The importance of markers HLA6 and CD68 in placenta tissues of recurrent pregnancy loss.

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ABSTRACT: *Introduction:* Recurrent pregnancy loss of unknown etiology is correlated with immunological factors during pregnancy. Changes in leukocyte subpopulations and *HLA (Human Leukocyte Antigen)* expression take place in pregnant uterus on both decidua basalis and decidua parietalis in order to carry the semiallogenic embryo. These changes affect the pregnancy course.
Objective: Our research is focused to enlighten the immunological changes that take place in the uterus of women with recurrent abortions of unknown etiology during first trimester of pregnancy.
Materials and methods: The miscarriage group was obtained from 25 women who miscarried between the ages of 35 to 42 years and controls consisted of 25 healthy women between the ages of 27 to 39 years, who had electively terminated their pregnancies during first trimester of pregnancy. The abortion was processed and specimens taken were studied, using immunohistochemical methods. Specimens were taken from decidua basalis and decidua parietalis. Monoclonal antibodies were used against *HLAG (Human Leukocyte Antigen G)* and CD68 (Cluster of Differentiation 68). The results were statistically analysed with Mann-Whitney test.
Results: HLA-G expression in decidua basalis from miscarriage group was found decreased.CD68 + cell expression was found increased in both deciduas from the miscarriage group.
Conclusion: The immunological profile of women with recurrent miscarriage is quite different comparing with controls. A possible role of CD68+cells in RPL was observed .Changes in HLA-G expression was observed.

Key Words: Recurrent miscarriage, CD68, HLA-G.

INTRODUCTION

Recurrent pregnancy loss (RPL) constitutes a challenge in the field of reproductive medicine as the etiology, in approximately 50% of cases of RPL, remains unknown. It has been assumed that immune cause is associated with RPL, with few available evidence-based diagnostic and treatment guidelines. The etiology of RPL is considered complicated, subdivided into embryological (mainly due to an abnormal embryonic karyotype) and maternal causes, affecting the endometrium and/or placental development (coagulation disorders, autoimmune defects, endocrine disorders and endometrial defects).

Various alterations take place in the maternal immunity system during gestation, ensuring the protection of the semi-allogenic fetus and the maintenance of pregnancy. More specifically, Human Leukocyte Antigen *(HLA*) gene expression status, lymphocyte populations and complement proteins are all submitted to specific changes in order a possible abortion to be avoided. It seems that trophoblast cells control monocyte migration and differentiation, producing a pro-inflammatory cytokine and chemokine profile, essential for their survival and growth[1].

The attack of maternal immune cells against paternal *HLA* class 1 antigens can be prevented by regu-

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lating firmly the expression of specific HLA class 1 molecules in subpopulations of trophoblasts[2]. The extravillous trophoblasts migrating into the deciduas display a unique pattern of *HLA* class 1b molecules, with *HLA-E, F* and *G* predominating. *HLA-G* activates different pathways in uterine NK cells and macrophages and alters their killer function by interacting with leukocyte inhibitory receptors (LIR's) on uterine natural killer cells (uNK) and macrophages and with the T cell receptor on CD8+ (Cluster of Differentiation) cells[3,4]. *HLA-G* expression in antigen presenting cells suppress T-cell proliferation, via apoptosis[5]). Polymorphism and methyliosis of *HLA-G* antigen are associated with recurrent pregnancy loss[6,7]. Moreover, sted thatHLA-G is not involved in T-cell suppression exclusively with its membrane-bound form, but also with a soluble isoform in maternal serum[8,9,10,11].

Regarding macrophages, their collaboration with trophoblast promotes the normal forming of placenta by enabling vascular remodeling and tissue homeostasis[12]. Decidual CD68+ cells (macrophages) excrete immunosuppressive agents (Prostaglandin E2), and downregulate inflammatory responses at the feto-maternal interface that help to prevent some uterine infections in pregnant women. The quick and effective removal of apoptotic cells by tissue macrophages prevents the release of self-antigens, and in the case of pregnancy, paternal alloantigens[13].

Our aim was to examine the immune profile of women with recurrent pregnancy loss of unknown etiology, during first trimester of their pregnancy, using immunological markers for CD68 factor as well as the *HLAG* expression in two groups of decidual tissues.

MATERIALS AND METHODS

The miscarriage group was obtained from 25 women, between the ages of 35 to 42 years, who miscarried during the 1st trimester of gestation and controls consisted of 25 healthy women, between the ages of 27 to 39 years, who had electively terminated their pregnancies, during the 1st trimester of gestation. All samples were collected after obtaining informed consent from patients. All 25 women from the miscarriage group had a history of at least three prior first trimester miscarriages of unexplained aetiology (normal parental karyotypes, intrauterine structural study, luteal phase endometrial biopsy, hormone concentrations and negative cervical cultures, lupus anticoagulant and antibodies to cardiolipin and phosphatidyl serine).

Pathology-examination

Tissues

Tissues were collected, immediately after miscarriage or elective abortion and washed with distilled water for removal of mucus and blood, then, were studied under stereomicroscope, so that tissues from decidua, villus chorion and parts of the embryo, could be distinguished and examined for formation abnormalities or placental lesions. Specimens were collected from the above stabilized in aqueous solution that consisted of neutral formalin 10% v/v for 12-24 hours and then were placed in automatic machine for further processing, including fixation, dehydration, xylene clarification and paraffin embedding. Then, sections in 3-5 mm transferred to positive charged and properly prepared glass plates, which were kept in an oven, at 37-40°C for 30-45 min. After this step, specimens were stained with haematoxylin-eosin solution (Harris) and the most suitable of them were selected for immunohistochemical study.

Immunohistochemistry

In all specimens, decidua basalis was identified using the antibody cytokeratin (CK7), which is positive in trophoblastic cells (Figure 1). Furthermore, for the discrimination between decidual and trophoblastic cells at the fetomaternal interface, duplicate sections were stained with a monoclonal antibody against prolactin, for the visualization of decidual cells (Figure 2).

The unstained specimens were further processed using an automatic machine (Bond Max) that carried out the following procedures. First, deparaffinization was performed in xylene. Afterwards, specimens were immersed in absolute alcohol, in decreasing concentration 100%, 96% and 70% v/v consecutively and were rinsed with distilled water. Antigen retrieval was performed by incubation in various temperatures, depending on the antibody that was examined each time.

Following this procedure, specimens were at first rinsed with PBS buffer, then incubated in H_2O_2 for

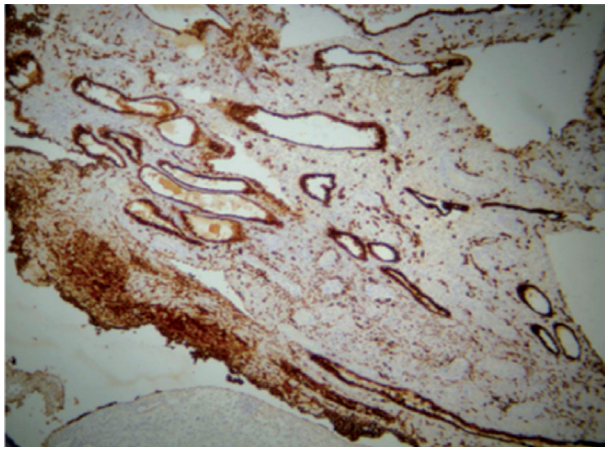

Figure 1. Control group. Decidua Basalis. CK7. Detection of trophoblastic cells. x16.

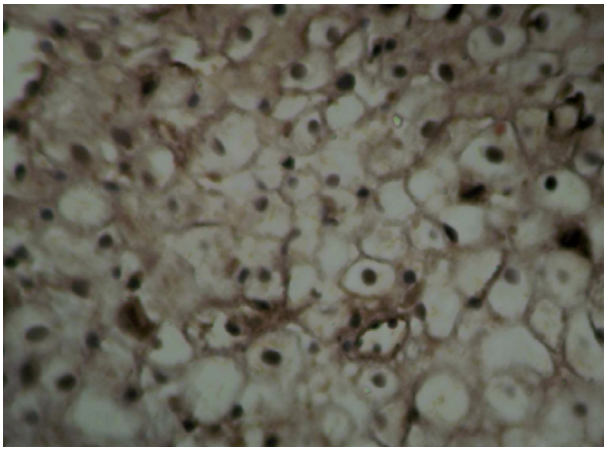

Figure 2. Decidua basalis. Prolactin detection in decidual cells. X160.

5 min, to quench endogenous peroxidase activity and finally rinsed again with PBS buffer. Thereafter, specimens were covered with a solution of the primary tonic monoclonal antibody, one of the five used in our study. These antibodies as mentioned above are: HLAG (clone MEM-G/2: sc-51676, Santa Cruz Biotechnology, Inc USA), CD68 (clone 514H12, Catalog No: PA0273, Leica Microsystems UK). Eventually, specimens were washed using WAS solution.

For the detection of the immunohistochemical staining, specimens were firstly immersed in Post-Primary solution. After being washed off specimens were immersed in Polymere solution and then in chromogen diaminobenzidine (DAB) solution. Finally, specimens were stained with Haematoxylin- eosin. Following the previous stages that were performed by the automatic processor, specimens were rinsed in tap water and dehydrated with escalating densities of ethanol solution (70, 96 and 100% v/v consecutively) and xylene. Then, they were covered with tape, placed in glass plates and immersed in Canada balsam.

The previously reported immunohistochemical staining procedure was repeated for each of the 2 antibodies that were examined in this study. The monoclonal antibody for CD68 was already set and ready to be used, while the monoclonal antibody for HLAG needed to be in dilution 1:50 correspondingly.

Microscopic evaluation was performed to the cells of the intermediate trophoblast on decidua basalis and decidua parietalis of recurrent miscarriage and elective abortion material. Specimens were examined using an optical Zeiss™ microscope and photographs were taken using a Contax™ camera, attached to the microscope. Totally, 100 specimens (50 from the implantation site and 50 from desidua) were examined. Intensity of staining was evaluated as negative (-), weak (+), moderate (++) and strong (+++). Finally, the results were statistically analysed and checked for their significance using the Mann-Whitney test.

RESULTS

Regarding the immunohistochemical staining for CD-68 cells, all sections (n = 25) of decidua basalis in control group were found negative (-) (Figure 3a). In contrast, the staining for CD-68 cells was detected positive in all sections (100%) of decidua basalis in miscarriage group (Figure 3b). Specially, 3 out of 25 (12%) sections of miscarriage group presented moderate (++) staining, with the rest of them (88%) to be strong positive (+++) (Figure 3b). Observing the sections of decidua parietalis of control group, CD68 cells were not identified in none of them (0%). On the contrary, intensity of immunohistochemical staining for CD-68 cells on decidua parietalis of women from miscarriage group was detected moderate (++) in all sections (n = 25). It's obvious the statistical significant

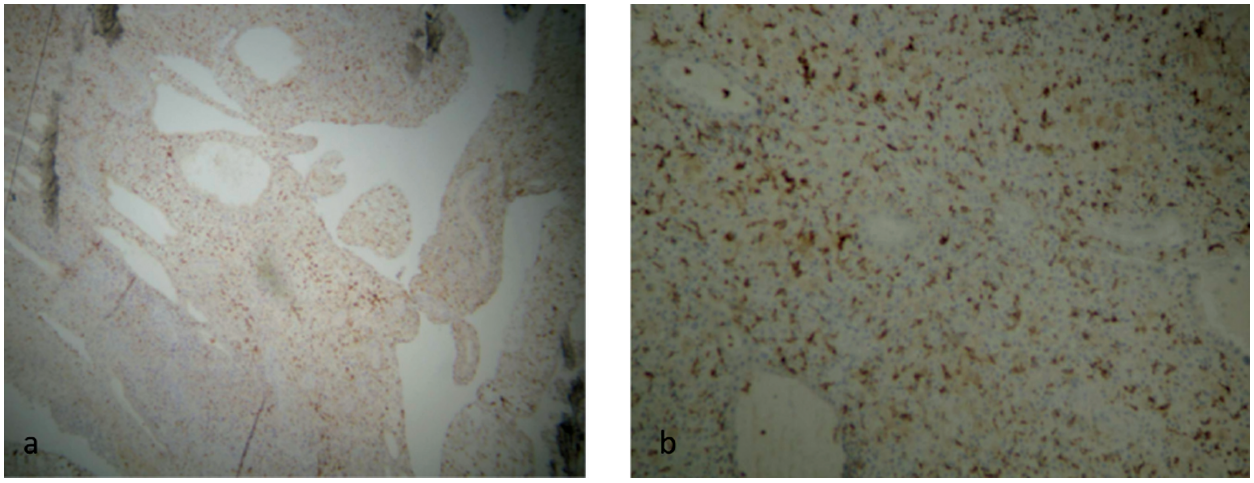

Figure 3. Staining for CD68+ cells in Decidua Basalis. a. Control group. Negative (-), x16. b. Miscarriage group. Strong (+++) intensity, x16.

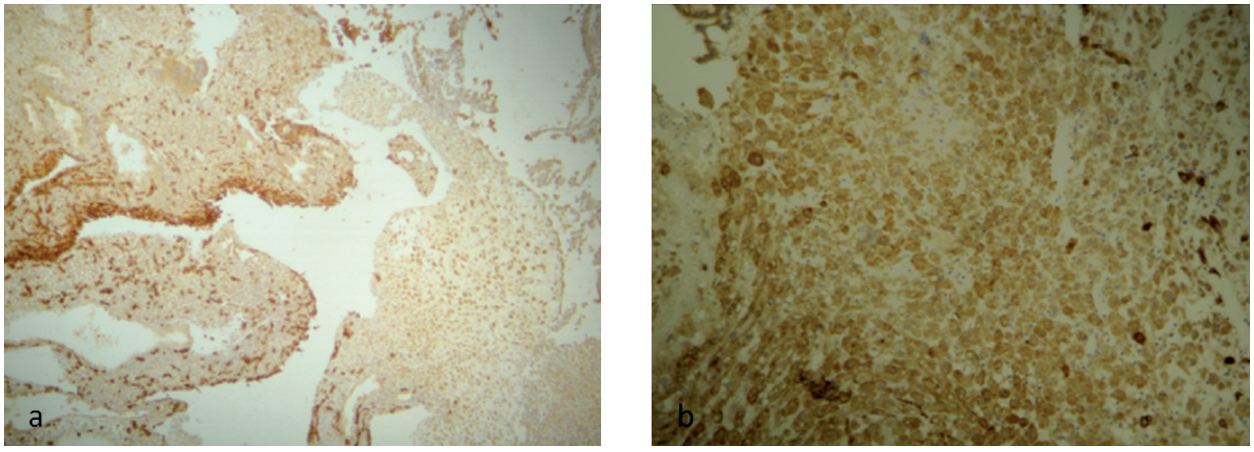

Figure 4. Staining for HLA-G in Decidua Basalis. x16. a. Control group. Weak (+) intensity. b. Miscarriage group. Negative (-) intensity*Clinical Skills Teaching in AUTH Medical School.c.*

difference between miscarriage and control group, in regards to inflitration of CD-68 cells in both basalis and parietalis decidua ($p < 0.05$) (Table 1).

Regarding immunohistochemical staining for HLA-G antibody in sections of decidual basalis, there was statistical significant difference between control and miscarriage group. All sections (n = 25) of decidua basalis in control group were found weak positive (+) (Figure 4a), while all sections in miscarriage group were negative (-) (Figure 4b). As for the sections from decidua parietalis, in both control and miscarriage group HLA-G antibodies were not found (Table 1).

DISCUSSION

Based on the results of our study, the immunological profile of women with recurrent pregnancy loss is quite different, comparing with that of controls. It seems that recurrent pregnancy loss syndrome is provoked by a 'malfunction' of the immunity system, which fails to protect the fetus. Previous reports concerning this syndrome, seem to support such an hypothesis, as they also detect alterations of the relative populations of the immunity cells in deciduas in contrast with controls.

It is known the role of macrophages (CD 68+ cells) in formation of feto-maternal interface. Pre-

Table 1. Intensity of staining for immunological cell markers in decidual tissues from miscarriage group and control group.

	Miscarriage Group	**Control Group**		**Miscarriage Group**	**Control Group**	
CD68	Decidua Basalis	Decidua Basalis	p	Decidua Parietalis	Decidua Parietalis	p
(-)	0(0%)	25(100%)	< 0.05	0(0%)	25(100%)	< 0.05
(+)	0(0%)	0(0%)		0(0%)	0(0%)	
(++)	3(12%)	0(0%)	< 0.05	25(100%)	0(0%)	< 0.05
(+++)	22(88%)	0(0%)		0(0%)	0(0%)	
HLA-G						
(-)	25(100%)	0(0%)	< 0.05	25(100%)	25(100%)	
(+)	0(0%)	25(100%)	< 0.05	0(0%)	0(0%)	
(++)	0(0%)	0(0%)		0(0%)	0(0%)	
(+++)	0(0%)	0(0%)		0(0%)	0(0%)	

vious studies highlight that macrophages, in human uterus, participate in apoptotic cell phagocytosis[13], interact with trophoblast to perform vascular remodeling in the uterus[14], increase monocyte migration at decidua basalis and induce a significant increase in the secretion and production of the pro-inflammatory cytokines and chemokines[1]. In our study, we detected a significant increase of CD68+ cell expression in both decidua basalis and parietalis of women with recurrent miscarriage, in contrast with controls. Based on the available reports, the number of macrophages in decidua of women with recurrent pregnancy loss seems to be similar with normal population[15]. An increase in macrophage population was observed only in non pregnant endometrium of women with recurrent miscarriage. Our research suggests a possible involvement of CD68+ cells in recurrent pregnancy loss pathophysiology.

HLA-G expression in antigen-presenting cells suppress T-cell proliferation and alters the killer function of uterine macrophages and NK cells, protecting the semi-allogenic fetus[3,4,5]. In our study, HLA-G expression was found decreased in decidua parietalis of women with RPL in comparison with women with elective abortions (control group). In decidua basalis, in both groups, *HLA-G* expression was found invariable. Our results, are in accordance with the relative previous reports, which claims that in decidua from women with recurrent miscarriage an increased NK cell marker expression of both CD56 and CD16 was accompanied by a decreased expression of HLA-G[16]. However, our conclusions should be interpreted with caution, as other reports support that *HLA* class 1b antigens, *HLA-E, HLA-F,* and *HLA-G,* are detectable only on some subpopulations of trophoblast[17,18,19,20,21]. In the same direction, Bhalla (2006) stated that *HLA-G* is expressed only in the extravillous trophoblast and there is no significant difference in expression pattern between the recurrent miscarriage and the control group[22].

Although the immunity system cells and their factors seem to involve actively on feta-maternal interface disorganization and unexplained recurrent pregnancy loss, a lot of research is yet needed in order to decrypt fully the multifactor etiology of this clinical entity.

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Η σημασία των δεικτών HLA6 και CD68 σε υλικό αποβολών.

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ΠΕΡΙΛΗΨΗ: *Εισαγωγή:* Οι αγνώστου αιτιολογίας αποβολές, κυρίως 1ου τριμήνου, σχετίζονται με ανοσολογικούς παράγοντες. Σαν απάντηση στο ημιαλλογενές έμβρυο, παρατηρούνται μεταβολές στους υποπληθυσμούς των λευκοκυττάρων και των HLA, τόσο στο φθαρτό όσο και στη θέση εμφύτευσης, οι οποίες επηρεάζουν την πορεία της κύησης.
Σκοπός: Η ανοσοϊστοχημική διερεύνηση των αλλαγών που συμβαίνουν στη μήτρα γυναικών με αγνώστου αιτιολογίας αποβολές, κατά το 1ο τρίμηνο της κύησης.
Υλικό-Μέθοδοι: Η ομάδα μελέτης περιελάμβανε 25 γυναίκες , ηλικίας 35-42 ετών με αποβολές και η ομάδα ελέγχου 25 υγιείς γυναίκες, ηλικίας 27-39 ετών, με τεχνητή διακοπή στο 1ο τρίμηνο της κύησης. Δείγματα από το φθαρτό και την θέση εμφύτευσης εξετάσθηκαν ανοσοϊστοχημικά , χρησιμοποιώντας τα μονοκλονικά αντισώματα έναντι των HLA-G και CD68. Τα αποτελέσματα αναλύθηκαν στατιστικά με το Manu-Whitney test.
Αποτελέσματα: Στην ομάδα μελέτης, η έκφραση του HLA-G στο φθαρτό βρέθηκε ελαττωμένη, ενώ η έκφραση του CD68 ήταν αυξημένη.
Συμπεράσματα: Ανοσοϊστοχημικά, βρέθηκαν σημαντικές διαφορές μεταξύ των ομάδων ελέγχου και μελέτης.

Λέξεις Κλειδιά: Καθ'έξιν αποβολές, HLA-G και CD68.

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