RCAS1 Decidual Immunoreactivity during Stillbirth: Immune Cell Presence and Activity

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Problem
Alterations in RCAS1 (a receptor-binding cancer antigen expressed on SiSo cells) expression in the placenta and decidua may be related to the regulation of the process of maternal immune tolerance against fetal antigens. Moreover, it has been demonstrated that the occurrence of the spontaneous beginning of stillbirth is related to a decrease in the placental expression of RCAS1. There are no data currently available on the immune processes in decidua during stillbirth. The aim of this study was to evaluate the RCAS1 immunoreactivity level in decidua and to identify the cytotoxic immune cells present during labor, induced after intrauterine fetal death either with a combination of oxytocin (OT) and prostaglandins or with OT alone; a further objective was to assess the potential impact of these molecular alterations on the effectiveness of stillbirth induction.

Methods
The immunoreactivity of RCAS1, CD3, CD56, CD69, and CD25 was assessed by immunohistochemistry in 31 decidual samples derived from patients in whom the stillbirth occurred before the onset of labor.

Results
The RCAS1 immunoreactivity level was higher in a statistically significant manner in decidual tissue samples derived from patients in whom OT alone proved insufficient to induce labor after the diagnosis of intrauterine fetal death but required additionally the use of prostaglandins when compared with samples from women in whom stillbirth was induced successfully with OT alone. However, we did not observe any differences either in CD56 and CD3 positive cell presence or in CD25 and CD69 antigen immunoreactivity in the respective decidua of these two groups of patients.

Conclusion
The level of RCAS1 in decidua seems to influence the effectiveness of stillbirth induction.

Introduction

Stillbirth continues to be an important public health issue. In recent decades, the development of healthcare has reduced the incidence of sudden infant death syndrome,[1] but the problem of stillbirth, which entails fetal death prior to labor, remains unresolved.[2] There is a significant difference in the incidence of stillbirth between the developed and the developing countries. The rate of stillbirth ranges from three per 1000 in developed countries when compared with 100 per 1000 births in developing countries.[2,3] Difficulty in accessing proper medical care is the most likely reason for the high stillbirth rate found in the developing countries. Moreover, overall improvements in medical care have influenced the outcome more for intra-partum than for ante-partum stillbirth.[4] Yet, regardless of the circumstances, delivery is always the most hazardous part of the medical care that the patient receives. In most cases, spontaneous labor will not occur after a diagnosis of intrauterine fetal death, but will have to be induced, and this induction has an effect on maternal morbidity. There is currently limited statistical information on the relationship between maternal mortality/morbidity and the course of stillbirth.[2] Thus, the method of labor induction together with access to medical care may significantly affect the outcome of treatment. Whether or not the method of effective labor induction is related to molecular changes at the maternal–fetal interface would seem to be an important issue given that such changes in physiological conditions determine the course of labor.[5] As with the physiological labor, normal uterine contractions with cervical remodeling are essential to the proper course of stillbirth. Myometrial activation may be stimulated via the paracrine and endocrine pathways by uterotonic agonists (including oxytocin (OT) and prostaglandins (PGs).[6] However, the beginning of labor is determined not only by the proper myometrial contractility pattern, but also, most likely, by the activity of the fetal adrenals and related cofactors,[7–9] not to mention molecular changes at the maternal–fetal interface that cause alterations in the level of maternal immune tolerance against fetal antigens.[5] As the involvement of endocrine signals from the fetal adrenals during the initiation of labor is well known,[7–9] we have focused our study on the maternal–fetal interface—that is, the interface of the maternal decidua and its immunomodulating activity.

The initiation of labor is associated not only with the increase in OT, oxytocin receptor (OTR), and PG expression,[10–18] but also with a complex molecular response leading to a brief activation of the maternal immune system with an accompanying capacity to restrict this very activation.[21–26] Szekeres-Bartho et al.[21] have observed an increase in lymphocyte activity during labor while Abadia-Molina et al.[22] have found lymphocytes with a prominent expression of antigens (such as $CD25^+$, $CD69^+$, and human leukocyte antigen class II-DR) in the decidua basalis during labor at term. During labor at term, an alteration in the distribution of NK cells (both $CD56^+ CD16^-$ and $CD56^+ CD16^+$) has been observed between the decidua basalis and decidua parietalis.[23] In recent reports, it has been suggested that the maternal immune system is not solely responsible for the proper initiation of labor, but fetal macrophages have also been determined to play an important role in this process.[27] At the beginning of stillbirth, both the fetal immune system and fetal adrenals probably fail to function properly. For this reason, we have chosen stillbirth as a way to analyze the involvement of the maternal decidua in immunoregulation during the course of labor. In our recent studies, we have demonstrated that the placenta retains some suppressory activity against maternal immune cytotoxic cells even after fetal death and, depending on this suppressory activity, either spontaneous stillbirth will occur or labor will have to be induced.[28] The proper function of the phenomenon of maternal immune tolerance, however, is conditioned by the suppressory activity of both the placenta and the decidua. The ability to suppress the activity of the immune cells present within the maternal–fetal interface, particularly during labor, is realized mainly through proteins originating from the decidual cells, as placental physiological suppressory activity diminishes.[29–31] In our previous study, we have demonstrated that RCAS1, one of the proteins in decidua, is responsible for the inhibition of activated cytotoxic immune cells in decidua during labor at term[29,30] and also during the course of such pathological conditions as preterm placental abruption[32] and pre-eclampsia.[33,34] Moreover, because of its ability to inhibit the growth and activation of NK cells and T and B lymphocytes,[35] RCAS1 has previously been shown to be responsible for the escape of cancer cells from host immunological surveillance.[36–42] It has also been shown that RCAS1 interaction with the receptor on the effector cell may lead to Fas-associated death

domain activation and may induce effector cell apoptosis through the caspases cascade.[43] In uterine cervical cancer, an increase in the apoptosis of lymphocytes (mainly $CD3^+$) surrounding RCAS1 positive-cancer cells and RCAS1-positive metastatic cancer cells in lymph nodes has been observed.[36] Recently, Han et al.[44] has determined that RCAS1 can reversibly inhibit the activity of cytotoxic immune cells *in vitro*; this indicates that the changes in its expression in the decidua and placenta may be associated with the reversible restriction of the activity of the immune system during labor. Alterations in RCAS1 expression in the placenta and decidua as well as its presence within the serum of pregnant women may be related to the regulation of immune tolerance against fetal antigens during pregnancy.[29] This seems to be one of the physiological homeostatic mechanisms in a woman's reproductive tract responsible for the proper activation of the immune system during labor. In our previous study,[28] we demonstrated that the occurrence of the spontaneous beginning of stillbirth is related to a decrease in RCAS1 placental expression when compared with cases in which labor needed to be induced following the diagnosis of intrauterine fetal death. The aim of this study, therefore, was to evaluate the RCAS1 immunoreactivity in decidua and to identify the cytotoxic immune cells present during stillbirth – induced either with a combination of OT and PGs or with OT alone – to assess the potential impact of these molecular alterations on the effectiveness of stillbirth induction.

Methods

Patients

The decidual tissue samples evaluated in our study were obtained from 31 pregnant women in whom stillbirth occurred before the onset of labor (ante-partum stillbirth). These patients were hospitalized during the period between December 2004 and December 2006 either in the Department of Gynecology, Obstetrics and Oncology at the Jagiellonian University, Krakow, Poland, or in the Clinical Department of Obstetrics and Gynecology at the State Hospital in Rzeszow, Poland. The main causes of fetal ante-partum death included fetal anomalies (congenital and karyotypic) (60% of cases), growth restriction (10%), placental thrombosis (10%), and unexplained stillbirth (20%). Furthermore, the patients were divided into two subgroups, according to the method of labor induction following the diagnosis of intrauterine fetal death. Group 1 consisted of patients in whom stillbirth was induced using an intravenous infusion of diluted OT (5 U in 0.5 L of normal saline), at an initial dose of 2 drops per min increased every 5 min by 2 drops per min until regular uterine contraction occurred; when OT proved insufficient (after 24 hr of observation), PGs were administered intravaginally. Group 2 consisted of patients in whom stillbirth was induced using OT, and Misoprostol (PGE_1) was used additionally until delivery occurred (a 200 μg in every 6 hr). The patients in whom fetal death was related to an infection acquired during the birth process (as confirmed by histopathological examination of the fetal membranes and chorionic plate) were excluded from this study. Patients with anti-phospholipids syndrome were also excluded from the study. The tissue samples derived from patients in whom stillbirth was induced were immediately fixed in 10% buffered formaldehyde solution and sent to the Pathomorphology Department of the Jagiellonian University. An experienced pathomorphologist (K.G.) evaluated the routinely stained (HE – hematoxylin and eosin) slides prepared from the paraffin-embedded tissue material and also selected sufficient material for further analysis. Last, selected paraffin blocks were cut and used for immunohistochemistry.

The consent of the patient was obtained in each case. Prior to this study, we also obtained the approval of the Jagiellonian University Ethical Committee for our research program (KBET/89/B/2005).

Immunohistochemistry

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

In each case, immunohistochemistry was performed by using 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, dilution 1:1000) in a moist chamber overnight. Further, the following antibodies were also used: CD56 (NCAM; NCL-CD56-504; Novocastra, MA, US) 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. The 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 for the positive one, but without the primary antibody. RCAS1 reactivity was evaluated in an entire slide from the decidua as follows: 0 (no reactivity), +1 (any staining pattern in up to 10% of the cells), +2 (positive staining in 11–30% of the cells), and +3 (more than 30% of positive cells). The various types of lymphocytes in the decidua were also evaluated. The number of immune cells in an entire specimen was counted and an average cell number per 1 hpf (high power field, objective magnification ×40) 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 – 11 to 20 positive cells/1 hpf; +4 – more than 20 positive cells/1 hpf. Because of the scarcity of CD25-, CD69-, CD56-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 groups of women studied, checked by performing Shapiro-Wilk test, showed that each of the women was different from normal. Non-parametric testing was therefore carried out. Statistical significance between the groups was determined by the Mann–Whitney *U*-test. The data were presented in terms of median value and intraquartile range.

Results

Clinical Comparison of the Two Groups of Patients Analyzed in Whom, Following the Diagnosis of Intrauterine Fetal Death, Labor was Induced Either with a Combination of OT and PGs or with OT Alone

As stillbirth can take place at any of the different stages of pregnancy, it seems appropriate to compare the parameters characterizing the course of pregnancy in the different groups of patients considered (Table I). The profile of the clinical parameters of patients enables us to compare the levels of the antigens studied in these two groups of patients, evaluated according to the method of stillbirth induction.

Immunohistochemical Analysis of the Immune Cells Present and their Activity

CD3-positive cells were identified in all the decidual tissue samples derived from patients in whom stillbirth was induced by oxytocin and in 85% of the decidual tissue samples derived from patients in whom stillbirth was induced by a combination of OT and PGs (Fig. 1).

CD56-positive cells were observed in 42% of the decidual tissue samples derived from those patients induced by OT and in 31% of the decidual tissue

Table I Clinical Characteristics of Patients Who Underwent Stillbirth in Relation to the Method of Labor Induction Following the Diagnosis of Intrauterine Fetal Death

Variables	OT alone (n = 16)	Combination of OT and PGs (n = 15)	P-value
Maternal age (median, IQR)	30 (11)	28 (9)	0.25
Parity (median, IQR)	2 (1)	2 (2)	0.83
Gestational age (median, IQR)	27.5 (8)	27 (3)	0.76
Fetal birth weight (median, IQR)	580 (670)	850 (630)	0.42
Duration of the labor – hours (median, IQR)	16 (6)	30.7 (30)	0.002
Number of diluted oxytocin infusion (median, IQR)	1 (0)	1 (1)	0.16
Number of PGE_1 doses (median, IQR)	0 (0)	1 (1)	<0.001

IQR, intraquartile range; OT, oxytocin; PGs, prostaglandins; PGE_1, misoprostol.

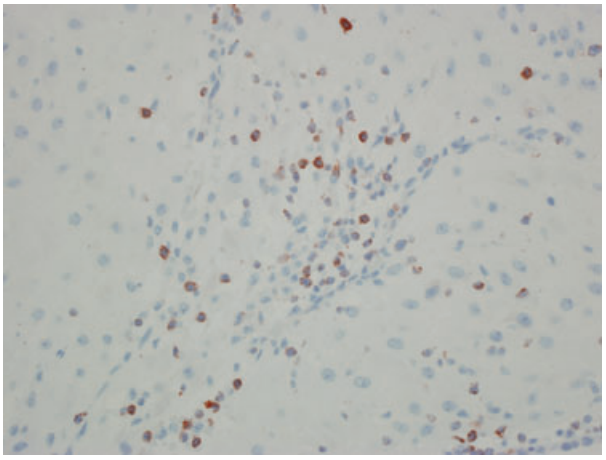

Fig. 1 Multiple CD3 lymphocytes in the infiltrate of deciduas (×40).

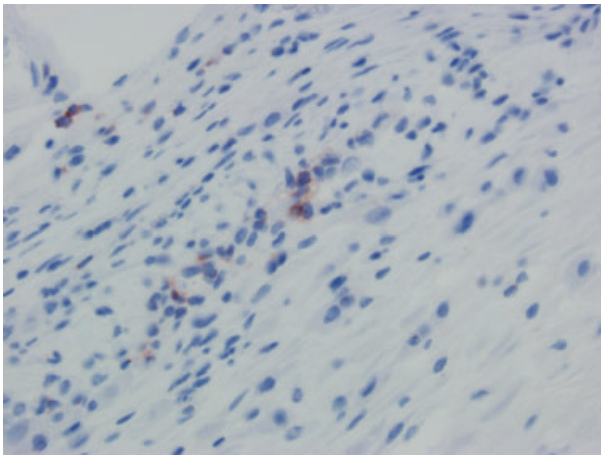

Fig. 3 Immunoreactivity of CD69 antigen in lymphocytes in deciduas (×60).

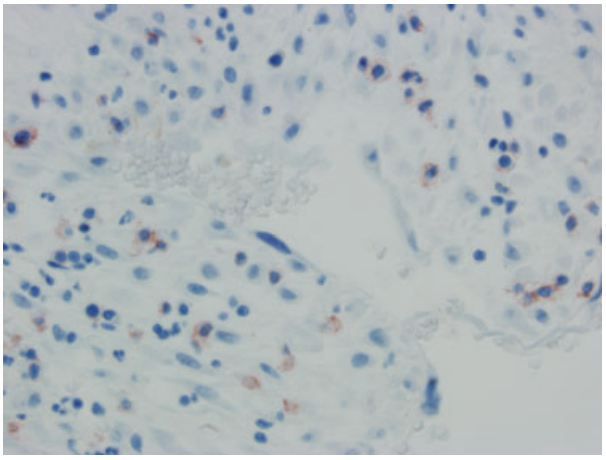

Fig. 2 CD56 positive cells in deciduas (×60).

samples derived from those induced by a combination of OT and PGs (Fig. 2).

CD69 antigen immunoreactivity was observed in 21% of the tissue samples derived from patients in whom stillbirth was induced by OT and in 31% of the tissue samples derived from patients induced by a combination of OT and PGs (Fig. 3). CD25 antigen immunoreactivity was comparably weak in both the groups studied (Table II).

We found no statistically significant differences in the immunoreactivity levels of the antigens CD3, CD56, CD25, and CD69 between the two examined groups that consisted of those patients who underwent stillbirth induced by OT and of those who underwent induction with a combination of OT and PGs (Table III).

Table II Immunoreactivity of CD3, CD56, CD69, and CD25 Antigens within Decidua-Derived from Patients Who Underwent Stillbirth in Relation to the Method of Labor Induction Following the Diagnosis of Intrauterine Fetal Death

		Immunoreactivity per cent (number of cases)			
	Antigens	0	+1	+2	+3
OT alone (n = 16)	CD3	–	38[a] (6)	31 (5)	31 (5)
	CD69	72 (12)	14 (2)	14 (2)	–
	CD56	62 (10)	38 (6)	–	–
	CD25	93 (15)	7 (1)	–	–
Combination of OT and PGs (n = 15)	CD3	13 (2)	26 (4)	20 (3)	41 (6)
	CD69	60 (9)	33 (5)	7 (1)	–
	CD56	67 (10)	33 (5)	–	–
	CD25	87 (13)	13 (2)	–	–

OT, oxytocin; PGs, prostaglandins.
[a]Percentage of cases (n, number of tissue samples).

Comparison of RCAS1 Alterations within Decidua Derived from Stillbirth According to the Method of Stillbirth Induction

RCAS1-positive cells (Fig. 4) were identified in 14% of the decidual tissue samples derived from patients in whom stillbirth was induced by OT and in 54% of the decidual tissue samples derived from patients in whom stillbirth was induced by a combination of OT and PGs (Fig. 4, Table IV).

We have identified statistically significant differences in RCAS1 decidual immunoreactivity level in patients in whom stillbirth was induced by OT when

Table III Analysis of Immunoreactivity of CD3, CD56, CD69, CD25 Antigens and RCAS1 within Decidua in Relation to the Method of Labor Induction Following the Diagnosis of Intrauterine Fetal Death

Variables	OT alone (n = 16)	Combination of OT and PGs (n = 15)	P-value
CD3 (median, IQR)	2 (2)	2 (2)	0.9
CD56 (median, IQR)	0 (1)	0 (1)	0.8
CD69 (median, IQR)	0 (0.5)	0 (1)	0.57
CD25 (median, IQR)	0 (0)	0 (0)	0.7
RCAS1 (median, IQR)	0 (0)	1 (1)	0.019

IQR, intraquartile range; OT, oxytocin; PGs, prostaglandins.

Table IV Immunoreactivity of RCAS1 within Decidua Derived from Patients Who Underwent Stillbirth in Relation to the Method of Labor Induction Following the Diagnosis of Intrauterine Fetal Death

	RCAS1 immunoreactivity per cent (number of cases)			
	0	+1	+2	+3
OT alone (n = 16)	86[a] (14)	14 (2)	–	–
Combination of OT and PGs (n = 15)	41 (6)	46 (7)	13 (2)	–

OT, oxytocin; PGs, prostaglandins.
[a]Percentage of cases (n, number of tissue samples).

compared with those in whom stillbirth was induced by a combination of OT and PGs (Fig. 5, Table III).

Discussion

The RCAS1 immunoreactivity level was significantly higher statistically in decidual tissue samples derived from patients in whom OT alone proved insufficient to induce labor following intrauterine fetal death, so PGs were also used, when compared with the RCAS1 immunoreactivity level of the samples derived from those in whom stillbirth was induced successfully with OT alone. However, we did not observe any differences either in CD56 and CD3 positive cell presence or in CD25 and CD69 antigen immunoreactivity in the respective decidua of these two groups of patients. To our knowledge, this is the first investigation to focus on RCAS1 decidual immunoreactivity in patients in whom stillbirth has been induced.

In our previous study, we showed that the spontaneous course of stillbirth is related to a lower level of RCAS1 placental expression than that is found in patients in whom labor needed to be induced after the diagnosis of intrauterine fetal death.[28] This difference indicates that the spontaneous course of stillbirth may result from the increasing activity of the maternal immune cytotoxic cells in response to the

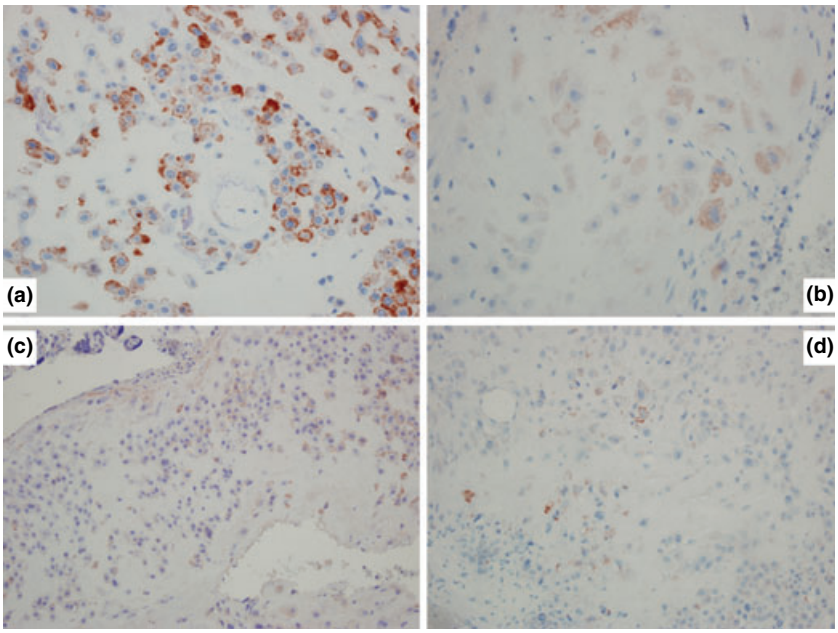

Fig. 4 Decidual RCAS1 immunoreactivity during stillbirth in relation to the method of labor induction following the diagnosis of intrauterine fetal death: a combination of oxytocin and prostaglandins (a,b,c) or oxytocin alone (d). (a) Moderate RCAS1 expression – an area of the decidua with the strongest positive reaction in the entire specimen (×40). (b) Weak expression of RCAS1 – an area of the specimen with the strongest RCAS1 expression in decidual cells (×40). (c) Moderate (+2) expression of RCAS1 in decidual cells (×20). (d) Weak (+1) expression of RCAS in decidual cells (×20).

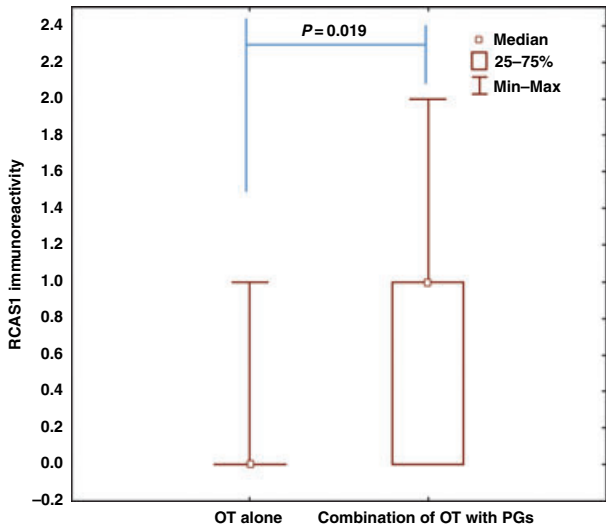

Fig. 5 Comparison of RCAS1 immunoreactivity level within decidua derived from patients who underwent stillbirth in relation to the method of labor induction following the diagnosis of intrauterine fetal death: a combination of oxytocin (OT) and prostaglandins (PGs) or oxytocin alone (OT).

decrease in expression of inhibitory factors (such as RCAS1) in the placenta.[30] The maternal immune tolerance during pregnancy phenomenon is controlled by both placental and decidual cells. We confirmed this finding in our previous study that analyzed RCAS1 expression in both eutopic and ectopic decidua with concomitant consideration of the presence and activity of the immune cells during cesarean section.[45] Investigating ectopic decidua allowed us to study the immunomodulating activity of decidua free from the suppressory influence that the placenta exerts on decidua within the uterine cavity. We clearly demonstrated that the activity of ectopic decidua is effective enough to inhibit the infiltrating immune cells during cesarean section.[45] Our reason for choosing the stillbirth was to show that decidua and its associated cofactors are essential to the course of labor, during which the function of both the fetal adrenals and fetal immune system is disrupted. Placental suppressory activity may also be observed during stillbirth, but probably does not exhibit the alterations that typically occur with the various stages of physiological labor. This is most likely related to the necessity for inducing stillbirth when the RCAS1 level in the placenta is observed to be still elevated even after intrauterine fetal death. Furthermore, it has been demonstrated that the initiation of vaginal labor at term is related to an increase in the number of immune cells infiltrating the decidua.[12,20–24,26,46–48] The number of these cells in the decidua has been found to be significantly higher following the spontaneous initiation of labor than after elective cesarean section.[23]The increase is most prominent during the spontaneous beginning of labor, whereas the further progression of labor is characterized by an actual decrease in the activity of immune cytotoxic cells in the decidua.[26,29] This decrease is a response to the increasing suppressory activity of decidual cells as labor progresses, because the inhibitory activity of the placenta diminishes with the advancement of labor.[30] This decidual function may be related to the expression of RCAS1. In this study, we have shown for the first time that the decrease in RCAS1 immunoreactivity, even when a comparable number of cytotoxic immune cells were present, enabled the induction of stillbirth by OT alone (mainly by inducing myometrial activation). By contrast, patients with a high level of cytotoxic immune cell suppression in the decidua and a correspondingly high level of RCAS1 immunoreactivity, needed an additional application of PGs to be induced following intrauterine fetal death. We speculate that, if alterations in RCAS1 levels in decidua are found, they will correlate with alterations in the number and activity of immune cells, and then stillbirth will spontaneously begin. However, these alterations in immune cells are related not only to decreased decidual RCAS1 levels, but also to the placental RCAS1 level, and in our study, we did not observe any differences in the number and activity of immune cells in the decidua according to the method of stillbirth induction.

The more the level of OTR expression in the myometrium rises with the increase in myometrial contractility,[10] the more stretching will occur with uterine contraction; this in turn will increase cyclooxygenase (COX-2) and PGs production.[11,12] However, alterations in the level of immune tolerance as the course of labor progresses are related to both OTR expression and an increase in COX-2 activity.[12,13] Interleukin-1beta (IL-1beta) increases the secretion of OT in decidua as well as the production of prostaglandins through COX-2, but at the same time decreases OTR expression.[14] During normal physiological labor, PGs would be released by the membranes in response to stretching and pro-inflammatory cytokine activity.[12,15,19,20,49] Furthermore, it has been shown that the typical blockade of immune responses during labor results in a decrease in PGs

production.[50,51] Prostaglandins are important mediators of the immune system reactions that accompany labor, such as immunoregulation and feto-placental communications.[19] On the one hand, interleukins (such as IL-1beta[49], IL-10[16], IL-6[17], IL-8[17], and TNF-alpha[14]) regulate the synthesis of PGs in a woman's reproductive tract during labor; on the other hand, however, prostanoids increase the production of cytokines.[52] Thus human labor, assisted by the increased synthesis of PGs in a woman's reproductive tract, is a complex molecular process, and its proper course is determined by the interaction of cytokines and PGs.[12,13] PGs enable the development of proper uterine contractile activity and the maturation of the uterine cervix[53] once the immune system has been stimulated at the beginning of labor.[53,54] PGs also might allow the molecular reaction initiated at the beginning of labor to be terminated by both the maternal and fetal immune systems. The results of our study indicate that the increase in PGs in a woman's reproductive tract even with a high level of cytotoxic immune cell inhibition in the decidua permits the molecular reaction to be triggered, although the number of CD3- and CD56-positive cells is almost stable in both cases of stillbirth, with higher and lower level of immune cell inhibition. In such cases, the application of OT alone during stillbirth proves insufficient because it does not affect the maternal immune system activity in the same way as PGs.

Conclusion

The level of RCAS1 in the decidua seems to influence the effectiveness of stillbirth induction.

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