMI 1640 containing 4.5% glucose and layered on the Percoll gradient (7). As reported by Kliman (1986) (7) when centrifuged over a Percoll gradient cytotrophoblast cells migrate in layers ranging from 1.062 to 1.048 densities. We adapted the protocol described by Nagamatsu et al (2004) and used three gradients of Percoll as table 1 shows (8).

Table 1. The composition of the three gradients used in Percoll separation of trophoblast and uterine mononuclear cells.

<table>
<thead>
<tr>
<th>Percoll gradient</th>
<th>Corresponding density</th>
<th>Percoll (ml)</th>
<th>PBS (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 %</td>
<td>1.062</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>40 %</td>
<td>1.048</td>
<td>2.4</td>
<td>3.6</td>
</tr>
<tr>
<td>20 %</td>
<td>1.028</td>
<td>1.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The tubes were centrifuged at 800xg, for 25 min, at 20°C. The layers obtained after centrifugation were separately collected, washed twice by centrifugation in RPMI1640 and viability and cell numbers were detected in a Burker-Turk microscopic counting camera.

Immunocytochemistry
In order to check the presence of the trophoblast cells in the upper layer, 2x10⁶ cells were centrifuged in a Hettich Universal 32R centrifuge, using Dako slides. Smears were air-dried at room temperature and then labelled, after rehydration, with mouse anti-human CK7 antibodies (Dako, OVTL 12/30 clone). The diaminobenzidine reaction was used for visualization and cell nuclei were counterstained with hematoxylin.

Immunohistochemistry
A small tissue fragment was fixed in phosphate-buffered 4% formalin (24 hours, room temperature) and embedded in paraffin. Sections were cut (4 µm), deparaffinized, rehydrated and labeled with mouse anti-human...
CK7 antibodies (Dako, OVTL 12/30 clone) in order to visualize trophoblast cells (9). Monoclonal mouse anti-human CD45 antibodies were used to label the leukocytes in order to examine their tissue distribution. For visualization diaminobenzidine and counterstaining with hematoxylin was used.

**Flow-cytometry investigation**

The cells from the upper layer obtained after Percoll gradient centrifugation of the trypsinized placental tissue were collected separately from the cells that migrated in layers 2 and 3 (fig. 3) which were pooled together. Intracellular labeling with appropriate monoclonal antibodies was meant to distinguish between trophoblast cells and leukocytes present in layer 1 and to establish the lymphocyte subpopulations present in the uterine mononuclear cells pooled in layers 2 and 3 (table 2).

| Table 2. The monoclonal antibodies used for immunophenotyping |
|-----------------------------|-----------------------------|
| **Layer 1**                  | **Uterine (layers 2 and 3) and peripheral blood mononuclear cells** |
| CK7(FITC)/CD45 (PE-Cy5)     | CD16 (FITC)/CD4 (PE)/CD3 (PerCP)/CD56 (APC) |
|                             | CD16 (FITC)/CD8 (PE)/CD3 (PerCP)/CD56 (APC) |
|                             | CD3 (FITC)/CD19 (PE)/CD56 (APC) |

The immunophenotype of peripheral blood mononuclear cells (PBMC) separated by Ficoll centrifugation of whole blood was used for comparison, in order to assess the risk of blood contamination of the uterine samples. For intracellular labeling cell membranes were fixed in 2% paraformaldehyde, then permeabilised with saponin and labeled with monoclonal antibodies (table 2). The four-color flow-cytometric investigation assessed the presence of trophoblast cells (CK7+CD45-) and the percentages of lymphocyte sub-populations: T cells (CD3+ and CD4+ or CD8+), NK cells (CD16+CD56+), NKT (CD16+CD56+CD3+), B cells (CD19+).

Acquisition was done for 20000 events using a FACSCalibur flow-cytometer and analysis used the CellQuest software.

**RESULTS AND DISCUSSIONS**

**Immunohistochemistry for trophoblast population**

Staining of the paraffin-embedded tissue showed the invasion of trophoblast cells between decidual cells, a normal process in the attempt of the fetus to reach a bigger, constant source of nutrients (fig. 1). Labeling showed that anti-CK7 antibody is not specific for the trophoblast only; epithelial cells from endometrial glands also bind this antibody, thus confirming the observations made by Blaschitz et al (1997) (10) (fig. 2).
Fig. 1 CK7-positive trophoblast cells (brown) that replaced the vascular endothelial cells or are scattered in the endometrial stroma between decidual cells (large, pale cells) in a normal 7-weeks pregnancy. Magnification x 20. Hematoxylin counterstaining.

Fig. 2 Epithelial cells of endometrial glands showed positivity to CK7 (7-weeks pregnancy). Magnification x20, hematoxylin counterstaining.

Preparation of trophoblast cells and uterine leukocytes suspensions
The trypsinized placental cells were layered on the Percoll gradient and centrifugation generated several layers (fig. 3).
Microscopic examination showed that the upper layer mainly contains polygonal cells resembling trophoblast, layers 2 and 3 contain various types of leukocytes and some trophoblast and decidual cells, layer 4 contains granulocytes, nucleated red blood cells (fetal), rare lymphocytes and decidual cells and layer 5 consists of red blood cells (1).

As Haigh et al (1999) reported that the cytokeratin-7 (CK7) intermediate filament is highly expressed throughout the trophoblast lineage, the presence of the trophoblast cells in the upper layer was assessed by labelling a smear with 2×10^4 cells with anti-CK7 antibodies (9).

The presence of the trophoblast between the cells isolated in the first layer was also checked by flow cytometry. The population showed variable granularity and positivity for CK7 (fig. 5 a and 5 b).
The endometrium contains a population of leukocytes whose number and subtypes vary during the menstrual cycle and in early pregnancy. The pattern of distribution of the leukocytes in the uterine tissues was detected by immunohistochemistry and confirmed the literature data. Thus Beier (1998) (11) described by immunohistochemistry a big number of NK cells around spiral arteries and endometrial glands and Kämmerer (2004) (12) described intense proliferation of uterine NK and immature dendritic cells in early-pregnancy decidua; the two populations are often in contact with each other and with the invasive cytotrophoblast cells, being involved in generating the inhibitory signals that induce tolerance to fetal antigens. Our tissue sections were labeled with antibodies directed towards the pan-leukocyte antigen CD45 (fig. 6).

The ratios of different endometrial lymphocyte subpopulations were established by flow-cytometry and revealed that NK cells are prevalent (60%) followed by T cells (30%). B cells are scarce in pregnant endometrium confirming the data reported by Kämmerer (2004) (12) (table 3). Our data confirm literature reports where NK cells represent 50 – 90 % of the uterine leukocytes (13). Only not the number makes the difference with the peripheral blood but also their phenotype: CD56+CD16- (fig. 7). The presence of this particular type of NK cells in the endometrium was explained by the migration of rare, atypical circulatory precursors that eventually differentiate and proliferate in the endometrium. Cooper et al. (2001) described two subsets of circulatory NK cells which are both phenotypically and functionally different:

**Fig. 5 a)** Volume (FSC) – granularity (SSC) diagram showing the flow-cytometric distribution of the cells that migrated in the first layer during Percoll gradient centrifugation. **b)** CK7-positive trophoblast cells (the orange histogram) separated in layer 1 compared to CK7-negative cells (red line histogram) separated in layers 2 and 3.
TROPHOBLAST AND LYMPHOCYTES AT FETAL-MATERNAL INTERFACE

those with low CD56 expression are specialized in cytolysis while NK cells with high CD56 expression are predominantly cytokine- secretory (14).

The decidual NK cells are involved both in cytokine secretion and in spiral arteries remodelling (15).

Fig. 6 CD45-positive leukocytes (brown) disseminated in the uterine stroma or aggregated near a blood vessel. Normal 7-weeks pregnancy. Magnification x 10. Hematoxylin counterstaining.

Table 3. Lymphocyte subsets in decidua compared with peripheral blood in 10 normal first-trimester pregnancies

<table>
<thead>
<tr>
<th></th>
<th>Uterine mononuclear cells</th>
<th>Peripheral blood mononuclear cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cells (CD56+CD16-)</td>
<td>61 ± 4.5%</td>
<td>15 ± 1.7%</td>
</tr>
<tr>
<td>NKT cells</td>
<td>16.5 ± 1.5%</td>
<td>5 ± 1.3%</td>
</tr>
<tr>
<td>(CD56+CD16-CD3+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells (CD3+)</td>
<td>27 ± 2%</td>
<td>68 ± 3%</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td>13.5 ± 1.5%</td>
<td>33 ± 4.5%</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td>14 ± 2%</td>
<td>29 ± 4.5%</td>
</tr>
<tr>
<td>B cells (CD19+)</td>
<td>&lt; 1%</td>
<td>5 ± 4.2%</td>
</tr>
</tbody>
</table>
Daniela Constantinescu, Carmen Cozmei, Laurette Graziella Cozma, et al

**Fig. 7 a)** Uterine NK cells express CD56 at a high level but are negative for CD16 and **b)** partially coexpress CD3.

NKT (natural killer T) cells are a small subgroup of T cells expressing NK markers and have immunomodulatory roles during pregnancy. Their main characteristic is the presence of an invariant receptor TCRαβ resulted in the majority of decidual NKT cells from the rearrangement Vα24 – Ja Q Vβ11. NKT phenotype is CD3+ CD161+ Vα24+ (16). We defined NKT cells as CD56+CD16+/-CD3+. Although Tsuda (2001) found eight times more NKT in first trimester decidua than in peripheral blood, the uterine percentages noticed by us in the cases investigated to date are only 3.3 times higher than the peripheral ones (16).

T cells were reported to account for 10% of the decidual lymphocytes; although we found bigger percentages, the T cell population is much reduced compared to the peripheral blood.

**CONCLUSIONS**

Our study has revealed a feasible method in order to separate trophoblast cells and uterine leukocytes from fetal-maternal interface. This method is likely to prove useful in further investigations that could elucidate some aspects of the role of maternal immunocompetent cells in pregnancy evolution and to outline some useful immune tests for detecting and monitoring pregnancies with high obstetrical risk.

**REFERENCES**

4. Zhou Y, Genbacev O, Fisher SJ: *The human placenta remodels the uterus by using a combination of molecules that govern vasculogenesis or leukocyte*


