ORIGINAL ARTICLE

RCAS1 Decidual Immunoreactivity in Severe Pre-Eclampsia: Immune Cell Presence and Activity

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Keywords
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Problem
Pre-eclampsia seems to be related to the disturbance of immune tolerance regulation during pregnancy. Receptor-binding cancer antigen expressed on SiSo cells (RCAS1) decidual level alterations were concomitant with changes in immune cell number and activity in decidua. As decidual immunomodulating activity participates in the development of immune tolerance during pregnancy, we aimed to evaluate the immunoreactivity level of decidual RCAS1 with respect to the presence and activity of immune cells.

Method of study
RCAS1, CD3, CD56, CD69, and CD25 immunoreactivity was assessed by immunohistochemistry in 30 decidual samples derived from patients with severe pre-eclampsia (sPE) and from a healthy control group.

Results
RCAS1 immunoreactivity was statistically significantly higher in decidual tissue samples derived from patients with sPE tissue than in those derived from healthy patients in whom elective cesarean section at term was performed. A statistically significantly lower number of $CD56^+$ and $CD3^+$ cells and lower immunoreactivity level of CD69 were found in patients with sPE compared with those from the control group.

Conclusion
The limited immune cells infiltration in decidua during sPE is associated with increase in RCAS1 decidual level.

Introduction

Eclampsia was described by Cook and Briggs in 1903 as peripartum convulsions. Hypertension preceding the occurrence of eclampsia complicates 2–7% of gestations, and most of these cases are related to primiparternity.[1,2] Pre-eclampsia is a major cause of maternal and fetal morbidity and mortality.[2,3] Although the mechanism of the development of this pregnancy-related disorder is linked to placental function, its etiology still remains unclear. Pre-eclampsia seems to be a maternal-paternal maladaptaion[1] but it has also been considered a chronic graft rejection.[4] This may suggest a possible relationship between the

development of pre-eclampsia and a disturbance in immune tolerance during pregnancy.

Trophoblast decidual invasion is an important process in normal gestation; the maternal immune cells present in decidua are responsible for the limitation of trophoblast invasion.[5,6] In pre-eclampsia, however, the trophoblast invasion is disturbed with concomitant abnormal maternal inflammatory response (maternal response to placentation).[7] Inappropriate maternal inflammatory response involves both maternal-fetal interface and the circulating trophoblastic cells.[8] This leads to the restriction of trophoblast cell invasion in decidua in pre-eclampsia in comparison to normotensive women,[9] as a result of an increase in trophoblast apoptosis in the placenta. In advanced pre-eclampsia with severe fetal growth restriction (FGR), the trophoblast apoptosis level has been statistically significantly higher.[10] Trophoblast cell apoptosis is concomitant with maternal immune cell activity, and in pre-eclampsia the regulation of immune cell activity is disrupted. In pre-eclampsia patients, the cytokine profile is balanced with respect to Th1 profile.[7,11,12] The evaluation of immune cell infiltration in decidua in severe pre-eclampsia (sPE) was the focus of our present study. Decidual cell activity completes the suppressive activity of placental cells during gestation.[13] The immunomodulating role of decidua is related to the expression of several proteins, including Fas-L, RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), and others.[14–16] RCAS1 has been shown to be responsible for tumor cell escape from host immunological surveillance in various cancers.[17–20] This protein has also been demonstrated in physiological conditions in placenta, palatine tonsils, bone marrow, and in the normal mucosa of women's reproductive tract.[21–25] RCAS1 expression in placenta is related with the phenomenon of maternal immune tolerance for fetal antigens during pregnancy.[22,25,26] Recently, RCAS1 has been discovered in decidua during the third trimester of pregnancy and in the fallopian tube during the implantation of an ectopic pregnancy. RCAS1 decidual level alterations were concomitant with the changes in immune cell number and activity in decidua.[13,27] RCAS1 presence in decidua seems to be related with the process of immune tolerance during pregnancy.[13] RCAS1 decidual expression has not been considered in pre-eclampsia so far. As pre-eclampsia seems to be related to immune tolerance process disturbance, we aimed to evaluate RCAS1 immunoreactivity with respect to immune cell presence and activity within the decidua of pre-eclamptic patients.

Materials and methods

Human Subject

In our study, we considered decidual tissue samples obtained from 30 pregnant women. These tissue samples were obtained from two groups of patients. The first group consisted of 18 patients on whom cesarean sections were performed because of sPE or eclampsia; these samples were obtained during surgery. The second group included 12 patients on whom cesarean sections at term were performed. For the purpose of this study, we examined the histories of 12,000 patients who delivered between 1999 and 2004 in the Department of Gynecology, Obstetrics and Oncology of the Jagiellonian University, Krakow, Poland. From this group of women, 30 patients were selected for our study. Patients with multiple pregnancies or existing pregnancy complications such as diabetes mellitus, as well as all cases of stillbirth, were excluded from this study.

Decidual tissue samples were retrieved from the archive files of the Department of Pathomorphology of the Jagiellonian University. Two experienced pathomorphologists (K.G. and A.L) independently evaluated the routinely stained (hematoxylin and eosin) slides prepared from paraffin-embedded tissue material and selected material adequate for further analysis. Chosen paraffin blocks were cut and used for immunohistochemical analysis. The patients' consent was obtained and prior to the study, the approval of the Jagiellonian University Ethical Committee (KBET/89/B/2005) was also obtained.

Severe Pre-Eclampsia patients – sPE Group

The patients included in our study presented clinical findings of pre-eclampsia, such as hypertension and proteinuria. Pre-eclampsia was regarded as severe if there were sustained rises in blood pressure to at least 170 mmHg (systolic) and simultaneously at least 110 mmHg (diastolic) accompanied by proteinuria (more than 500 mg every 24 hr).

Eclampsia was defined as the onset of convulsions in women who had pre-eclampsia with concomitant severe CSN symptoms (e.g. altered mental status, headaches, blurred vision or blindness). Study group consisted of two subgroups including 14 patients with

sPE and four patients with eclampsia. In 14 cases of sPE and eclampsia patients, a concomitant FGR established as a fetal weight less than 10th percentile according to the Polish standard for birth weight and gestational age was observed.[28] The patients with pre-eclampsia or eclampsia in our study delivered by cesarean section and the surgical procedure was performed in all cases with an unripe cervix or a cervical dilatation less than 1 cm. As significant differences in soluble HLA-G and sHLA-DR in maternal serum level have been shown in pre-eclampsia in comparison to patients with HELLP syndrome,[29,30] patients with HELLP syndrome were also excluded from our study. In the analyzed placentas, the following histological changes were found (in routinely HE stained slides): pale infarcts (50% of cases), ecchymoses (10% of cases), fibrosis or hyaline changes (50% of cases), thrombotic occlusion of vessels (10%), and no pathological changes were detected in 10% of cases. Additionally, in 30% of the cases both infarcts and fibrosis of the villi were observed.

Control Group

Our control group consisted of 12 women delivering by cesarean section at term because of fetal distress syndrome or fetal malpresentation. Again, this group consisted of patients on whom the cesarean section was performed with an unripe cervix or with a cervical dilation of less than 1 cm. Patients with intra-amniotic infection confirmed by histopathological examination of the fetal membranes and chorionic plate or other symptoms of intrauterine infections (C-reactive protein levels >7 mg/dl) were excluded from this study.

Immunohistochemistry

CD3 and CD56 antigens were selected for the identification of lymphocytes within decidua because CD3 positive cells represent the T-cell population, while the CD56 antigen recognizes NK cells. CD69 and CD25 antigens were chosen for the evaluation of immune cell activity by the immunohistochemistry method because of expression changes observed in decidua.[6,31]

Immunohistochemical analysis was performed in the Pathomorphology Department of the Jagiellonian University. Four-micrometer slides from each case, including the endometrium, prepared routinely for immunohistochemistry, were stained to visualize the expression of RCAS1 and CD3, CD69, CD25, CD56-positive cells (lymphocytes).

In all cases, immunohistochemistry was performed by applying the Envision method using Dako Autostainer (DAKO, Glostrup, Denmark). 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. Moreover, the following antibodies were applied: CD56 (NCAM; NCL-CD56-504, Novocastra, Norwell, MA, USA) in dilution 1:100, 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. Visualization of reaction products was performed using AEC (3-amino-9-ethyl-carbazole) as a chromogen (AEC Substrate Chromogen ready-to-use, DAKO) for 10 min at room temperature. Sections were counterstained with hematoxylin and mounted in glycergel. As a positive control for RCAS1, a breast cancer specimen was used. For the negative control, the same specimen and method were used as applied for the positive one, but without the primary antibody. RCAS1 immunoreactivity and numbers of lymphocytes were evaluated in decidua. RCAS1 expression was evaluated in an entire slide from the decidua as it is presented in Table I. Immune cells were calculated in an entire specimen (the same area as RCAS1) according to the manner presented in Table I.

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.

Results

We analyzed the clinical parameters pertaining to the course of gestation and labor for the patients with sPE and the control group (Table II).

Table I The Scale Used for Evaluation of RCAS1, CD3, CD56, CD69, and CD25 Antigens Immunoreactivity

	Immunoreactivity level				
	0	+1	+2	+3	+4
Immune cells antigens: CD3, CD56, CD69, CD25	Lack of positive cells or only single positive cells in the entire specimen	1–5 positive cells per 1hpf	6–10 positive cells/1hpf	11–20 positive cells/1hpf	More than 20 positive cells per 1hpf
RCAS1	No reactivity	Any staining pattern in up to 10% of the cells	Positive staining in 11–30% of the cells	More than 30% of positive cells	–

Table II Clinical Characteristic of Patients

Variables	Pre-eclampsia (n = 18)	Cesarean section-control group (n = 12)	P-value
Maternal age (average ± S.D.)	32 ± 3.6	28.1 ± 4	NS
Parity (median ± S.E.M.)	1.5 ± 0.15	1 ± 0.2	NS
Gestational age (median ± S.E.M.)	30.5 ± 0.69	37.5 ± 1.7	0.03
Newborns birth weight (average ± S.D.)	1250 ± 500	3039 ± 328	0.01
Apgar score (median ± S.E.M.)	6.5 ± 0.5	8.5 ± 0.4	0.02
Cervical dilatation (median ± S.E.M.)	0 ± 0.9	0 ± 0.1	NS
Systolic blood pressure (median ± S.E.M.)	180 ± 3.5	115 ± 1.79	<0.01
Diastolic blood pressure (median ± S.E.M.)	117 ± 1.4	75 ± 1.65	<0.01

S.D., standard deviation; S.E.M., standard error of the mean; NS, no statistically significant differences ($P > 0.05$).

We have not observed any statistically significant differences in clinical parameters among the groups examined, such as maternal age, parity, and cervical dilation (evaluated just before surgery), which fact enabled us to compare RCAS1, CD3, CD56, CD69, and CD25 immunoreactivity levels in these groups. The peripheral leukocyte number in sPE group did not differ significantly from those observed in controls (12.09×10^9/L (±2.97) versus 10.8×10^9/l (±2.9) retrospectively).

Analysis of RCAS1 Immunoreactivity Level in Decidua

RCAS1 immunoreactivity was observed in 95% of patients with pre-eclampsia, while RCAS1 immunoreactivity in the control group was confirmed in 66% of patients within decidua (Fig.1, Table III).

Statistically significantly higher RCAS1 immunoreactivity level was identified in sPE than in the control group ($P = 0.02$).

No differences in RCAS1 immunoreactivity in decidua derived from patients with sPE and eclampsia were identified.

Analysis of Immune Cells Presence and their Activity

CD3 positive cells were observed in decidua in 44% of patients with sPE while their presence in decidua was identified in all patients from the control group. CD56 positive cells were identified in decidua only in 5% of patients with sPE and in 59% patients from the control group. CD69 antigen immunoreactivity was revealed in 27% of patients with sPE and in 75% of patients from the control group. CD25 antigen immunoreactivity was not observed in patients with sPE, while it was found in 16% of patients from the control group.

No differences were observed in the number of CD56 and CD3 positive cells and the immunoreactivity of CD69 and CD25 antigens in decidual tissue

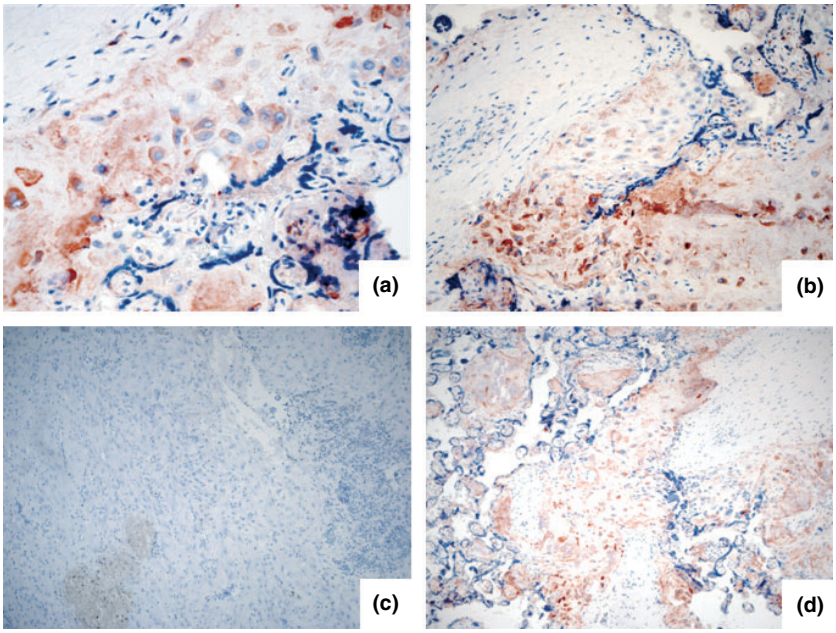

Fig. 1 Decidual receptor-binding cancer antigen expressed on SiSo cells (RCAS1) immunoreactivity. (a) Strong RCAS1 reaction in decidua derived from patients with severe pre-eclampsia (sPE). Obj. magn. ×60. (b) Strong RCAS1 reaction in decidua derived from patients with sPE. Obj. magn. ×40. (c) Week RCAS1 immunoreactivity in decidua derived from control group. Obj. magn. ×20. (d) Moderate RCAS1 immunoreactivity in decidua derived from control group. Obj. magn. ×40.

Table III Analysis of RCAS1, CD3, CD56, CD69, and CD25 Antigen Immunoreactivity Level in Decidua

		Immunoreactivity				
Groups	Antigen	0	+1	+2	+3	+4
sPE	RCAS1	5.5 (1)	17.5 (3)	27 (5)	50 (9)	–
(*n* = 18)	CD3	55 (10)	45 (8)	–	–	–
	CD56	94.5 (17)	5.5 (1)	–	–	–
	CD69	73 (13)	27 (5)	–	–	–
	CD25	100 (18)	–	–	–	–
Control	RCAS1	33 (4)	33 (4)	8 (1)	26 (3)	–
group	CD3	–	8 (1)	–	66 (8)	26 (3)
(*n* = 12)	CD56	40 (5)	26 (3)	8 (1)	26 (3)	–
	CD69	26 (3)	40 (5)	–	8 (1)	26 (3)
	CD25	83 (10)	17 (2)	–	–	–

RCAS1, receptor-binding cancer antigen expressed on SiSo cells; sPE, severe pre-eclampsia.

samples derived from patients with sPE and eclampsia. These results allowed to compare the whole sPE group with the control group and the results are presented in Fig. 2.

The Analysis of RCAS1 Immunoreactivity Levels in Relation to the Immune Cells Concentration and their Activity

Significant negative correlation was observed between RCAS1 immunoreactivity level and the number of CD3 positive cells ($g = -0.48$, $P = 0.09$) in decidua obtained from patients with sPE. Additionally in this group of patients, statistically significant negative correlation was identified between CD69 antigen immunoreactivity level and RCAS1 immunoreactivity in decidua ($g = -0.67$, $P = 0.02$). On the contrary, in the control group, a significant positive correlation between RCAS1 immunoreactivity and CD69 antigen immunoreactivity in decidua was identified ($g = 0.51$, $P = 0.06$).

Discussion

RCAS1 immunoreactivity was statistically significantly higher in decidual tissue samples derived from patients with sPE tissue than in those derived from healthy patients on whom elective cesarean sections at term were performed. A statistically significantly lower number of $CD56^+$ and $CD3^+$ cells and immunoreactivity level of CD69 were found in patients with sPE, compared with those from the control group.

To our knowledge this is the first investigation focused on RCAS1 immunoreactivity level and the number and activity of immune cells in decidua of patients with pre-eclampsia.

As far as immune response during pre-eclampsia is concerned, our results confirm the characterization of the endometrial leukocyte population of patients

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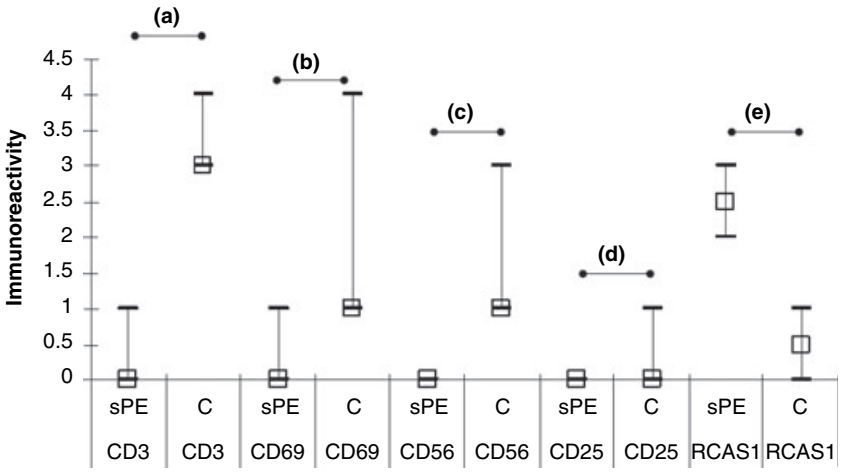

Fig. 2 Comparative analysis of RCAS1, CD3, CD56, CD69, and CD25 immunoreactivity in decidua sampled during cesarean section because of severe pre-eclampsia (sPE) and in the control group (C – cesareans section at term). Each vertical line represents the 25–75% range of data. The median (□) was presented for each group. Statistically significant differences were observed in the immunoreactivity level of the following antigens: CD3 – (a) sPE versus C ($P < 0.001$); CD69 – (b) sPE versus C ($P < 0.01$); CD56 – (c) sPE versus C ($P = 0.001$); CD25 – (d) sPE versus C ($P = 0.07$); RCAS1 – e sPE versus C ($P = 0.02$).

with pre-eclampsia presented by Eide, Stallmach, Wilczynski, and Bachmayer.[11,32–34] However, while following their line of thought, we differed on two points. First, unlike Eide, Wilczynski, and Stallmach,[11,32,33] we evaluated decidual tissue samples derived from cesarean sections performed because of sPE or the presence of the symptoms of elampsia. Second, unlike Bachmayer and Stallmach, who analyzed patients after both cesarean section and vaginal delivery,[33,34] we analyzed only the patients who had cesareans. Additionally, we considered patients who did not differ in the range of cervical ripening, during cesarean; in both groups, the cesarean sections were performed without labor (i.e. with an unripe uterine cervix). The possible influence of molecular changes associated with the initiation of labor[35] on the results of the performed analysis was excluded. Consequently, like these researchers, we identified the disturbance of immune cell infiltration in the decidua of pre-eclamptic patients in comparison with the healthy control group. In the Eide report, the number of CD56-positive, CD3-positive cells, and CD69 antigen expression were lower in pre-eclampsia than in normal pregnancy, and the lowest level was identified in women with pre-eclampsia with FGR.[32] However, Stallmach has observed an increase in immune cell infiltration in decidua in patients with pre-eclampsia with FGR.[33] Similarly, Bachmayer has indicated an increase in CD56 positive cells in the decidua of pre-eclamptic patients.[34] Wilczynski, who has analyzed the tissue derived from curettage by flow cytometry, has identified a lower number of CD3-positive cells with a concomitantly higher number of $CD56^+$ $CD16^+$ positive cells in pre-eclamptic patients in comparison with normal pregnancy.[11] Tissue samples in this analysis included both decidua basalis and decidua parietalis, while Sindram-Trujillo has indicated more prominent NK ($CD56^+$ $CD16^+$) cells infiltration in decidua parietalis than in basalis during the peripartum period.[11,36] This may explain the differences observed in the Wilczynski study. Contrary to these studies, Darmochwal-Kolarz has discovered a lower number of CD56 and CD94 positive cells in the decidua of pre-eclamptic patients and, as with our report, has analyzed patients with severe hypertension (higher than 160/100 mmHg).[37] In our study, only those patients with severe hypertension (higher than 170/110 mmHg) with concomitant FGR were included. This condition may be the reason for the observed decidual immune infiltration deficit in patients with sPE. Pre-eclampsia is associated with Th1 profile dominance.[3,7,38] The lack of dNK infiltration as well as dNK activity may be caused by disturbed IL-12/IL-15 and IL-18 profiles.[4,39,40] A positive correlation between the remodeling of the spiral artery and reduced NK cell number has been described previously.[15,32] Pre-eclampsia, however, seems to be similar to chronic graft rejection and is associated with an increase in immune response.[4] This suggests that the immune cells infiltration deficit in decidua concomitant with sPE, observed in the present study, may result from the deregulation of immune tolerance process. These changes in immune tolerance process might be related to decidual cells activity.

The decrease in immune cell infiltration in decidua from sPE patients observed in our study is

associated with RCAS1 immunoreactivity increase. Decidua may act as a buffer between maternal immune cell activity and placental cells.[13,27] Because immune cell infiltration depends mainly on the activity of decidual cells,[41] the observed decrease in immune cells infiltration in pre-eclamptic patients may be accompanied by the growth of selective suppression activity of decidual cells (by an increase of RCAS1 immunoreactivity). It has been shown previously that RCAS1 interaction with the receptor on the effector cell may lead to FADD (Fas associated death domain) activation and through the caspases cascade induce effector cell apoptosis.[24] The evaluation of apoptosis within the population of lymphocytes surrounding RCAS1 positive cells has already been described. Sonoda has shown that RCAS1 over-expression in uterine cancer cells and in metastatic cells in the lymph nodes is correlated with a growing number of apoptotic lymphocytes (mainly $CD3^+$).[19] RCAS1 expression has also been found in normal endometrium and tubal mucosa.[21] In normal endometrium, the growth of RCAS1 expression during the mid-secretory phase was concomitant with the growth of immune cell infiltration.[25] Inversely, in endometrial cancer, RCAS1 over-expression is accompanied by immune cell infiltration decrease.[16] Nakashima has indicated that while RCAS1 may induce apoptosis of cytotoxic lymphocytes, it mainly leads to the strong suppression of lymphocyte growth.[17] This second RCAS1 action (suppression of lymphocyte growth) seems to be more prominent and decisive for reproductive tract homeostasis in physiological conditions, whereas in patients with retained placental tissue and in patients with sPE, RCAS1 over-expression in decidua is accompanied by a dramatic drop in immune cell infiltration.[13] These interactions are similar to those identified in endometrial cancer.

The present study does not establish precise cause-and-effect relations between RCAS1 immunoreactivity and immune cell disappearance, but shows that increased RCAS1 immunoreactivity level in decidua is accompanied by a prominent restriction of immunological cell infiltration within decidua.

The present study had certain apparent limitations. First, the small number of patients involved does not allow the consideration of the possible influence of applied therapy on the obtained results. We reduced the impact of applied therapy by including in our study only those patients in whom the presence of severe symptoms of pre-eclampsia or symptoms of eclampsia were observed. Additionally, patients included in the control group differed with regard to gestational age. Including the patients of the same gestational age necessitates comparing the patients with pre-eclampsia to the patients with pre-term delivery, although the complex etiology of pre-term delivery may complicate the analysis of the results. In our previous study, the level of immune tolerance during pregnancy was independent of the fetal maturity, and that is why the control group included patients delivering by cesareans at term.[42] The level of immune tolerance changes during labor and depends on the progress of the labor. Because the patients in the pre-eclampsia group delivered by cesarean without labor, the control group included patients with unripe cervices. The second limitation was inherent in the laboratory technique. Immunohistochemical methods do not properly allow the differential expression of the levels of an antigen and do not permit the evaluation of more than one CD marker on a single cell. This technique was nevertheless adopted because the key problem in our study is the evaluation of the presence of immune cells within decidua basalis, and immunohistochemistry in fact allows the identification of immune cells in relation to other tissue structures. Indeed, this method has been used successfully for the identification of cells within decidua by other researchers.[41]

Conclusion

The limited immune cells infiltration in decidua during sPE is associated with an increase in RCAS1 decidual level.

Acknowledgments

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