ORIGINAL ARTICLE

Cycle dependent RCAS1 expression with respect to the immune cells presence and activity

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Abstract

INTRODUCTION: The number of cytotoxic immune cells grows in the endometrium during the secretory cycle phase. RCAS1 is a protein inhibiting the activated immune cytotoxic cells. The expression of RCAS1 has been confirmed in endometrium. The aim of the present study was to evaluate the RCAS1 expression alterations with respect to the menstrual cycle changes and the number and activity of cytotoxic immune cells.

MATERIAL AND METHODS: RCAS1, CD25, CD69, CD56, CD16, CD68 antigens expression was assessed by immunohistochemistry in endometrial tissue samples which were obtained from 33 patients. Tissue samples were classified according to the menstrual cycle phases, with division of the cycle into three phases: proliferative (8 cases), periovulatory (10 cases), and secretory (15 cases) ones.

RESULTS: A significantly higher RCAS1 expression was observed in the periovulatory and the secretory menstrual cycle phases than in the proliferative phase. The changes in RCAS1 expression were combined with significant differences in the number of immune cells and their activity. The highest level of CD69 antigen expression was observed during the periovulatory cycle phase, while the highest level of CD25 antigen expression was observed during proliferative phase, the number of CD56 positive cells was at the highest level during the secretory cycle phase. No significant differences in the number of macrophages and CD16 antigen expression were observed with respect to the menstrual cycle phases.

CONCLUSION: RCAS1 endometrial expression may favor the coexistence of active lymphocytes and endometrial cells.

Introduction

The endometrium with decidual changes is a phenomenon playing a crucial role in reproduction. During decidualization the number of immune cells grows and the endometrium is ready for the ovum implantation (implantation window). Epithelial endometrial cells are surrounded then by a high number of mononuclear cells including NK, macrophages, T lymphocytes and other cells [7,18,25,33,34,37]. The presence of immune cells is necessary for the ovum implantation and further development of pregnancy. Many chemokines and particles appearing in the endometrium at

the time of the implantation window are typified by immunomodulating activity. They include: interleukins (IL-1, Il-15, IL13, IL-2), Galectin-9, LIF (Leukemia inhibitory factor), RANTES (mRNA transcripts encoding regulated on activation, normal T-cell-expressed and -secreted), CAP, metalothionein, Fas-L, prolactin and others [2,11,13,16,20,21,27,28,33,40,41,50]. Recently, the alterations in RCAS1 (Receptor associated cancer antigen presenting on SiSo cells) expression within the reproductive tract epithelium including the fallopian tube, and the endometrium were reported [17,44].

RCAS1 is a protein responsible for tumor escape from the host immunological surveillance [5,26,30,38,39] but its expression was also demonstrated in many physiological conditions participating in the regulation of cytotoxic cells activity. It was also described in the bone marrow, palatine tonsils and the placenta [1,8,24,29,43,45,46]. Similarly RCAS1 expression has been disclosed in immune mediated diseases including endometriosis, nasal polyps and liver diseases [8,10,48]. It was therefore concluded that RCAS1 expression in the healthy reproductive tract epithelium might be associated with the effect on immune cytotoxic activity [44].

The aim of the present study was to evaluate RCAS1 expression alterations with respect to the immune cells identification and activity through the assessment of such antigens as CD56, CD68, CD16, CD25, CD69 and with respect to the menstrual cycle phases, i.e. proliferative, periovulatory and secretory ones.

Material and Methods

Human subject

Eutopic human endometrium tissues were obtained from 33 non-menopausal fertile women, aged 30–47 years. These patients underwent hysterectomy because of a benign gynecological indication (leiomyomas). No patient included in our study received any hormonal treatment. The surgical procedure was performed in the Gynecology and Infertility Department of the Jagiellonian University in Krakow, Poland. All tissue samples were verified by the standard histology method using the hematoxylin and eosin staining technique following a formalin fixation. Tissue samples were classified according to the menstrual cycle phases, with division of the cycle into three phases: proliferative (8 cases), periovulatory (10 cases), and secretory (15 cases) ones. The endometrial samples from uterine corpus included the entire thickness of the endometrium (basal and superficial part, composed of stromal cells and glandular epithelial cells).

Patients' consent was obtained in all cases. The approval of the research program by the Jagiellonian University Ethical Committee was obtained prior to the study (KBET/89/B/2005).

Immunohistochemistry

Immunohistochemical analysis was performed in the Pathology Department of the Jagiellonian University. Five-micrometer slides from each case, encompassing the endometrium, prepared routinely for immunohistochemistry, were stained to visualize the expression of RCAS1 and CD16, CD56, CD69, CD25, CD56-positive cells (mainly lymphocytes) as well as CD68+ cells, that is macrophages.

In all cases immunohistochemistry was performed applying the Envision method using Dako Autostainer. For RCAS1 immunostaining the slides were treated with the mouse monoclonal antibody Anti-RCAS1 (Medical and Biological Laboratories, Naka-ku Nagoya, Japan in DAKO Antibody Diluent with Background Reducing Components-DAKO, Denmark, dilution 1:1000) in the moist chamber overnight. To immunolocalize the immune infiltrate cells the monoclonal antibodies were applied: CD56 (NCAM; NCL-CD56-504, Novocastra) in dilution 1:100, CD69 (NCL-CD69, Novocastra) in dilution 1:25, CD25 (Interleukin-2 Receptor, NCL-CD25-305, Novocastra) in dilution 1:25, CD16 (NCL-CD16, Novocastra) in dilution 1:40, CD68 (Klone PG-M, Dako) in dilution 1:50, according to the manufacturer's instructions. Visualization of reaction products was performed using AEC (3-amino-9-ethyl-carbazole) as a chromogen (AEC Substrate Chromogen ready-to-use, DAKO, Denmark) for 10 minutes at room temperature. Sections were counterstained with hematoxylin and mounted in glycergel. For all antibodies a tonsil specimen was taken as the positive control. All stainings were performed with the same procedure but with the omission of the primary antibody as a negative control.

RCAS1 expression was evaluated in an entire slide, in the glandular epithelium and the stromal cells, as follows: 0 – no reactivity; +1 – weak, when any cytoplasmic staining pattern (also granular in perinuclear region) was observed (in up to 10% of positive cells); +2 – marked cytoplasmic (sometimes together with membranous) staining in 11–30% of the cells); +3 – high expression (more than 30% of positive cells).

The immune cells were calculated in an entire specimen, and an average cell number per 1hpf (high power field, objective magnification x40) was calculated. Variable scales were used to evaluate semiquantitatively the number of cells, depending on their general number in the specimen. So, CD25+, CD56+ and CD69+ cells being very scarce were estimated as follows: 0 – lack of positive cells; +1 – single positive cells in the specimen; +2 – 1–5 positive cells per 1hpf ; +3 – more than 5 positive cells/1hpf. For more abundant CD68+ and CD16+ cells the other scale was used: 0 – lack of positive cells; 1+ – 1–5 positive cells per 1hpf, 2+ – 6–10 cells/1hpf; 3+ – 11–20 positive cells /1hpf; 4+ – more than 20 positive cells per 1hpf.

Statistical analysis

The distribution of variables in the examined groups of women checked with the use of the Shapiro-Wilk test showed that all of them were different from normal. Therefore, non-parametric testing was employed. Statistical significance between the groups was determined by the Kruskal-Wallis analysis of variance (ANOVA) test. The Mann-Whitney U test was then used as applicable. The Spearman rank test was used to evaluate interclass correlation coefficients. All calculations were carried out with the use of STATISTICA software v. 6 (StatSoft, USA, 2001).

Results

Analysis of RCAS1 immunoreactivity

RCAS1 immunopositivity was revealed in 67% of endometrial tissue samples. RCAS1 positivity was visible in the endometrial epithelium without positive reaction in the proper stromal cells (Figure 1, Table 1).

A significantly lower RCAS1 expression in the endometrium was identified during the proliferative menstrual cycle phase than during the periovulatory one ($p<0.01$) and than during the secretory cycle phase ($p<0.01$). RCAS1 expression was at comparable levels during the secretory and the periovulatory cycle phases.

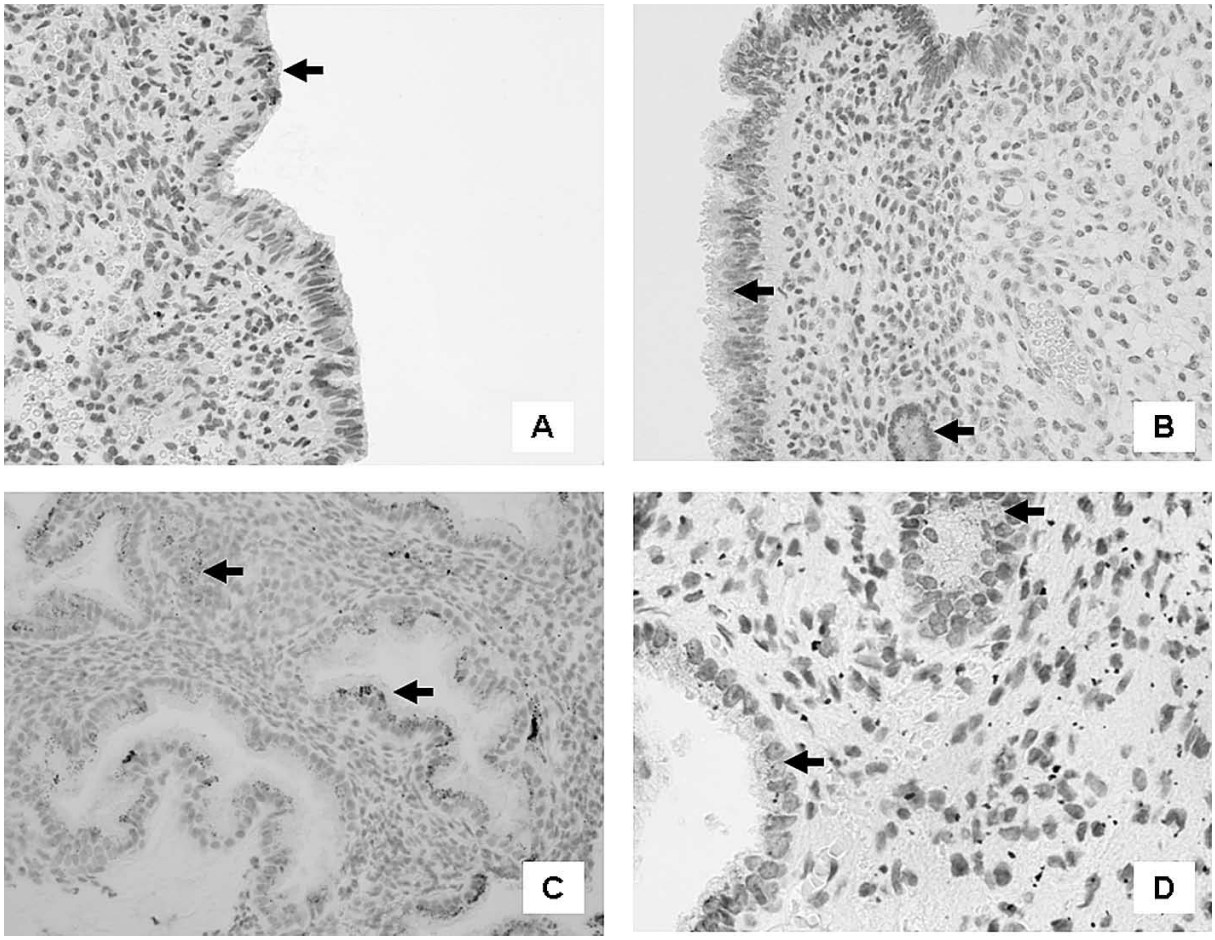

Figure 1. RCAS1 expression in: proliferative (A), periovulatory (B), and secretory (C,D) endometrium.
A - Weak positive glandular reaction in proliferative endometrium (horizontal arrow). Obj. magn. x20;
B - Strong immunoreactivity in glandular epithelium of periovulatory endometrium (horizontal arrows). Obj. magn. x20.
C - Strong immunoreactivity in glandular epithelium of secretory endometrium (horizontal arrows). Obj. magn. x20;
D - Strong immunoreactivity in glandular epithelium of secretory endometrium (horizontal arrows). Obj. magn. x60.

Table 1. RCAS1 expression in the endometrium referring to menstrual cycle changes.

Groups	RCAS1 Immunoreactivity			
	0	+1	+2	+3
Proliferative phase (n=8)	87 (7)	13 (1)	-	-
Periovulatory phase (n=10)	10 (1)	50 (5)	40 (4)	-
Secretory phase (n=15)	20 (3)	60 (9)	20 (3)	-

Table 2. Immune cells number and their activity regarding to menstrual cycle changes.

Menstrual cycle phase	Antigen	Intensity of staining percentage (number of cases)				
		0	+1	+2	+3	+4
Proliferative (n=8)	CD56	37.5 (3)	37.5 (3)	25 (2)	-	-
	CD16	-	25 (2)	62 (5)	13 (1)	-
	CD68	-	13 (1)	87 (7)	-	-
	CD25	-	38 (3)	62 (5)	-	-
Periovulatory (n=10)	CD69	87 (7)	-	13 (1)	-	-
	CD56	40 (4)	20 (2)	40 (4)	-	-
	CD16	-	-	90 (9)	10 (1)	-
	CD68	-	-	80 (8)	20 (2)	-
	CD25	30 (3)	30 (3)	40 (4)	-	-
Secretory cycle (n=15)	CD69	30 (3)	20 (2)	40 (4)	10 (1)	-
	CD56	7 (1)	14 (2)	65 (10)	14 (2)	-
	CD16	-	14 (2)	47 (7)	32 (5)	7 (1)
	CD68	-	28 (4)	65 (10)	7 (1)	-
	CD25	53 (8)	47 (7)	-	-	-
	CD69	65 (10)	14 (2)	21 (3)	-	-

Analysis of immune cells presence and their activity

A significantly higher CD69 expression was identified in the periovulatory phase in comparison to the proliferative one (p=0.02), while CD69 expression was at a comparable level during the periovulatory and the secretory cycle phases. CD25 antigen expression was at a comparable level in the proliferative and periovulatory phases, while during the secretory phase it was significantly lower than in the proliferative one (p<0.001), being also lower than in the periovulatory phase (p=0.056). The number of CD56 positive cells was highest during the secretory cycle phase, significantly higher than during the proliferative and the periovulatory phases (p=0.01 and p=0.02, respectively). However, the number of CD56 positive cells was at a comparable level during the periovulatory and proliferative phases. No significant differences in the number of macrophages (CD68 immunoreactivity) and CD16 antigen expression were observed with respect to the menstrual cycle phases. (Table 2).

Discussion

A statistically significantly higher RCAS1 expression was observed in the periovulatory and the secretory menstrual cycle phases than in the proliferative phase. The immune system of the female reproductive tract is unique and is restricted by hormonal changes. The NK cells population present in the endometrium is represented mainly by $CD56^{+}CD16^{-}$ cells [18,33]. The highest number of CD56 positive cells was found in our study in the secretory cycle phase and no CD16 antigen expression increase was disclosed with respect to hormonal changes during the menstrual cycle phases. Macrophages represent about 15% of endometrial cells and their number does not alter with respect to the menstrual cycle phases [7,18]. In our study CD16 and CD68 antigens expression did not alter. Additionally, a correlation between CD16 and CD68 expressions during the secretory and the periovulatory phases was found (R=0.73, p=0.001). Hormonal changes during the menstrual cycle are accompanied also by the alterations of the immune cell activity changes. Although CD25 and CD69 antigens are not highly specific (CD25 expression in the endometrium may result from the presence of CD4+CD25+ regulatory T (treg) cell suppressing the immune cells response [14]; CD69 is an antigen related to increase of activity of various immune cells), the expression of activation markers in the endometrium (CD69, CD25, CD71, HLA-DR) seems to be closely associated with the NK cells population [15]. The intensity of CD69 antigen expression was highest during the proliferative phase and decreased gradually through the menstrual cycle [22,42]. In our previous report a significantly higher CD69 antigen expression was observed during the secretory cycle phase when compared to the proliferative one, but without considering the periovulatory phase. In the present study we considered three cycle phases, including the secretory, the proliferative and the periovulatory ones, and the highest level was found during the periovulatory cycle phase. CD69 antigen expression has to be discriminated from CD25 antigen expression on the same decidual lymphocytes [4]. CD56 positive cells within the endometrium possess IL-2alfa receptor (CD25) and remain rather activated than in a resting state [33]. In our study CD25 was statistically significantly lower in the secretory than in the proliferative cycle phase. This finding is

in agreement with the data published by Ho et al., who disclosed a higher CD25 expression in the proliferative than in the secretory phase [15]. Down-regulation of IL-2 receptor expression in decidual lymphocytes is observed despite the evidence of lymphocyte activation [4]. Thus, an increase of CD69 expression during the periovulatory phase in comparison to the proliferative one with a concomitant lower CD25 expression might result from a selective CD25 suppression [4]. A similar phenomenon was reported on the lymphocytes in human cervical cancer [36]. The host immune tolerance in neoplasms seems to be similar to the maternal immune tolerance during pregnancy [6]. According to Chao the differences in immune markers expression might result from certain inhibitory mediators derived from the feto-placental unit or other decidual components [4]. RCAS1 expression increases gradually with menstrual cycle changes in the endometrium. The role of this protein in the creation of the maternal immune tolerance during pregnancy has been reported recently. RCAS1 expression was identified within the trophoblast and the placenta [29,47]. This protein might be a factor responsible for this selective suppression quoted above.

A gradual increase of RCAS1 expression within the endometrium during the periovulatory and the secretory phases might be secondary to the increasing cytotoxic activity during the menstrual cycle phases until it reaches the level necessary fort the ovum implantation. Differences in the expression of some cytotoxic activity markers (CD25, CD69) might probably result from differences in the expression of factors responsible for selective inhibition of cytotoxic cells. i.e. RCAS1. Endometrial cells modulate local activity of immune cells by their activation and inhibition. A higher IL-15 secretion was reported during the secretory cycle phase. This cytokine is crucial for NK cells proliferation and survival [19]. The growing increase of IL-13 simultaneous with IL-15 (which is responsible for the inhibition of Th1 cytokines production) [32]. Decidualization begins in every ovulation in which the immune system combined with the endometrium reaches proper activity enabling implantation and preventing abortion. CD69 and CD25 expression on lymphocytes was also demonstrated in the decidua during spontaneous abortion [31,15]. On the other hand, a high amount of NK cells and high pre-conceptional NK cells activity are associated with an increased pregnancy loss [35,49]. Alterations in cytotoxic cells number and the activity in the endometrium are associated with the endometrial reproductive function. The aim of such a phenomenon is to cumulate the adequate number of activated cytotoxic cells within the endometrium. The balance between the increasing intensity of the immune response and endometrial cells, independently of the presence or lack of ovum implantation, seems to be maintained by the expression on endometrial cells factors or their secretion affecting the immune cells activity to the extracellular matrix. These factors include Fas-L, DcR3, IL-11, of a well-known role in this process, but also RCAS1 [12,23,50].

In conclusion, RCAS1 endometrial expression may favor the coexistence of active lymphocytes and endometrial cells.

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