The immunohistochemical analysis of RCAS1 in the endometrium during menstrual bleeding

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Abstract

Introduction: The presence of the suppressive microenvironment of the endometrium is crucial for ensuring the proper course of reproductive processes. This phenomenon is related to the intensity of the expression of proteins such as RCAS1 that are able to suppress the immune cytotoxic cells infiltrating the endometrium. The aim of our study has been to gather information on the immunoreactivity level of RCAS1 within endometrial cells during menstrual bleeding. Methods: We analyzed the immunoreactivity levels of RCAS1 in the endometrial cells during both the late secretory cycle phases and menstrual bleeding. We also analyzed the immunoreactivity of such antigens as CD69, CD25, and CD3 within the immune cells infiltrating the endometrium. Results: RCAS1 immunoreactivity levels in the endometrium were significantly higher during menstrual bleeding than during the late secretory cycle phases. During menstruation, the immunoreactivity level of CD69 decreases while the immunoreactivity level of CD25 increases in comparison to those levels found in the endometria of women in the late secretory cycle phases. Conclusion: The intensity of the suppressive profile of the endometrium related to RCAS1 expression changes significantly during menstruation as compared to the late secretory cycle phases.

Key words: RCAS1, endometrium, menstruation, CD25+, CD69+
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ceably decrease [7, 8]. This phenomenon is directly connected with the intensity of the suppressive profile of the endometrium. Little is known, however, about the profile of the endometrium during menstruation. We have chosen RCAS1 for our analysis because it is an important factor in the homeostasis of the endometrium. RCAS1 is a protein that may stimulate the suppression and apoptosis of both T lymphocytes and NK cells [18, 19]. The presence of these proteins has been observed in the endometrium and decidua as well as within the tumor microenvironment and various types of neoplasms. For example, RCAS1 has been identified within the microenvironment of different types of adenocarcinoma and squamous cell carcinoma [19, 20]. Specifically, Sonoda et al. have demonstrated that the expression of RCAS1 within cervical cancer cells is related to the presence of lymph node metastases and to cancer nest growth [20]. Moreover, the degree of RCAS1 expression within the endometrium changes according to the particular menstrual cycle phase and is higher in the secretory than in the proliferative cycle phases. Fluctuations in RCAS1 concentration levels within the peripheral blood of the women have been observed, and the highest concentration level has been found during menstruation [21]. No such studies, however, have been performed for analyzing the immunoreactivity levels of RCAS1 within the endometrium during menstruation. For our study, we therefore decided to measure the immunoreactivity levels of RCAS1 within the endometrium at the start of menstruation. In order to more effectively analyze the changes in the levels of RCAS1 we chose two different antigens, CD25 and CD69, and measured the immunoreactivity levels of both of these within the mononuclear cells infiltrating the endometrium during menstruation.

Materials and methods

Patients

The tissue samples evaluated in our study were obtained from 19 patients on whom a diagnostic biopsy of the endometrium was performed following a diagnosis by ultrasound examination of endometrial hypertrophy. The ultrasound diagnosis was performed on those patients who had been suffering from inappropriate menstrual bleeding. No hormonal treatment was administered prior to the diagnosis. The patients included in the study averaged 41 ± 3 years of age. All of them had been pregnant prior to the age of 30. In the study we included only those patients in whom upon histopathological verification, no endometrial hypertrophy was found and in whom no additional pathological disturbance in the endometrium was observed. Based on histopathological verification, we chose for our analysis those tissue samples taken from the endometria of patients in the late secretory and menstrual phases.

The tissue samples were collected during the surgical procedures performed in the Department of Gynecology and Oncology of the Lukaszczyzk Oncological Center in Bydgoszcz, Poland between November, 2009 and December, 2010. The immunohistochemical staining was performed in the Department of Pathomorphology of the Jagiellonian University, Kraków, Poland. Prior to the present study we obtained the approval of the Nicolaus Copernicus University Ethical Committee for our research program (KB/54/2009), Bydgoszcz, Poland.

Immunohistochemistry

Immunohistochemical analysis was performed in the Pathomorphology Department of the Jagiellonian University, manually, with application of the Ultravision LP-Value Detection System (Thermo Scientific, Lab Vision Corporation, Fremont, CA, USA). For visualization of reaction products AEC (3-amino-9-ethyl-carbazole) as a chromogen (AEC substrate Chromogen ready to use, DAKO, Denmark) was used for 10 min at room temperature.

Sections were counterstained with Meyer’s hematoxylin and mounted in glycergel.

As a positive control for RCAS1 a ductal breast cancer specimen was used. The staining was performed on breast cancer slides with the same procedures but omitting the primary antibodies as a negative control.

Four-micrometer slides cut from curettage material routinely fixed in 10% solution of formaldehyde and embedded in paraffin were stained to visualize the expression of RCAS1 secretion in the endometrium of secretory and menstrual cycle phases. 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 a moist chamber overnight.

Further, the following antibodies were also used: CD69 (NCL-CD69; Novocastra) in dilution 1:25, CD25 (interleukin-2 receptor, NCL-CD25-305; Novocastra) in dilution 1:25, CD3 (NCL-CD3p, rabbit polyclonal antibody; Novocastra) in dilution 1:100, according to the manufacturer’s instructions.

A semi-quantitative interpretation of immunohistochemical results was carried out by an experienced
A percentage of RCAS1-positive endometrial glandular cells was evaluated at least in 5 hpfs (high power field – objective magnification × 40, Nikon Eclipse 50i Microscope). Depending on percentage of positive cells the reaction was classified as: 0 – lack of immunopositive cells; +1 – 1-10% of the glandular cell with positivity for RCAS1, +2 – 11-30%, +3 – more than 30% of the positive cells. The various types of lymphocytes in the endometrium were also evaluated. The number of immune cells in an entire specimen was counted and an average cell number per 1 hpf calculated. The following scale was used to evaluate the number of CD3-positive lymphocytes semi-quantitatively: 0 – lack of positive cells or only single positive cells in the entire specimen; +1 – 2 to 5 positive cells / 1 hpf; +2 – 6 to 10 positive cells /1hpf, +3 – more than10 positive cells/ 1 hpf. Because of the scarcity of CD25- and CD69-positive lymphocytes, the other three-pointed scale was applied to evaluate their number: 0 – lack of positive cells, +1 – presence of single cells, up to two per 1 hpf, +2 – more than two positive lymphocytes per 1 hpf.

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 the women 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

RCAS1 immunoreactivity
RCAS1-immunopositive endometrial cells were found in all of the tissue samples from menstrual endometria (Fig. 1 a, b) and in 88% of the samples from late secretory endometria. Moreover, when a direct comparison was made of the samples taken from women in the late secretory cycle phases with those taken from women during the menstrual period, it revealed a statistically significantly increase in the RCAS1 immunoreactivity level during menstrual period (Fig. 2).

CD25, CD69, and CD3 immunoreactivity
CD25 immunopositive mononuclear cells were found in 55% of the tissue samples derived from menstrual endometria and in 40% of the samples from late secretory endometria. CD69-positive immune cells were found in 33% of the tissue samples taken from women during their menstrual period, and in 66% of the tissue samples taken from those in the late secretory cycle phases. Furthermore, we found CD3-positive immune cells in all the tissue samples derived from menstruating women, and in 50% of the samples taken from women in the late secretory cycle phases. Moreover, when a direct comparison was made between the samples from the women in the late secretory cycle phase and those from the women having their menstrual period, it revealed a significant increase in the CD25-positive immune cell infiltration. Likewise, we observed a slight increase in the CD3-positive cell infiltration to the endometrium in the menstruating women.

Fig. 1a. Moderate immunoexpression in RCAS1 in menstrual endometrium (ca. 30% of glandular cells with weak positivity). Obj. magn. × 20
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Fig. 1b. Strong RCAS1 expression in the breakdown part (on the right part of the photo) of menstrual endometrium, weaker (on the left) in parabasal glands of endometrium. Obj. magn. × 20

Fig. 2. RCAS1 immunoreactivity level differences correlated with the menstrual cycle phases (LS – late secretory, M – menstrual period)

Fig. 3. CD25 immunoreactivity level differences correlated with the menstrual cycle phases (LS – late secretory, M – menstrual period)

Fig. 4. CD69 immunoreactivity level differences correlated with the menstrual cycle phases (LS – late secretory, M – menstrual period)

By contrast, the decrease in the infiltration of CD69-positive cells correlated with hormonal fluctuations occurring between the late secretory cycle phases and menstrual period.

Discussion

We found statistically significant increase in the immunoreactivity levels of RCAS1 in the endometria during menstruation in comparison to the levels observed in the tissue samples of women during the late secretory cycle phases. To our knowledge, this is the first investigation to focus specifically on the immunoreactivity levels of RCAS1 within the endometrium during menstruation. For our study, we performed RCAS1 immunoreactivity analysis on the endometrium...
in correlation with the menstrual cycle phases. We analyzed the tissue samples from the endometria of women in the proliferative and secretory cycle phases (that is, the early-, mid- and late-proliferative and secretory phases) in detail [22, 23]. Moreover, RCAS1 soluble form concentration levels in the blood serum were assessed in the women during all the menstrual cycle phases, including the menstrual period. We found in our previous study that the RCAS1 soluble form concentration level in the blood was the highest in women during menstruation [21]. Likewise this study showed that the RCAS1 immunoreactivity level was significantly higher during menstruation than in the late secretory cycle phases. RCAS1 helps to inhibit the maturation and apoptosis of the cytotoxic immune cells such as those lymphocytes and T, B, or NK cells [18] that infiltrate the endometrium. An increase in RCAS1 expression is thus generally linked with the strong suppression of the cytotoxic mononuclear cells infiltrating the tumor microenvironment [18-20, 24-26]. Similarly, in the endometrial microenvironment, an increase in the degree of RCAS1 expression was observed in comparison to the proliferative and secretory cycle phases and was associated with an increase in the selective suppression of the cytotoxic immune cells infiltrating the endometrium [22]. During the subsequent menstrual cycle phases, not only does the level of immune cell activity change, but so does the type of immune cell dominating the infiltration. For example, in the secretory cycle phase, dNK cells are pre-dominant in the endometrium, but during menstruation, neutrophils and macrophages dominate [15-16, 27]. Moreover, RCAS1 has been observed on the surface of macrophages in the tumor microenvironment in different types of cancerous tumors (such as brain, ovarian, and salivary gland tumors) and in metastatic tumors [12, 28-31]; additionally, it has been found in the organs of patients with different types of chronic inflammatory diseases, such as those of the liver [32] and nasal mucosa [33], and it has furthermore been observed in women with ovarian endometriosis [30]. Thus far no such macrophages have been found in the normal endometrium of women during the proliferative and secretory menstrual cycle phases [30]. In our study, however, we did observe RCAS1-positive macrophages in menstrual endometria. While RCAS1 macrophages are generally involved in regulating erythropoiesis [19], they are also crucial for the development of the suppressive microenvironment surrounding the tumor cells [27] and for the suppressive profile of the microenvironment of the endometrium. In our study we observed a significant decrease in CD69 antigens levels accompanied by an increase in CD25 antigen levels when we compared the levels found in the tissue samples derived from women during the menstrual period with those of women in the late secretory cycle phases. CD69 markers represent immune cell activity, and the level seems be linked with RCAS1 level, as a result of the role of RCAS1 plays in the suppression of the immune response [24-26]. The increase in RCAS1 is followed by a decrease in CD69 antigen expression as has been observed in decidua during placental abruption [9]. However, given that suppressive lymphocyte T (Treg) cells are also CD3 and CD25+ , CD25 cells are not the only markers of immune cell activity [34]. Probably the increasing infiltration of CD3+ cells in conjunction with the increase in CD25+ immune cells is connected with an infiltration of Treg cells to the endometrium. Treg cells participate in generating the suppressive profile of the endometrium and are also a primary factor in creating the suppressive profile in different organs and tumors [1, 2, 5, 34-36]. In our study we found CD3-positive cells in all the endometrial tissue samples derived from women during menstruation. Further detailed study is needed, however, in order to elucidate the precise role CD3 positive cells play in the homeostasis of human endometrium. In sum, the suppressive profile of the endometrium serves to maintain this organ’s homeostasis even as it is being massively infiltrated by active immune cells, and RCAS1 is a major participant in the regulation of this suppressive phenomenon.

Conclusion

It appears that the intensity of the suppressive profile of the endometrial microenvironment may be related to RCAS1 expression within endometrial cells. Moreover, the intensity of this phenomenon fluctuates during menstruation in response to changes in the immune cell infiltration to the endometrium.

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References


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