Immunoregulatory Effects of Human Amnion Epithelial Cells on Natural Killer and T Cells in Women with Recurrent Spontaneous Abortion (RSA)

Tekrarlayan Gebelik Kaybı Olan Kadınlarda İnsan Amniyon Epitel Hücrelerinin Doğal Öldürücü ve T Lenfositleri Üzerine Olan Etkisi

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Introduction: Unexplained Recurrent Spontaneous Abortion (URSA) is the most common immunological complication during pregnancy and defined as two or more sequential miscarriages before the 20th week of gestation, which affects approximately 1% to 5% of couples.1-3 Many causes have

Materials and Methods: Peripheral Blood mononuclear cells (PBMC) were obtained from 14 URSA patients and co-cultured with isolated hAECs. NK cells and T cells were identified using anti-CD56 and anti-CD3 monoclonal antibodies (mAb). The expression of the activating receptor CD69 and the degranulation marker CD107a on NK cells and T cells were detected using specific mAb and analyzed by flow cytometry.

Results: We found that CD69 activating receptor expression on NK cells and T cells was significantly decreased by incubation with hAECs in a dose-dependent manner (p=0.049). Also, the degranulation marker CD107a was significantly downregulated on NK cells and T cells following incubation with hAEC (p=0.003).

Conclusion: Our results suggest hAECs have immune regulatory effects on activation and cytoxicity of NK and T cells. Potential therapeutic application of hAECs for dysregulated NK and T cells immunity should be investigated in the future.

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been postulated to play a role in the etiology of RSA, however in more than half of RSA cases, the etiology is unknown which, this condition is defined as Unexplained Recurrent Spontaneous Abortion (URSA).\textsuperscript{[4–7]}

Immunological factors in both innate and adaptive immunity may be involved in RSA pathogenesis.\textsuperscript{[8–11]} It has recently been shown that URSA is similar to an alloimmune condition\textsuperscript{[9]} and evidence of in vitro assays has shown that immune cells such as T (CD3\(^+\)) and NK (CD56\(^+\)) cells are responsible for embroytotoxicity. Also, the numbers and activities of these cells are suppressed in normal pregnancies.\textsuperscript{[12–15]} NK cells are effector lymphocytes of the innate immune system which are present in peripheral blood and in the uterine and play an important role in maintaining pregnancy.\textsuperscript{[16,17]} These cells are the most abundant population of lymphocytes (about 70–90\%) in the uterine implantation site.

According to the cell surface markers, NK cells are generally divided into CD56\(^{dim}\)/CD16\(^{-}\) and CD56\(^{bright}\)/CD16\(^{-}\)NK cells groups. CD56\(^{bright}\)/CD16\(^{-}\)NK cells are mainly found in the uterine (UNK) whereas NK population in peripheral blood (PNK) are principal in the uterine (UNK) whereas NK population in peripheral blood and in the uterine and play an important role in maintaining pregnancy.\textsuperscript{[16,17]} These cells are the most abundant population of lymphocytes (about 70–90\%) in the uterine implantation site.

Human Amniotic Epithelial Cells (hAECs) with immunomodulatory effects are the candidate as a novel stem cell therapy.\textsuperscript{[25,26]} These cells release some factors with anti-inflammatory properties which can inhibit immune cells in both the innate and adaptive immune systems.\textsuperscript{[27]} Numerous recent investigations have demonstrated that hAECs are able to produce soluble factors such as MIF (migration inhibitory factor), TRAIL, TNF-\(\alpha\), FasL, TGF-\(\beta\) (Transforming Growth Factor-\(\beta\)) and PGE2 (prostaglandin E2). It has also been shown, that freshly isolated hAECs have low immunogenicity because they express low levels of HLA-class I and lack HLA-class II expression. These cells have stem cell-like properties and are not tumorigenic.\textsuperscript{[25,26,28–34]} According to the above-mentioned descriptions, hAECs have features that may act as an immunomodulatory agent for different diseases. So this has motivated us to investigate the effect of hAECs on activation and cytotoxic activity of NK and T cells by in-vitro co-culture of these cells with peripheral blood NK cells and T cells from women with (RSA).

Materials and Methods

Isolation of hAEC

Fourteen human placentas were collected from women with elective normal cesarean deliveries (37–40 weeks). After explaining the purposes of the research, oral and written informed consent was obtained. Amniotic membrane was mechanically stripped from corion and placed in RPMI 1640 (Gibco) medium culture containing penicillin/streptomycin (Gibco), then was transferred to the laboratory. Human Amniotic Epithelial Cells (hAECs) were isolated using the procedure as previously defined\textsuperscript{[35–37]}, with minor modifications. Briefly, an amniotic membrane was rinsed 2 or 3 times with phosphate-buffered saline (PBS) and washed amnion was cut into small pieces and was digested thrice in 0.05\% trypsin (Gibco): EDTA at 37°C in a shaking water bath for 20 min. Following digestion, trypsin was inactivated with fetal bovine serum (FBS) (Gibco) and the hAECs were collected by centrifugation (1800 RPM, 5 min) and suspended in DMEM/F12 culture medium (Gibco). Isolated hAECs were typically ≥95\% pure with calculated for cytokeratins 7, 8/18 by flow cytometry. The cells were counted and their viability was determined using trypan blue and finally plated in flat-bottomed tissue culture plates to perform co-culture experiments.

 Patients and Samples

Human peripheral blood samples were collected from 14 women with unexplained RSA who had two or more consecutive RSA. The average age of patients was between 21–41 years. After explaining the purposes of the study, oral and written informed consent was taken and the study was approved by the ethics committee from the Isfahan University of Medical Sciences. URSA patients with immune diseases, infectious diseases, metabolic diseases, congenital thrombophilia, autoimmune diseases and patients under immunotherapy were excluded from this study.

PBMC isolation, activation and co-culture with hAEC

Peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Hypaque (Biosera, France) density gradient centrifugation, washed three times and then resuspended in RPMI 1640 medium culture. 1 × 10\(^6\) PBMCs/well...
were cultured alone or co-cultured with isolated hAECs in RPMI1640 (Gibco) supplemented with 10% FBS (Gibco), 2 mM glutamine, Penicillin (100 U/mL) and Streptomycin (100 μg/mL) (Gibco) and IL-2 (100unit/mL) (eBiosciences, USA) at ratios of 1:5, 1:1, 5:1 hAECs/PBMCs for 3 days. The cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO₂. All co-cultured were compared to PBMCs culture in complete medium plus IL-2 cytokine alone without hAECs.

**Mixed lymphocyte reactions (MLR)**

Human Amniotic Epithelial Cells (hAECs) alloreactivity was assessed by mixed lymphocyte reaction (MLR). In brief, responder PBMCs were cultured in a 96-well flat-bottom tissue culture plate along with hAECs as stimulator at 1:1, 1:2, 1:3 ratios for 3.5 and 8 days in duplicate. The proliferative allogeneic reaction was determined using a proliferation BrdU cellular ELISA Kit (Abnova. Taiwan). Positive control consisted of PBMCs plus anti-CD3 and anti-CD28 (all ebiosciences, USA) alone. Negative controls consisted of PBMC alone and hAE cells alone in culture media.

**Flow cytometry**

After incubation for 3 days, the cells were washed three times with PBS and blocked by a human FC blocker solution (Miltenyi, Biotec, USA). Cells (2–3×10⁶) were resuspended in 1 ml PBS with 1% FBS, and were stained by use of the following conjugated mAbs for 45 min at 4 °C in the dark: mouse anti-human CD3-FITC, mouse anti-human CD56, mouse anti-human CD69, CD107a (all eBiosciences San Diego, CA, USA). The following isotype controls were used: Mouse IgG1 K-FITC, Mouse IgG1 K-PE, Mouse IgG1 K PE-Cy5 (all eBiosciences San Diego, CA, USA). Flow cytometry was carried out on a BD FACSCalibur cytometer (Becton Dickinson, San Jose, CA) and 20,000 cells were counted in each experiment. Data analysis was performed with the CellQuest software (Becton Dickinson). In order to identify lymphocyte populations, we first gated on cells based on forward (FSC) and side scatter (SSC) properties. This population was gated further as NK cells and T cells, on viability as assessed by CD56 positivity, CD3 negativity, and CD56 negativity, CD3 positivity respectively.

**Assay of CD107a degranulation**

Cytotoxic granules in NK cells contain different lytic enzymes such as perforin, granzyme and the lysosome-associated membrane proteins CD107a/LAMP-1. CD107a is a marker of NK cells cytotoxic activity which its surface expression has been frequently used as a marker for degranulation. Cytokine-stimulated (IL-2, 150 U/mL) PBMCs were co-cultured with human amniotic epithelial cells at ratios of 1:5, 1:1, 5:1 hAECs/PBMCs for 3 days. The cells were then stained with PE-conjugated anti-human CD107a mAb and incubated for 5 h in a humidified incubator with 5% CO₂ at 37°C. Monensin (Biolegend) was added to each well at a final concentration of 6 μg/mL during the last 4 h of the culture to inhibit the degradation of CD107a from the cell surface and ensure the detectability of this marker after stimulation. To determination of the background level of degranulation unstimulated PBMCs were similarly treated in parallel and IL-2 (100 U/mL) stimulated PBMCs served as the positive control. Following 5 h incubation, cells were harvested and stained with FITC-conjugated anti-human CD3, PE-Cy5-conjugated anti-human CD56 (eBiosciences San Diego, CA, USA) for NK and T cell detection. Populations of NK cells were defined as lymphocytes that were CD3-negative and were further defined by their expression of CD56. Cd107a expression on CD3-CD56⁺ NK cells and CD3⁺ CD56⁻ T cells was examined on a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA).

**Flow cytometric analysis of CD69 expression**

CD69 expression was assessed on NK and T cells by means of flow cytometry in order to identify NK and T cells activation. The detection of activated cells was assayed by using FITC-anti-CD3, PE-Cy5-anti-CD56 and PE-anti-CD69 (all eBiosciences San Diego, CA, USA) in cold PBS plus 1% BSA. A cell gate region was drawn around lymphocytes in order to exclude debris. The NK cells were defined as lymphocytes that were CD3-negative and were further defined by their expression of CD56. The expression of surface CD69 was assessed in the CD3⁻ CD56⁺ NK cells and CD3⁺ CD56⁻ T cells gated population by FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA).

**Statistical analysis**

All statistical analyses were performed using SPSS Statistics version 20.0 (2011, SPSS Inc., Chicago, IL, USA). A two-tailed t-test was used to analyze the results between study subjects and controls. The statistical comparisons among groups (doses) were performed using univariate analysis.
of variance. The results were reported to be statistically significant if the \( P \) value was <0.05.

**Results**

**Purity of hAECs and their Effects on proliferative allogenic response in PBMCs**

Isolated hAECs were typically ≥95% pure with calculated for cytokeratins 7, 8/18 by flow cytometry (Fig. 1a). We analyzed the *in vitro* allogenic effect of hAECs in co-cultured assay that incorporated hAECs together PBMCs. In contrast to the highly proliferative reaction induced by PMA/I and anti-CD3/anti-CD28 stimulation in positive control (\( p=0.0001 \)), hAECs did not stimulate a proliferative allogenic response when cultured with PBMCs in different doses and days (Fig. 1b).

**Downregulation of CD107a expression in NK cells by Human amnion epithelial cells (hAECs)**

The cell surface expression of CD107a on NK cells (CD3−CD56+) incubated with hAECs was significantly decreased at a ratio of 5:1 (22.5±14.2) for 3 days compared with control (32±17.5). Moreover, the comparison between 3 different ratios was shown that the expression of CD107a on CD3−CD56+NK cells was significantly lower in co-cultured hAECs and PBMCs at the ratio of 5:1 (\( p=0.007 \)) as compared with two other ratios (1:1 and 1:5) (Fig. 2). Also, we did not find a significant difference between CD56 dim and CD56bright NK cells in CD107a expression. The range of values is shown in Table 1.

**Suppression of CD69 expression in NK cells by Human amnion epithelial cells (hAECs)**

To evaluate the effect of hAECs on NK-cell activation potential, PBMCs were co-cultured with hAECs cells for 3 days. Flow cytometric analysis revealed a significant decrease in CD69 expression on NK cells at high concentration of hAECs in co-cultured group (5:1) (35.2±18.5) compared to control (45.9±22.5) (\( p=0.049 \)). However, no significant differences were observed in CD69 expression between 3 co-cultured ratios (Fig. 3).

**Downregulation of CD107a expression in T cells by Human amnion epithelial cells (hAECs)**

We also assessed the impact of the hAECs on the T cell surface expression of CD107a. Flow cytometry analysis shown a significant decrease in CD107a expression on T cells at 1:1 (10.8±4.1) (\( p=0.003 \)) and 5:1 (9.7±3.1) (\( p=0.003 \)) ratios as compared with control (13.73±3.3) (Fig. 4). No significant changes in CD107a expression were observed between 3 different co-cultured groups.

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**Figure 1. A, B. Purity of hAECs and their allogeneic effects on PBMCs.** **A:** Isolated hAECs were analyzed with pan-cytokeratin markers (7, 8/18) labeled with FITC and the purity of cells was typically ≥95%. **B:** PBMCs were co-cultured with different numbers of hAECs for 3 days. A BrdU cellular ELISA kit was used to measure alloreactive response of PBMCs. Stimulated PBMCs with anti-CD3/CD28 antibodies were used as positive control. The depicted results are representative of 4 independent experiments. All data show mean ± SEM.
Suppression of CD69 expression in T cells by Human amnion epithelial cells (hAECs)

To confirm whether hAECs suppress T cell activation, we explored the expression level of CD69 in T cells after IL-2 stimulation followed by the treatment with or without hAECs (Fig. 5). As shown in Figure 5, the expression of CD69 on CD3+CD56− T cells was significantly decreased in all co-cultured groups (1:5, 1:1, 5:1) (8±3.2, 6.9±2.9, 5.7±2.9).

Table 1. Percentages of CD69+ and CD107a+ in NK, T, and NKT cell subsets in co-culture with hAECs

<table>
<thead>
<tr>
<th>Cell subsets</th>
<th>1:5</th>
<th>1:1</th>
<th>5:1</th>
<th>Control</th>
<th>1:5</th>
<th>1:1</th>
<th>5:1</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3−CD56+</td>
<td>45.1% (17.6-92)</td>
<td>47.6% (13.7-77)</td>
<td>35.2% (8.3-62)</td>
<td>43.9% (11.5-89)</td>
<td>31.8% (4.5-20)</td>
<td>31.1% (4.6-18.5)</td>
<td>22.5% (5.6-19.6)</td>
<td>32% (11.5-66)</td>
</tr>
<tr>
<td>CD3+CD56-</td>
<td>8% (2.5-15.5)</td>
<td>6.9% (2.1-13.5)</td>
<td>6.5% (1.5-13)</td>
<td>9.7% (4.1-15.5)</td>
<td>12.4% (4.2-25)</td>
<td>10.8% (4.1-19.9)</td>
<td>9.7% (4.3-17)</td>
<td>13.7% (6.7-20)</td>
</tr>
</tbody>
</table>

*Intra-group significant difference (p≤0.05) between control and co-cultured hAECs/PBMCs at ratio of 5:1
*bIntra-group significant difference (p≤0.05) between control and co-cultured hAECs/PBMCs at ratio of 1:1

Figure 2. A, B. Effect of hAECs on NK-cells CD107a expression (A and B). Freshly isolated PBMCs from women with RSA (n=14) were co-cultured with 3 proportions of hAECs (1:5, 1:1, and 5:1). A: CD107a expression on CD56+/CD3− NK cells was analyzed by flow cytometry with gating strategy. Representative FACS dot plots of CD107a analysis are presented. In order to identify lymphocyte populations, we first gated on cells based on forward (FSC) and side scatter (SSC) properties. This population was gated further as NK cells on viability as assessed by CD56 positivity and CD3 negativity. Also, isotype control has indicated as negative population. Numbers represent the percentages of CD107a+ CD56+/CD3− NK cells. B: Percentage of CD107a+ cells within CD56+/CD3− NK cells was analyzed by flow cytometry. (**p<0.001)

Figure 3. A, B. Effect of hAECs on NK-cell activation (A and B). Freshly isolated PBMCs from women with RSA (n=14) were treated with indicated ratios of hAECs and without hAECs as control. (A, B) CD69 expression on CD56+/CD3− NK cells was analyzed by flow cytometry with gating strategy. Representative FACS dot plots of CD69+ NK-cells analysis are presented. In order to identify lymphocyte populations, we first gated on cells based on forward (FSC) and side scatter (SSC) properties. This population was gated further as NK on viability as assessed by CD56 positivity and CD3 negativity. Numbers indicate percentages of CD69+ CD56+/CD3− NK cells. (C, D) Percentage of CD69+ cells within CD56+/CD3− NK cells and CD56+/CD3+NKT cells was analyzed by flow cytometry. (*p<0.05)
6.5±2.9 respectively) as compared with control (9.7±3.43). Also, no significant difference between the three hAECs ratios in co-cultured groups was observed.

**Discussion**

Several studies have recently shown that hAECs possess potent immunosuppressive and immunomodulatory properties. These cells like other fetal-derived placental cells evade maternal immune detection and secrete factors known to suppress maternal immune reactions against the fetal semi-allograft. Additionally, the presence of pathogenic autoantibodies such as anti-phospholipid antibodies (APAs), anti-thyroid antibodies (ATAs), and the pathologic role of cellular immunity, NK cells and, NKT cells have been reported in women with RSA. According to immunomodulatory properties of hAECs,
We examined whether co-culture of hAECs with PBMCs of URSA patients could modulate expression of early activation marker CD69 and a cytotoxic activity marker CD107α on NK cells and T cells.

To our best of knowledge, for the first time, we found that CD107α and CD69 expression was significantly decreased on CD3-CD56+ NK cells and CD3-CD56+ T cells after treatment with hAECs dose dependently. CD107α is a marker of degranulation on lymphocytes such as CD8+ T cells, NK cells. This marker is upregulated on these cells following stimulation and its induction is accompanied by cytokine secretion and target cell lysis. In addition, CD107α may express on the large piece of activated NK cells that may degranulate in the absence of cytokine secretion. Also, we found that CD56dim and CD56bright NK cells both express CD107α however, there is some discrepancy between various reports. Alter et al. have shown a significant percentage of CD56bright NK cells becomes CD107α upon target cell-mediated activation. On the other hand, Penack et al. have reported that CD56dim NK cells, are the majority of the CD107α-cells and are not cytotoxic at all towards K562 targets. Consistent with our results, Nagler et al. have persuasively described that freshly isolated purified CD56dim NK cells display a very strong cytotoxicity towards K562 and that even CD56bright NK cells kill the same target to some degree. Also, studies have shown both CD56 dim and CD56bright NK cell subsets expressed CD69 as an activation marker.

Kwak-kim et al. have shown that the expression of CD107α on surface of NK cells, is higher in women with recurrent pregnancy losses (RPL) as compared with normal controls and 1.25 (OH) 2 D3 treatment has reduced this marker. According to studies, URSA patients have an excessive expression of CD69 on NK cells and T cells as compared to normal fertile women and this can one of the main risk factors of immunological disturbances during implantation. Our results are in agreement with those reported by Li et al. in that a significant downmodulation of CD69 gene expression in NK cells has been shown after co-culturing with amnion-derived cells. Ramhorst et al. showed that women with RSA have high percentage of CD3+CD69+ T cells in peripheral blood as compared with normal fertile women which after alloimmunization with parental leukocytes in these patient, CD3 + CD69+ cells reached similar to those observed in normal fertile women. Also, consistent with our finding Motedayyen et al. have found hAECs suppress activation of naïve CD4+ T cells from women with RSA.

There is a bidirectional regulation between NK cells and other immune cells especially CD4+ T cells in autoimmune disorders and dysregulation between these cells is possibly happened in RSA to change the immunosuppressive environment required for fetal tolerance. Some immunomodulatory characteristic of hAECs such as low levels of HLA Class IA, lack of costimulatory molecules CD80/86 and secretion of TGF-β and IL-6 have indicated in different studies. The in vitro assessment of hAECs allogenic effects in our study indicated that hAECs did not stimulate a proliferative allogeneic response when cultured with PBMCs. According to previous studies, since hAECs cells do not express HLA class IA and HLA-DR molecules, our consequence is expected.

Human AECs express HLA-G on their surface or in soluble form and Lim et al. have indicated term hAECs express higher levels of HLA-G compared to preterm hAECs. The highest expression of HLA-G is in the placenta, where its main role involves protection of the semi-allogenic fetus from allo-reactive immune responses. The binding of HLA-G to killer immunoglobulin-like receptors (KIRs) and/or immunoglobulin-like transcript (ILT) on CD4+ and CD8+ T cells and NK cells inhibits proliferation of these cells. HLA-G modulate the cytotoxic activity of NK cells and also could inhibit harmful NK cell-mediated cytotoxicity. So decrease of CD69 and CD107α expression after co-culture with hAECs may be related to HLA-G expression by these cells.

Additionally, Shen et al. have shown hAECs express Programmed death-ligand 1 and 2 (PD-L1 and PD-L2). Engagement of PD-L1 and PDL-2 with their receptor PD-1 on T cells induces an inhibitory signal that suppresses TCR-mediated activation and T cell proliferation. Additionally, Sieverat et al. reported that hAEC transplantation induces a M2 macrophage phenotype related with reduction of established hepatic fibrosis. M2 macrophages produce high levels of anti-inflammatory cytokines such as IL-10, TGF-beta and low levels of IL-12. Therefore, it seems that hAECs exert their immunomodulatory effects on NK cells and T cells both directly by expressing HLA-G, PD-L1/PD-L2 and indirectly through production of anti-inflammatory cytokines by M2 macrophages.

However, yet there is still very limited data on the cytotoxicity role of NK cells and T cells and immune regulatory effects of hAECs in RSA. Further study is
needed to investigate hAECs effect on cytotoxic activity of NK cells and T cells in women with URSA.

Different studies have demonstrated hAECs that hAECs due to their anti-inflammatory properties could be used to alleviate the burden of some diseases such as MS, inflammation-induced fetal lung injury and liver disease. [30,66,67]

So, it seems that hAECs may interfere with the early phase of T and NK cells activation and could be used for attenuate immunologic responses in RSA however more detailed studies are needed to link the observed phenotypic changes with a biological implication of CD69 and CD107a downregulation in the control of RSA. However, our study was limited by a small study population, the absence of a pathophysiologic investigation of T cells, NK cells and NKT cells, and no data regarding cytokines, activatory and inhibitory receptors. Also, due to limitation in flow cytometry staining (we had 3 colors flow cytometry) we could not assess CD107a expression on CD3-CD16bright NK cells and CD3+CD8- T cells.

Conclusion

According to this study hAECs are capable of decreasing the activation and cytotoxic activity of CD56+CD3- T cells and CD56+CD3- NK cells and this could make hAECs an attractive candidate of cellular therapy for women with URSA.

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Ethics Committee Approval: This study was approved by the ethics committee of the Isfahan University of Medical Sciences.

Informed Consent: After explaining the purposes of the study, oral and written informed consent was taken.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The Authors declare no conflict of interest.

Author Contributions: Concept: NE, Design: NE, FK, Supervision: NE, AR, Resources: NE, Materials: NE, BR, Data collection and/or processing: FK, HM, Analysis and/or interpretation: NE, FK, Literature search: FK, HM, Writing manuscript: FK, NE, Critical review: NE, AR.

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