Identification of cytotoxic lymphocytes in decidua at term

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Abstract

Introduction: Although maternal immune tolerance for fetal antigens is necessary for the development of the fetus within the uterus, maternal immune system activity is nevertheless required for the proper course of labor. We have decided to focus on identifying the population of cytotoxic cells (CD56 and CD57 positive cells) that is in the decidua at the start of labor. Material and methods: For our analysis we have chosen to look at the decidua basalis. Thus decidua basalis tissue samples were collected from patients during cesarean section. We chose to focus specifically on the different zones of the decidua basalis (at the uterine and placental sites), analyzing tissue samples derived from patients during the different phases of labor. In total we examined tissue samples obtained from 31 patients and typified the population of cytotoxic lymphocytes in these tissues using flow cytometry. Results: In our study we observed significant changes in the population of cytotoxic cells, such as CD56 and CD57 positive mononuclear cytotoxic cells, within the overall population of lymphocytes at the spontaneous beginning of labor. The percentage of both CD56 and CD57 positive cells within the larger population of lymphocytes was the highest when uterine cervical dilation reached 2 cm. As labor advanced, the percentage of these cells decreased. Conclusion: The changes we found within the population of both CD56 and CD57 positive cells would seem to demonstrate changes in immune system activity and strongly suggest that such changes are necessary for the spontaneous beginning of labor.

Key words: cytotoxic lymphocytes, decidua, CD56+, CD57+

Introduction

Decidua plays a crucial role in the proper physiological course of labor. During the development of pregnancy this tissue is responsible for creating and maintaining the balance between maternal immune system activation and the immunosuppressive activity of fetal cells, such as trophoblasts [1]. Because the ovum implants in decidua, which is a specialized mucous membrane, the process of trophoblast invasion can be continued without any negative effect on the mother. This requires further explanation. Prior to ovulation, maternal immune cells begin to infiltrate the decidua as part of the proper preparation of this mucous membrane for future implantation [2, 3]. While on the one hand, these immune cells are responsible for the inhibition of trophoblast cell invasion, on the other hand, they help to create the maternal immune tolerance against fetal antigens [4, 5]. The maternal immune system recognizes the fetal antigens, but it is the trophoblasts that are actually able to suppress the potential attack of the maternal immune system through the expression of non-classical MHC antigens (such as HLA-G) [6-8] or through other molecular mechanisms linked to trophoblast cell membranes [9-12] and changes in the suppressive micro-environment of these invading trophoblast cells [1]. According to physiological principles, every immune cell activation causes the initiation of subsequent suppressive mechanisms [13], and in the development of pregnancy, one of the most important suppressive mechanisms is the recruitment of Treg cells to the endometrium [14-22]. The population of these cells increases over the course of pregnancy up until the initiation of labor. Recently, we described a decrease in Treg cell infiltration that can be detected in accordance with progression of labor [23]. Most likely, this decrease is associated with a cytotoxic immune cell activation that can be recognized at the spontaneous beginning of labor [11, 24-27]. Although maternal immune tolerance for fetal antigens is necessary for the development of the fetus within the uterus, maternal immune system activity is nevertheless required for the proper course of

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While there are many controversial components and processes involved in the activation of the maternal immune system at the beginning of labor, we have decided to focus specifically on identifying the population of cytotoxic cells that is in the decidua at the start of labor.

**Materials and Methods**

**Patients**

For our analysis we have chosen to look at the decidua basalis. Thus decidua basalis tissue samples were collected from patients during cesarean section. These tissue samples were then divided into two groups. The first group was sampled from patients during the curettage of the uterine cavity, just after the evacuation of the placenta from the uterine cavity. The second group of tissue samples was derived from the maternal portion of the placenta. Examining these two different groups of decidua basalis samples has allowed us to analyze the distribution of CD56 and CD57 positive cytotoxic cells in the decidua basalis. In our study we focused specifically on the different zones of the decidua basalis (at the uterine and placental sites), analyzing tissue samples derived from patients during the different phases of labor. In our model we used the tissue samples taken during cesarean sections that were performed in the subsequent phases of labor. We distinguished three groups of tissue samples. The first group of samples was derived from patients having elective cesarean sections without uterine cervical ripening. The second group was derived from patients during the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm). Finally, the third group consisted of tissue samples derived from those patients who had had cesarean sections during advanced labor. In all, we examined the tissue samples of 31 patients. The patient’s consent was obtained in each case. Prior to the present study we also obtained the approval of the Jagiellonian University Ethical Committee for our research program (KBET/135/B/2007). Patients with hypertension and diabetes mellitus, as well as patients with intra-uterine infections as confirmed by histopathological examination, were excluded from the study.

**Decidual mononuclear cell isolation**

The decidua and endometrium were cut into small fragments and disintegrated by being smashed through a 40 m cell-strainer. The realized cells were then spun down and the resulting pellet was subjected to ammonium chloride lysis in order to get rid of contaminating red cells. Following lysis, the cells were washed in PBS. Next the cells were re-suspended in PBS, counted, and used either immediately for staining or frozen for future analysis.

**Flow cytometry**

The cell phenotype was analyzed with the panel of mAb – CD57 FITC and CD56 APC (Pharmingen). Briefly stated, to the $1 \times 10^6$ cells suspended in 60 µl of staining buffer (PBS, 2% FBS) 20 ml of each mAb (CD56, CD57) was added. Next the cells were incubated in the dark for 30 min at 4°C. After incubation the cells were washed twice in PBS. The stained cells were then washed and collected using the FACSC anto-cytometer (Becton Dickinson; USA), and finally were analyzed with FACS Diva software (Becton Dickinson; USA). Each time $3 \times 10^4$ events were saved for analysis. Logical gates were used to analyze particular populations of cells.

**Statistical analysis**

The distribution of variables in the study groups of women checked with the use of the Shapiro-Wilk test showed that each of them was different from normal. The statistical significance between the groups was determined by the Kruskal-Wallis test, one-way analysis of variance by ranks. The Mann-Whitney U test was then used as applicable. All statistical analyses were carried out with the Statistica 8.0 software program. A $p$ value < 0.05 was considered indicative of statistical significance.

**Results**

**CD56 positive cells**

We observed statistically significant differences between the two zones of the decidua basalis with respect to the percentage of CD56 positive cells within the overall population of lymphocytes in the decidua. We found that the percentage of CD56 positive cells increased depending on the portion of the placenta from which the tissue was taken (the percentage of these cells was higher at the placental site).

The percentage of the CD56 positive cells within the population of lymphocytes changed significantly as labor progressed. These changes were observed in both zones of the decidua basalis at the uterine and placental sites.

The increase in the percentage of these cells at the beginning of spontaneous labor was statistically significant, but as labor advanced, the percentage appreciably decreased. The highest percentage of CD56 positive cells was observed when the dilation of the uterine cervix reached 2 cm.
Fig. 1. The characterization of the CD56 positive cell population within the overall lymphocyte population in decidua basalis at the uterine site as labor progresses: A) elective cesarean sections without uterine cervical ripening; B) the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm); C) advanced labor.

Table 1. The population of CD56 positive cells in both zones of the decidua basalis at the uterine and at the placental sites as labor progresses: A) elective cesarean sections without uterine cervical ripening; B) the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm); C) advanced labor.

<table>
<thead>
<tr>
<th>Labor phases</th>
<th>Decidua at the uterine site Median (IQR)</th>
<th>Decidua at the placenta site Median (IQR)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 10)</td>
<td>4.4 (5.5)</td>
<td>15.5 (9.4)</td>
<td>0.009</td>
</tr>
<tr>
<td>B (n = 12)</td>
<td>8.25 (5.25)</td>
<td>25.2 (15.45)</td>
<td>0.017</td>
</tr>
<tr>
<td>C (n = 9)</td>
<td>4.8 (1.8)</td>
<td>14.9 (13)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

CD57 cells positive cells

We observed statistically significant differences between the two zones of the decidua basalis with respect to the percentage of CD57 positive cells within the overall population of lymphocytes in the decidua. We found that the percentage of CD57 positive cells increased according to the portion of the placenta from which the tissue was taken and was higher at the placental site.

Table 2. The population of CD57 positive cells in both zones of the decidua basalis at the uterine and at the placental sites as labor progresses: A) elective cesarean sections without uterine cervical ripening; B) the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm); C) advanced labor.

<table>
<thead>
<tr>
<th>Labor phases</th>
<th>Decidua at the uterine site Median (IQR)</th>
<th>Decidua at the placenta site Median (IQR)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 10)</td>
<td>1.1 (1.2)</td>
<td>1.7 (2.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>B (n = 12)</td>
<td>3.4 (2.4)</td>
<td>4.8 (2.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>C (n = 9)</td>
<td>1.1 (0.5)</td>
<td>3.2 (3.4)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Additionally, the percentage of CD57 positive cells within the overall population of lymphocytes changed as labor progressed. These changes were observed in both zones of the decidua basalis at the uterine and placental sites.

At the beginning of spontaneous labor, the percentage of CD57 positive cells increased, but as labor advanced, the percentage appreciably decreased; the differences observed, however, were not statistically significant. The highest percentage of CD57 positive cells was noted when the dilation of the uterine cervix reached 2 cm.
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Fig. 3. The characterization of the CD57 positive cell population within the overall lymphocyte population in decidua basalis at the uterine site as labor progresses: A) elective cesarean sections without uterine cervical ripening; B) the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm); C) advanced labor

Discussion

In our study we observed significant changes in the population of cytotoxic cells, such as CD56 and CD57 positive mononuclear cytotoxic cells, within the overall population of lymphocytes at the spontaneous beginning of labor. The percentage of both CD56 and CD57 positive cells within the overall population of lymphocytes was the highest when uterine cervical dilation reached 2 cm. As labor advanced, however, the percentage of these cells decreased.

There is still no precise definition of the beginning of labor, particularly from a clinical point of view. Moreover, while in clinical practice it is easy to recognize the progression of labor, there is no accurate way to predict the spontaneous beginning of labor, and for this reason we have elected to identify and analyze the population of cytotoxic lymphocytes within the decidua basalis at the beginning of labor. To our knowledge, this is the first investigation to focus on the alterations in both CD56 and the CD57 positive cells in the decidua relative to the progression of labor.

Immune cells infiltrate the endometrium and decidua. The presence of these cells in the endometrium and their proper level of activity is crucial for the successful course of such processes as implantation, menstruation, abortion, and labor [1, 20]. The majority of immune cells are uterine NK cells and macrophages, but T cells are also present in the endometrium [2, 3]. Although the roles of both NK cells and macrophages in the endometrium have been widely described [20, 28], the regulation of immune cell activity in the endometrium remains unclear. In particular, the role of T cells in the endometrium has not been well elucidated [21]. Recently, however, both the presence of T regulatory cells and the important role they play by infiltrating decidua in the proper development of maternal immune tolerance against fetal antigen has also been described [14-17]. Although suppressive mechanisms within decidua have been well documented, there is still not enough evidence about the effector part at the maternal-fetal interface, and this component of the immune system within the decidua seems to be linked with T cell population. Sindram et al. has investigated the distribution of mononuclear cells in different sections of the decidua – in both decidua basalis and decidua parietalis – during labor [24-26]. In this study, significant differences were observed between the decidua basalis and the decidua parietalis with respect to the population of such cells as CD56(dim)CD16(+) NK cells and CD3(+) T cells expressing CD25, and (HLA)-DR [24-26]. Furthermore, significant differences were...
found between the samples derived from women undergoing spontaneous labor and those from women undergoing elective cesarean section. By contrast, in our study we focused on the different zones of the decidua basalis, analyzing tissue samples derived from patients during the different phases of labor. In our model we used the tissue samples taken during cesarean sections that were performed in the subsequent phases of labor. We distinguished three groups of tissue samples. The first group of samples was derived from patients having elective cesarean sections without uterine cervical ripening. The second group was derived from patients during the spontaneous beginning of labor with regular uterine contractions and the beginning of uterine cervical ripening (when the degree of dilation increased from 1 to 2 cm). Finally, the third group consisted of tissue samples derived from those patients who had had cesarean sections during advanced labor. We have previously used this same phasic model successfully in our studies [23, 29]; for example, when we stained the population of lymphocytes in decidua for analysis by immunohistochemistry. In that study we found that the beginning of labor is associated with the accumulation of both CD56 and CD3 positive cells within the decidua basalis [29]. Immune cell activation during the beginning of labor was also found. By Szekers-Bartho et al. [5] and Abadía-Molina et al. [27]. The progression of labor was associated with a decrease in both CD56 and CD57 positive cells within decidua, and this process was observed in each of the two zones of the decidua basalis. Suppression of the cytotoxic immune response in accordance with the progression of labor has already been described [11, 12, 23]. In our previous studies we demonstrated that the advancement of labor is associated with an increase in immunoreactivity within the decidua of the particular proteins (such as RCAS1) responsible for the suppression of cytotoxic lymphocytes and with an increase in the concentration in the blood sera of soluble forms of proteins such as HLA-G and RCAS1 [8, 11, 12]. Both RCAS1 [30-33] and HLA-G [34, 35] are well known to be strong suppressive factors, and the presence of these proteins within the decidua microenvironment is responsible for the inhibition of the activated immune cytotoxic cells and even for their apoptosis. Additionally, we observed the accumulation of suppressive macrophages (CD14 + B7H4+) within the decidua as labor progressed [23]. Most likely, the decrease in cytotoxic immune cell infiltration in accordance with the progression of labor is a result of the accumulation of suppressive factors.

To sum up, the changes we found within the population of both CD56 and CD57 positive cells would seem to demonstrate changes in immune system activity and strongly suggest that these changes are necessary for the spontaneous beginning of labor.

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References
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