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H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

   なし

2. 実用新案登録

   なし

3. その他

   なし

厚生労働科学研究費補助金（成育疾患克服等次世代育成基盤研究事業）
分担研究報告書

流産・不育症の病理所見について

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研究要旨
当センターの流産症例につき、流産物の病理学的解析を行った。流産物の病理標本のためのチェック項目リストを作成した。異常病理所見を抽出し、それらに従って流産の分類を試みた。習慣性流産と単発の流産との比較検討などは次年度以降に行う予定である。今年度はパイロット的に行い、フィブリン沈着がびまん性に見られる例に流産を繰り返す症例が見られた。

A．研究目的

繰り返す IUFD(intrauterine fetal death)は胎児側の要因と母体側の要因ともに検索する必要がある。劣性遺伝の奇形症侯群などが胎児に見つかる事もある。母体側要因として膠原病やその他の免疫異常が関連する事も多い。抗リン脂質抗体である抗カルジオリピン抗体(ACA)が高値を示す症例があるが、これらの胎盤を検索した結果、絨毛の周囲のトロフォブラストにフィブリンが沈着し、その部位に IgG の沈着が見られる事を以前に報告した。この梗塞像は、従来からいわれていた虚血性病変とは病因論的に違うと考え、perivillous fibrin deposition(PVFC)と呼んでいる。この場所での免疫反応の結果生じたものと推定される。

流産物の病理検査は、母児の異常を後方視的にも検索できる重要なものである。臨床所見や血液の臨床検査とともに病理検体からの情報を解析し、習慣性流早産の病態の研究を行った。

B．研究方法

流産の標本を臨床経過と将来に比較検討すべく、チェックリストを利用した（表-1)。絨毛・絨毛間腔・脱落膜の異常所見をそれぞれ記載することとした。

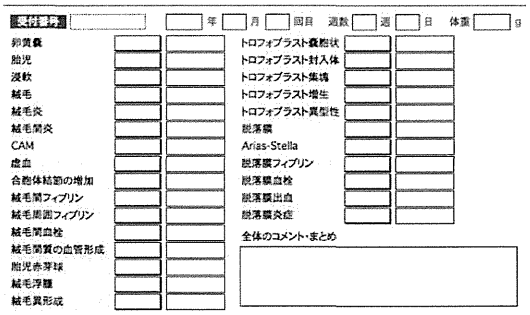

表 1-流産物のチェックリストの一様式

これらのチェックリストによる病理標本の検討結果及び別に行った染色体の結果を基に流産原因を 5 型に分類した。

（倫理面への配慮）

本研究は診療情報の一部としての胎盤病理検査結果を用いて解析を行ったもので、個人情報の保護に配慮した。

C．研究結果

流産の原因を病理標本及び染色体検査の結果から、次の 5 型に分類した。

①染色体異常などによる流産
②感染症（急性、慢性）が関連する流産
③自己抗体などに関連して、止血・凝固異常が関連すると考えられる流産
④子宮・胎盤系の血流異常(虚血病変)
⑤その他

単発流産における代表的な所見は、虚血様所見、絨毛間血栓、異形成絨毛、絨毛浮腫などである（図 1）。

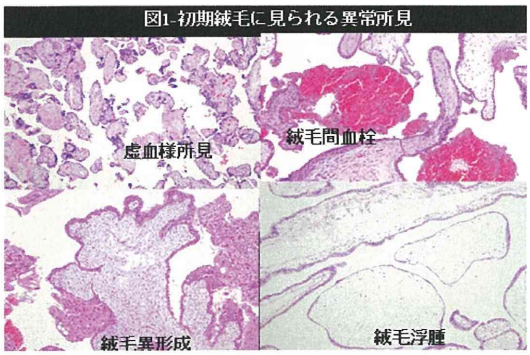

図 1-単発流産における代表的な病理所見

①染色体異常は、初期流産の半分以上を占めると言われている。妊娠中期以後では、異形成絨毛がよく見られる所見であるが、初期絨毛では異形成絨毛が見られないものも多い。特に最も多い 16 トリソミーでは異形成絨毛を伴わない。トロフォブラストの小嚢胞状変化は、従来より部分胞状奇胎に特徴的な所見と言われ、三倍体の胎盤所見として記載されているがこれも説得性のある所見とは言えない。部分胞状奇胎の診断には、流産の標本の解釈においても染色体の分析が十分になされていないことが問題と考えられる。

②流産児の病理検査における感染症は、中期以後の胎盤所見と見方が異なる。絨毛膜羊膜炎という形よりも、瀰漫性の細胞浸潤、あるいは膿瘍という形で流産が起こる。中期よりも比較的頻度は低い。

③絨毛周囲性のフィブリン沈着(perivilous fibrin change=PVFC)は、絨毛周囲に、trophoblast に密着し、しばしば trophobalast に変性が見られ、その直上にフィブリン沈着が見られるものである(図 2)。IgG など免疫グロブリンの沈着が認められることも多い。

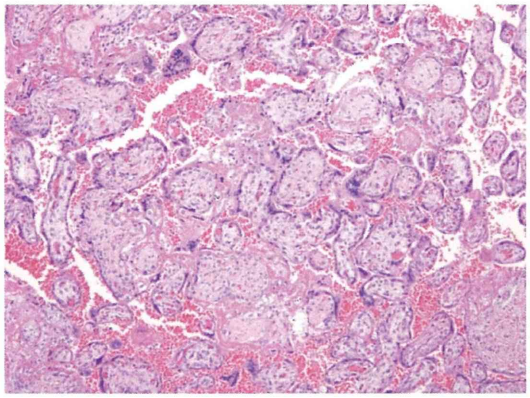

図 2-絨毛周囲性のびまん性フィブリン沈着(PVFC)

胎盤病理所見から習慣性流早産が窺われる所見として、Rohr's fibrin(massive intervillous fibrin deposition)がある（図 3）。絨毛間のフィブリンが、広範囲に、時には胎盤の全面に沈着する。胎盤は全体にかたく、貧血状で、実質臓器の様相を呈する。数ケ所から標本を作成し、絨毛間にフィブリンが瀰慢性に沈着する所見であった。PVFC とは異なり、組織学的には

瀰漫性の絨毛間フィブリンである。母体の膠原病や凝固異常症と関連する場合も見られる。繰り返す流早産の原因となり、次回妊娠の管理が重要である。

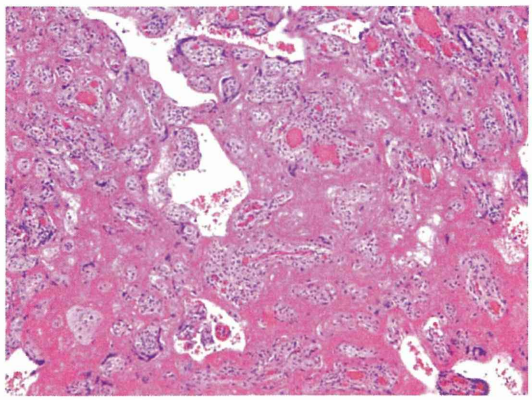

図 3 -絨毛間のびまん性フィブリン沈着(Rohr)

④絨毛の虚血所見は、妊娠中期以後の胎盤では、妊娠高血圧症との関連で確立されているが、流産物でも比較的高頻度にその所見が認められた。現在では、母体の異常との因果関係では、結論を出せなかった。

⑤その他の所見として、トロフォブラストの小嚢胞状変化、栄養膜細胞巣外皮、絨毛間腔内細胞浸潤などが目立つ症例が散見されたが、その臨床的意義などは不明であった。

D．考察

結果でも示したが、妊娠中期以後では、異形成絨毛がよく見られる所見であるが、初期絨毛では異形成絨毛が見られないものも多い。特に最も多い 16 トリソミーでは異形成絨毛を伴わない。以前に当科で行った初期絨毛の染色体検査と絨毛の病理変化の関連を示す（表 2,3)。初期絨毛では、絨毛の異形成よりも浮腫状変化がそれを示す可能性がある。

表2. 習慣性流産とその他の症例との染色体検査結果の比較

	全症例(%)	習慣性流産(%)	その他(%)
正常核型	81(44.3)	32(40.0)	49(47.6)
Trisomy	67(36.6)	30(37.5)	37(35.9)
Monosomy	16(8.7)	6(7.5)	10(9.7)
Triploidy	5(2.7)	1(1.3)	4(3.9)
Tetraploidy	3(1.6)	3(3.8)	0(0.0)
その他転座など	11(6.0)	8(10.0)	3(2.9)
	183	80	103

表3. Trisomyのうちわけと組織所見Dysmature像の有無

		Dysmatureと診断した症例:	
Trisomyのうちわけ	件数※	初回診断	再検時
16 trisomy	18(12)	0	3
21 trisomy	9(8)	0	1
15 trisomy	7(4)	1	1
4 trisomy	5(1)	0	0
8 trisomy	4(2)	1	1
22 trisomy	4(2)	0	1
18 trisomy	3(3)	2	3
13 trisomy	2(2)	1	2
20 trisomy	2(1)	1	1
7 trisomy	2(2)	0	2
2,3,9,10,14 trisomy	1*5(1)	0	0
2つ以上のtrisomy	6(1)	0	1
Total	67(38)	6	16

※( )内は、絨毛の評価が可能であった件数

習慣性流早産と関連する病理所見として、絨毛間フィブリン(PVFC)と絨毛周囲性フィブリン(Rohr fibrin)がある。これらは、別々の成り立ちのものか，あるいは程度の差を示すものか更に検討を要する。多数の症例を臨床経過や検査所見と比較検討することにより明らかにしたい。

流産における病理所見を解析する上での問題点を以下に示す。

1. 流産はもともと、異常状態なので、コントロール設定が困難である。
2. 組織観察のみ。胞状奇胎などわずかな例外を除いて肉眼所見が

ほとんど役立たない。

3. 出血と絨毛間血栓との類似。流産物排出の際の artifact が出血あるいは血腫としてみられる可能性があり、これと意味のある絨毛間血腫を鑑別する手段を考慮する必要がある。
4. 胎盤遺残とフィブリンの課題。稽留流産などでどのような病理変化が生じるか？検討の必要がある。
5. 初期流産における虚血所見は、特定の臨床状態と結びつかず、現時点では、臨床的な意義が不明である。今後、多数の例で検討する予定である。

以上の問題点を次年度以降に検討する予定である。

E．結論

今年度は、流産病理の解析方法を作成すること、その基本的な病理所見を提示することにとどまった。次年度以降、臨床経過や検査所見を比較検討することにより、習慣性流早産の病理所見を明らかにしていきたい。

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G．研究発表

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H. 知的財産権の出願・登録状況
(予定を含む。)

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

Ⅲ.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Characterization of regulatory T cells in decidua of miscarriage cases with abnormal or normal fetal chromosomal content

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ABSTRACT

Decreased regulatory T (Treg) cells have been reported in cases of recurrent pregnancy loss. To understand the role of Treg cells in human pregnancy, we have studied the frequency, localization and characterization of Treg cells in the decidua. The frequency of $Foxp3^+$ cells among $CD3^+CD8^-$ cells at the decidua basalis in cases of miscarriage with a normal embryo karyotype ($n = 10$) was significantly lower than in normally progressing pregnancies ($n = 10$). However, those frequencies in miscarriage with an abnormal embryo karyotype were similar to normally progressing pregnancies. Next, we used flow cytometry to study Treg cell expression of the proliferation marker Ki67 and functional Treg marker CCR5. The frequency of $Foxp3^+CD4^+$ T cells in miscarriage with a normal embryo ($n = 10$) was significantly lower than those in normally progressing pregnancies ($n = 15$) and in miscarriage with an abnormal embryo ($n = 14$). In miscarriage with a normal embryo, the population of $Ki67^-Foxp3^+CD4^+$ T cells was significantly smaller than in normal pregnancy. However, the frequencies of $Ki67^+Foxp3^+CD4^+$ cells and $CCR5^+Foxp3^+CD4^+$ cells were not different between the three groups. These data suggest that increased $Ki67^-$ Treg cells in the decidua basalis may play an important role in the induction of immune tolerance, and that immune-medicated pregnancy loss may be caused by decreased $Ki67^-$ Treg cells in the implantation site.

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1. Introduction

Regulatory T (Treg) cells were discovered as $CD4^+CD25^+$ T cells (Sakaguchi et al., 1995). It was subsequently clarified that Foxp3 is the master gene controlling the differentiation of Treg cells (Hori et al., 2003). In 2004, Aluvihare and colleagues reported that Treg cells might mediate maternal tolerance in mice during pregnancy (Aluvihare et al., 2004). Adoptive transfer of Treg cells purified from normal pregnant mice prevented fetal loss in CBA/J mated with DBA/2J mice, but transfer of Treg cells from non-pregnant mice was ineffective (Zenclussen et al., 2005). Recent data showed that Treg cells specific for male antigen (H-Y) contribute to tolerance of male fetuses in syngeneic pregnancy in mice (Kahn and Baltimore, 2010). To establish fetal antigen-specific Treg cell induction, seminal plasma plays an important role in mice (Robertson et al., 2009). In humans, our group was the first to show that Treg cells accumulate in the decidua in normal pregnancy, and the population of Treg cells is decreased in miscarriage (Sasaki et al., 2004). Women who experienced repeated miscarriage were shown to have a reduced frequency of Treg cells and reduced suppressive capacity of Treg cells

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(Arruvito et al., 2010; Wang et al., 2010). A decreased Treg cell pool was observed in preeclamptic cases (Sasaki et al., 2007) and normal pregnancies just prior to delivery (Zhao et al., 2007). Selective migration of fetus-specific Treg cells from the peripheral blood to the decidua in human pregnancy has also been reported (Tilburgs et al., 2008).

To understand the role of Treg cells in human pregnancy, we have studied the frequency and localization of Treg cells at the implantation site in cases of miscarriage with normal chromosomal content and with abnormal chromosomal content using immunohistochemical staining. Furthermore, we have attempted to identify a suitable marker for immune-modulating Treg cells involved in the maintenance of pregnancy in humans using flow cytometric analysis. It has been reported that chemokine receptor $CCR5^+$ Treg cells play an important role in the induction of fetal-derived alloantigen-specific tolerance in mice (Kallikourdis et al., 2007). $CCR5^+$ Treg cells effectively inhibited the proliferation of $CD4^+CD25^-$ cells stimulated by cross-linking with anti-CD3, suggesting that $CCR5^+$ Treg cells are a functional Treg subset. Our recent data shows that proliferating, paternal antigen-specific $Ki67^+$ $CCR5^+$ Treg cells accumulate in the uterine draining lymph node before implantation and in the uterus after implantation in mice (Shima et al., 2010a). Against this background, we have studied the expression of Ki67 and CCR5 in $CD4^+Foxp3^+$ Treg cells in humans, although there is no information on whether $Ki67^+$ Treg cells or $CCR5^+$ Treg cells are functional, or not.

2. Materials and methods

2.1. Cases of normal pregnancy and miscarriage

This study was approved by the Ethics Committee of the University of Toyama. We enrolled 15 subjects with normal pregnancy, 14 with miscarriage with abnormal chromosomal content [trisomy ($n=12$) including trisomy 15 ($n=3$), trisomy 16 ($n=3$), trisomy 21 ($n=2$), trisomy 22 ($n=2$), trisomy 6 ($n=1$) and trisomy 13 ($n=1$); translocation ($n=1$) and monosomy ($n=1$)], and 10 with miscarriage with normal chromosomal content by cytometric analysis. An additional $n=10$ subjects with normal pregnancy, $n=10$ with miscarriage with abnormal chromosomal content, and $n=10$ with miscarriage with normal chromosomal content were included for other analyses. No subjects had risk factors such as uterine malformation, thyroid dysfunction, genetic abnormalities, or antiphospholipid antibody syndrome. Decidual samples were obtained from pregnant subjects undergoing induced abortion, representing 'normal pregnancy', and from patients with miscarriage, representing 'miscarriage'. Gestational age was calculated from the diameter of the gestational sac by echosonography or the last menstrual period. When miscarriage was diagnosed, dilation and curettage were performed. We selected missed abortion cases and excluded inevitable abortion cases because immunological components differ between missed abortion (at an early stage of abortion) and inevitable abortion (at a late stage of abortion) (Nakashima et al., 2010).

Chorionic villi were sampled from miscarriage cases for cytogenetic analysis. Karyotypes determined from examination of miscarriage cases were abnormal in 10 cases and normal in 10 cases for the immunohistochemical study (Table 1), and abnormal in 14 cases and normal in 10 cases for the flow cytometric study (Table 2). In both studies, half of the chorionic tissue was used for the karyotype determination test, and half for pathological examination. Clinical backgrounds in the three groups are shown in Tables 1 and 2. The numbers of previous miscarriages in the women miscarrying with an abnormal embryo or miscarrying with a normal embryo were significantly higher than in normal pregnancy (Tables 1 and 2). In the immunohistochemical study, the number of gestational weeks at sampling in cases of miscarriage with an abnormal embryo was significantly less than that in cases of normal pregnancy (Table 1), but the numbers of gestational weeks at sampling were similar between the three groups in flow cytometric analysis (Table 2). We obtained written informed consent from all the subjects.

2.2. Flow cytometry

Decidual mononuclear cells (leukocytes) were purified by the Ficoll Hypaque method after homogenization and filtration through a 32 μm nylon mesh as previously reported (Saito et al., 1999).

The following monoclonal antibodies (mAbs) were used in this study: anti-CD4 (PerCP-Cy50.5; BD Bioscience, USA)

Table 1
Clinical background in normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo in immunohistochemical examination.

	Normal pregnancy ($n=10$)	Miscarriage with an abnormal embryo ($n=10$)	Miscarriage with a normal embryo ($n=10$)
Age (year)[a]	29 (25–38)	34 (26–43)	34 (26–38)
Gravidities[a,b]	2 (1–3)	2.5 (1–9)	4.5 (1–12)
No. of liveborn children[a]	0 (0–2)	1 (0–3)	0 (0–1)
No. of miscarriages[a,b]	0 (0–0)	2 (1–8)[†]	4 (1–12)[†]
Stillbirth[a]	0 (0–0)	0 (0–1)	0 (0–1)
Gestational weeks[a]	8 (7–10)	6 (5–8)[†]	8 (7–9)
BMI	19.1 (17.9–25.3)	21.1 (17.5–26.3)	21.0 (18.6–25.5)
Smoker	1 (10%)	0	0

[a] Median (range).
[b] Gravidities and miscarriages included.
[†] $P<0.05$ vs. normal pregnancy.

Table 2
Clinical background in normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo in flow cytometry examination.

	Normal pregnancy ($n=15$)	Miscarriage with an abnormal embryo ($n=14$)	Miscarriage with a normal embryo ($n=10$)
Age (year)[a]	27 (17–39)	36 (30–45)[†]	34 (29–39)
Gravidities[a,b]	2 (1–6)	4.5 (1–6)	4 (2–5)
No. of liveborn children[a]	0 (0–4)	0 (0–2)	1 (0–3)
No. of miscarriages[a,b]	0 (0–1)	4 (1–6)[††]	3 (1–5)[††]
Stillbirth[a]	0 (0–0)	0 (0–1)	0 (0–1)
Gestational weeks[a]	7 (6–9)	7 (6–11)	6 (6–10)
BMI	19.6 (17.4–20.8)	19.1 (17.1–33.7)	21.5 (17.7–25.8)
Smoker	2 (20%)	0	0

[a] Median (range).
[b] Gravidities and miscarriages included.
[†] $P<0.001$ vs. normal pregnancy.
[††] $P<0.0001$ vs. normal pregnancy.

and anti-CCR5 (PE; BD Bioscience, USA) for cell surface markers, and anti-Foxp3 (FITC; eBioscience, USA) and anti-Ki67 (Alexa Fluor647; BD Bioscience, USA) for intracellular markers. Decidual mononuclear cells were first stained with anti-CD4 mAb and anti-CCR5 mAb, then fixed and permeabilized by incubation for 30 min with fixation/permeabilization buffer (eBioscience, USA), and then stained with anti-Foxp3 mAb and anti-Ki67 mAb. Flow cytometry analysis was performed on a BD FACScan II (BD Bioscience, USA).

2.3. Immunohistochemical staining

To avoid maternal blood contamination, decidual tissues were washed in phosphate-buffered saline (PBS) and then fixed in 10% neutral buffered formalin for 48 h and embedded in paraffin. We stained paraffin-embedded sections immunohistochemically using mouse monoclonal anti-cytokeratin 7 antibody (Novus Biological, USA). Decidual tissues were classified as decidua basalis (presence of cytokeratin-positive extravillous trophoblasts (EVT)) or decidua parietalis (no cytokeratin-positive EVTs) as previously reported (Michimata et al., 2002). We checked the cytokeratin-positive EVT in decidual tissue in the cases shown in Table 2, but we could not obtain enough samples to evaluate Treg cells in the decidual basalis and decidua parietalis because most of the decidual sample was used for flow cytometric analysis. Therefore, we collected additional samples from other cases for immunohistochemistry, including $n=10$ normal pregnancy, $n=10$ miscarriage with abnormal chromosomal content, and $n=10$ miscarriage with normal chromosomal content (Table 1).

After deparaffinization, antigen retrieval was performed by autoclaving at 120 °C for 10 min. Sections were washed in PBS and incubated for 10 min in 10% normal goat serum prior to application of primary antibodies.

Sections were incubated overnight at 4 °C with mouse mAbs including anti-human CD3 (5 μg/ml; Novocastra, UK), anti-human CD8 (5 μg/ml; Dako Japan, Tokyo) and anti-human Foxp3 (eBioscience, USA). Immunohistochemistry was performed using the Envision system (Dako Japan, Tokyo) and diaminobenzidine (Sigma, USA). Commercially available CD4 monoclonal antibody was not effective for staining in paraffin-embedded tissue samples, so the numbers of $CD4^+$ T cells were calculated by subtracting the $CD8^+$ cell count from the $CD3^+$ cell count, as reported previously (Michimata et al., 2002).

2.4. Statistical analysis

The data were analyzed statistically by the Mann–Whitney U test using a statistical software package (SAS version 9.1; SAS Institute, USA). For all statistical analyses, $P<0.05$ was considered significant.

3. Results

3.1. Frequency of $Foxp3^+CD3^+CD8^-$ cells in the decidua basalis and decidua parietalis in immunohistochemical study

CK7-positive extravillous trophoblasts were observed in the deciduas (Fig. 1). We classified tissue as decidua basalis where EVT was present and decidua parietalis where EVT were absent. Surface staining for CD3 and CD8 and nuclear staining for Foxp3 were detected.

The frequency of $Foxp3^+$ cells among $CD3^+CD8^-$ cells in the decidua basalis of miscarriage cases with a normal embryo was significantly lower ($P=0.03$) than in cases of normal pregnancy (Fig. 2). However, the frequency in the decidua basalis of miscarriage cases with an abnormal embryo was similar to cases of normal pregnancy (Fig. 2). Interestingly, the frequencies of $Foxp3^+$ cells among $CD3^+CD8^-$ cells in the decidua parietalis were similar in normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo. This data suggests that reduced accumulation of Treg cells in the area of implantation (the decidua basalis) occurs in cases of miscarriage with a normal embryo.

3.2. Frequency of $Foxp3^+$ cells, $Ki67^+Foxp3^+$ cells, and $CCR5^+Foxp3^+$ cells among $CD4^+$ cells in flow cytometric study

The frequency of $Foxp3^+$ cells among $CD4^+$ cells in miscarriage was significantly lower ($P=0.0051$) than that in

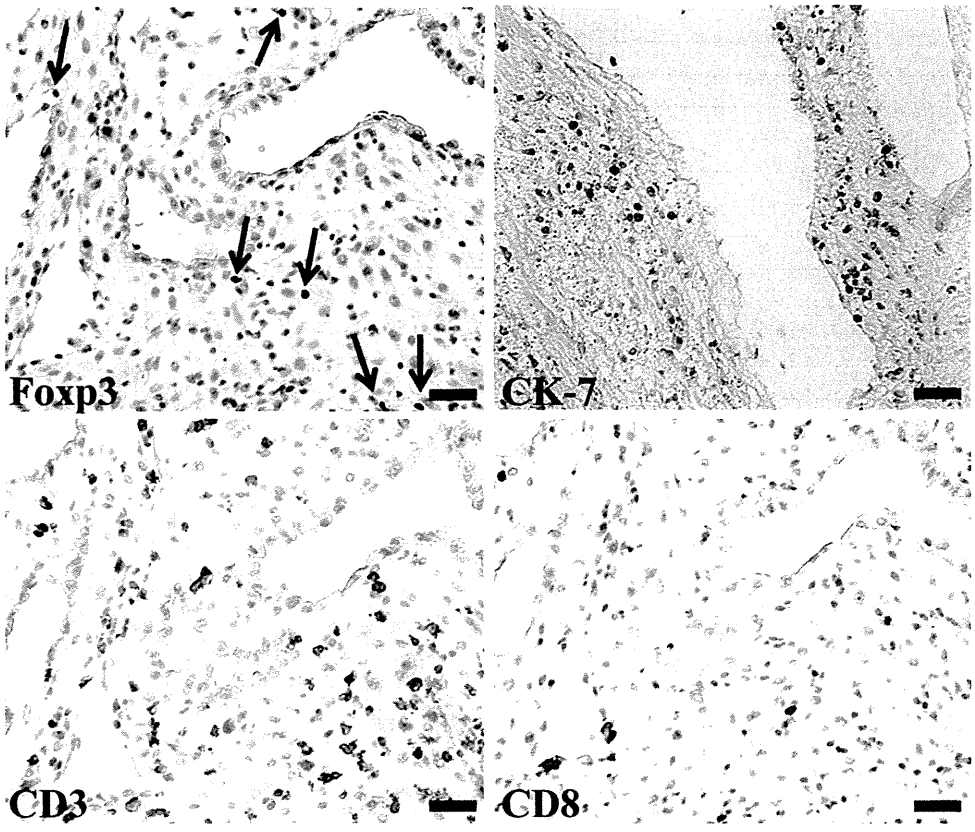

Fig. 1. Immunohistochemical staining for Foxp3, CD3, CD8, and CK7 in the decidua of cases of normal pregnancy. Magnification: 200×, scale bar: 30 μm.

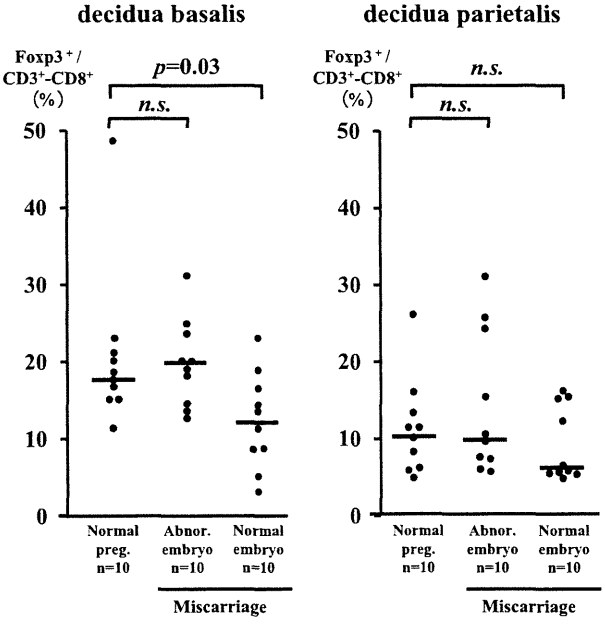

Fig. 2. Frequencies of $Foxp3^+$ cells amongst $CD4^+$ ($CD3^+CD8^-$) cells in the decidua basalis (left) and decidua parietalis (right) of cases of normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo, analyzed by immunohistochemistry. n.s. = not significant; horizontal line is the median value.

normal pregnancy cases (Fig. 3 left). Next, we divided miscarriage cases into those with abnormal fetal chromosomal content and those with normal fetal chromosomal content (Fig. 2 right). The frequency of $Foxp3^+$ cells among $CD4^+$ cells in cases of miscarriage with a normal embryo was significantly lower than in cases of normal pregnancy ($P = 0.007$), and than in cases of miscarriage with an abnormal embryo ($P = 0.0465$).

The frequency of Ki67-$Foxp3^+$ cells (non-proliferating Treg cells) among $CD4^+$ cells in cases of miscarriage with a normal embryo was significantly lower ($P = 0.0033$) than in cases of normal pregnancy, but this frequency in cases of miscarriage with an abnormal embryo was similar to the frequency in normal pregnancy (Fig. 4). The frequency of proliferating $Ki67^+Foxp3^+$ cells among $CD4^+$ cells was rather low compared with $Ki67^-Foxp3^+$ cells among $CD4^+$ cells, and these frequencies were similar in cases of normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo (Fig. 4).

In mice, $CCR5^+$ Treg cells have been reported to be functional Treg cells (Kallikourdis et al., 2007). However, the frequencies of $CCR5^+Foxp3^+$ cells among $CD4^+$ cells in the decidua were similar in cases of normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo (Fig. 5). There was no tendency toward a relationship between Treg frequency and any specific karyotype.

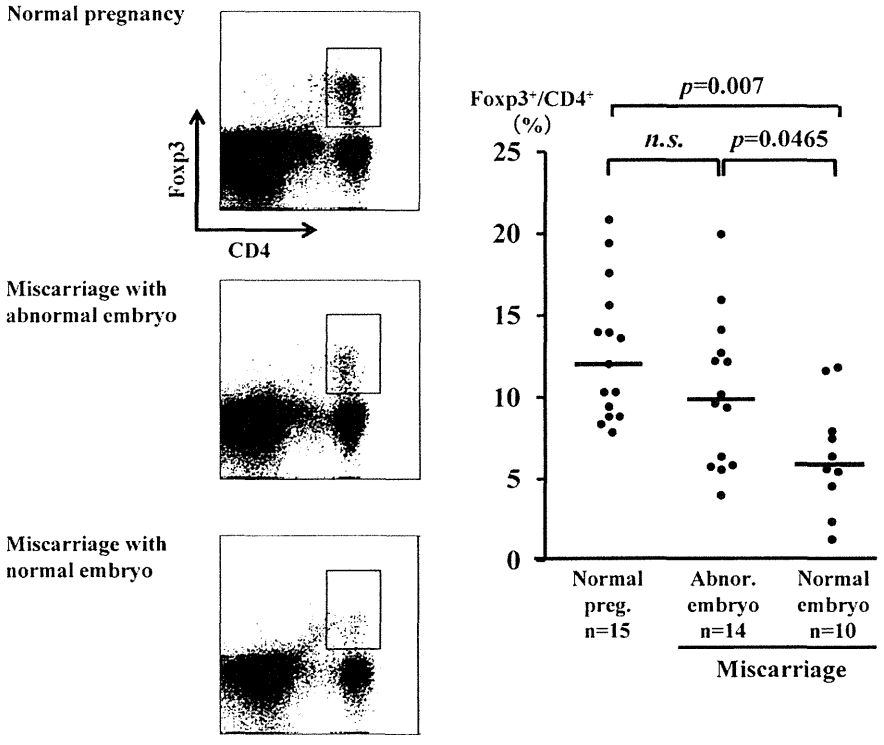

Fig. 3. Frequencies of Foxp3$^+$ cells amongst CD4$^+$ cells in the decidua of cases of normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo, analyzed by flow cytometry. Representative flow cytometric data (a) and frequencies of Foxp3$^+$ cells amongst CD4$^+$ cells in each group (b) are shown. Horizontal line is the median value.

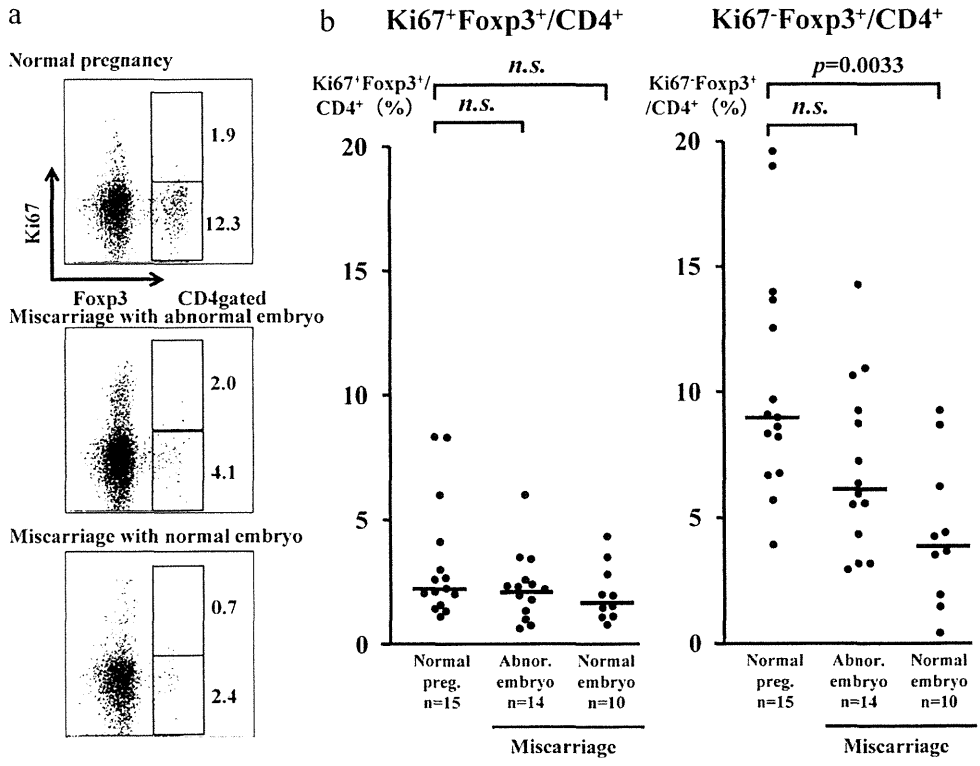

Fig. 4. Frequencies of Ki67$^+$Foxp3$^+$ Treg amongst CD4$^+$ cells (left) and Ki67$^-$Foxp3$^+$ Treg cells among CD4$^+$ cells (right) in the decidua of cases with normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo, analyzed by flow cytometry. Representative flow cytometric data (a) and frequencies of Ki67$^+$Foxp3$^+$ Treg cells among CD4$^+$ cells in each group (b) are shown. n.s. = not significant; horizontal line is the median value.

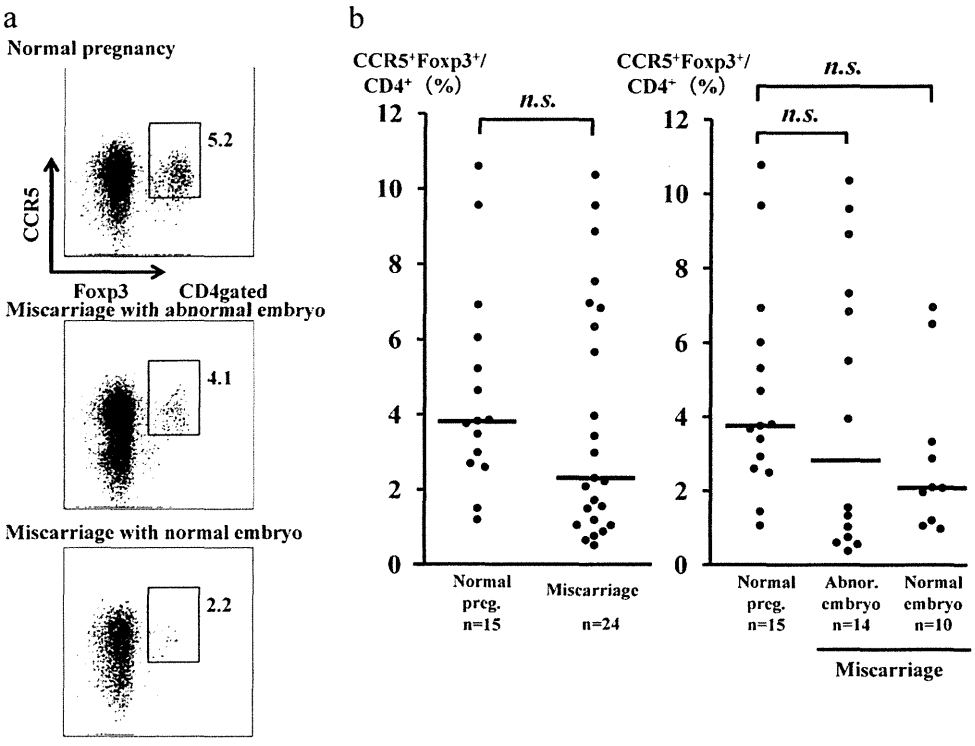

Fig. 5. Frequencies of CCR5$^+$Foxp3$^+$ Treg cells amongst CD4$^+$ cells in the decidua of cases of normal pregnancy, miscarriage with an abnormal embryo, and miscarriage with a normal embryo, analyzed by flow cytometry. Representative flow cytometric data (a) and frequencies of CCR5$^+$Foxp3$^+$ Treg cells among CD4$^+$ cells in each group (b) are shown. n.s. = not significant; horizontal line is the median value.

In summary, reduced accumulation of Ki67$^-$ Treg cells at the implantation area occurred in cases of miscarriage with normal fetal chromosomal content.

4. Discussion

Treg cells inhibit cytokine production in both CD4$^+$ T cells and CD8$^+$ T cells, cytotoxic activity of NK cells, and dendritic function and maturation, resulting in induction of tolerance (Sakaguchi, 2005; Akbar et al., 2007; Shevach, 2009; Corthay, 2009). Loss of Treg cells during early pregnancy induces implantation failure and abortion in mice (Aluvihare et al., 2004; Darrasse-Jèze et al., 2006; Shima et al., 2010b; Rowe et al., 2011). Transfer of exogenous Treg cells from pregnant mice to abortion-prone mice decreased the abortion rate and induced LIF and TGFβ synthesis (Zenclussen et al., 2005, 2006; Yin et al., 2012).

In humans, Treg cells in the decidua were found to be decreased in miscarriage cases (Sasaki et al., 2004; Jin et al., 2009) and in unexplained spontaneous abortion (Yang et al., 2008; Mei et al., 2010; Wang et al., 2010; Lee et al., 2011). Furthermore, low levels of circulating CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are predictive of a miscarriage risk in newly pregnant women with a history of failure (Winger and Reed, 2011), suggesting that the level of peripheral Treg cells may serve as a superior pregnancy marker. Dysregulation of Treg cells in patients with unexplained recurrent miscarriage has also been reported (Wang et al., 2011; Arruvito et al., 2010). Tilburgs and colleagues have reported that cases of HLA-C mismatched pregnancy exhibit a decidual lymphocyte response to fetal cells and contain functional Treg cells in decidual tissue, whereas cases of HLA-C matched pregnancy do not (Tilburgs et al., 2009). These findings support the idea that Treg cells also play an important role in the maintenance of pregnancy in humans.

The etiology of recurrent pregnancy loss (RPL) is unknown in 40–60% of cases (Clifford et al., 1994). If immune dysfunction can cause miscarriage, immunological change may occur at the implantation site (decidua basalis) of RPL cases with a normal embryo (Saito et al., 2011). In this study, we first showed that the accumulation of Treg cells in the decidua basalis was decreased, especially in cases of miscarriage with a normal embryo. Furthermore, the frequency of Treg cells remote from the implantation site (decidua parietalis) was shown to be similar to that in normal pregnancy. These findings suggest that a proportion of cases of RPL with a normal embryo are associated with immune etiologies.

A recent paper showed the heterogeneity of Treg cells, with subsets including CD45RA$^-$Foxp3^{low} resting Treg cells, CD45RA$^-$Foxp3^{high} activated Treg cells, CD4$^+$CD25$^+$Foxp3$^+$ conventional Treg cells, CD4$^+$CD25$^-$Foxp3$^+$ Treg cells, HLA$^-$DR^{high}CD45RA$^-$ Treg cells, HLA-DR^{low+}CD45RA$^-$ Treg cells, HLA-DR$^-$CD45RA$^-$ Treg cells, and naïve HLA-DR$^-$CD45RA$^+$ Treg cells (Miyara et al., 2009, Nishioka et al., 2006; Arruvito et al., 2010; Steinborn et al., 2012).

Kallikourdis et al. (2007) reported that paternal alloantigen enhanced the accumulation of CCR5$^+$ effector Treg

cells in the murine pregnant uterus. CCR$^+$ Treg cells might be a marker of Treg cells activated to become functional by paternal antigen in the mouse. Shima et al. (2010a) also reported that paternal antigen-specific Ki67$^+$ proliferating Treg cells expressed CCR5 on their surface. These findings suggest that CCR5$^+$ proliferating T cells might induce paternal antigen-specific tolerance in humans. In this study, frequencies of CCR5$^+$ Treg cells did not change in miscarriage cases. Guerin et al. reported that CCR7$^+$ Treg cells accumulated in the preimplantation mouse uterus (Guerin et al., 2011). We are planning to study the population of CCR7$^+$ Treg cells in the decidua of miscarriage cases.

Treg cells expressing the proliferation antigen MK167 increase in the human decidua, and these Treg cells display a more suppressive phenotype with more frequent expression of Foxp3, HLA-DR, and CTLA than in blood (Mjösberg et al., 2010). A recent paper reported that populations of memory Treg cells, which expand in a second pregnancy, contribute to maintaining pregnancy in mice (Rowe et al., 2012). However, in the women enrolled in this study who have children, the median numbers of live born children were not significantly different between the three groups (Tables 1 and 2). Therefore, an expanded Treg cell population in second pregnancy was not evident in this study. Although reagents are not available for detecting human memory Treg cells, they could be partially responsible for maintaining pregnancy in human. Future studies are required to clarify this.

Unexpectedly, this study showed that the frequencies of CCR5$^+$ Treg cells and Ki67$^+$ Treg cells are similar in cases of normal pregnancy, miscarriage with a normal embryo, and miscarriage with an abnormal embryo. However, the frequency of Ki67$^-$ non-proliferating Treg cells in miscarriage with a normal embryo was significantly lower than that in normal pregnancy. Our results may be explained as follows. A high amount of IL-2 can induce proliferation of Treg cells, but these proliferated T cells lose their regulatory function. But when IL-2 is then removed from the culture medium, they regain an immunosuppressive state (Takahashi et al., 1998). Decidual Treg cells might therefore regain their suppressive function after stopping proliferation. Foxp3 is expressed in T cells when T cells are activated and proliferating in human (Allan et al., 2005). Those two papers suggest that Ki67$^+$Foxp3$^+$ T cells might be activated T cells and Ki67$^-$ Foxp3$^+$ cells might be functional Treg cells in the human. Further studies are necessary to clarify this. These data might indicate that the phenotype of paternal antigen-specific Treg cells differs between mice and humans.

Recent data shows that a Foxp3 enhancer, conserved noncoding sequence 1 (CNS1) is essential for peripheral Treg cells, and is present only in placental mammals. During pregnancy, peripheral Treg cells specific to a model paternal alloantigen were generated in a CNS1-dependent manner and accumulated in the placenta (Samstein et al., 2012). Suitable markers for peripheral Treg cells have not been identified. We should clarify the population of peripheral Treg cells when appropriate markers have been developed. Thus, further studies are needed to identify the surface markers of paternal alloantigen-specific Treg cells, or functional Treg cells that play essential roles in the maintenance of pregnancy in humans.

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Impaired autophagy by soluble endoglin, under physiological hypoxia in early pregnant period, is involved in poor placentation in preeclampsia

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Abbreviations: AGTR1-AA, angiotensin II receptor, type 1 receptor agonistic autoantibody; ATG4B, autophagy-related 4B; COMT, catechol-O-methyltransferase; EVT, extravillous trophoblast; HIF1A, hypoxia inducible factor 1, alpha subunit; HLA-G, major histocompatibility complex, class I, G; HUVECs, human umbilical vein endothelial cells; KRT7, cytokeratin 7; MAP1LC3B, microtubule-associated protein 1 light chain 3 beta; NP, normal pregnancy; PE, preeclampsia; sENG, soluble endoglin; sFLT1, soluble fms-related tyrosine kinase 1; TGFB1, transforming growth factor, beta 1; TNF, tumor necrosis factor-alpha

In early pregnancy, trophoblasts and the fetus experience hypoxic and low-nutrient conditions; nevertheless, trophoblasts invade the uterine myometrium up to one third of its depth and migrate along the lumina of spiral arterioles, replacing the maternal endothelial lining. Here, we showed that autophagy, an intracellular bulk degradation system, occurred in extravillous trophoblast (EVT) cells under hypoxia in vitro and in vivo. An enhancement of autophagy was observed in EVTs in early placental tissues, which suffer from physiological hypoxia. The invasion and vascular remodeling under hypoxia were significantly reduced in autophagy-deficient EVT cells compared with wild-type EVT cells. Interestingly, soluble endoglin (sENG), which increased in sera in preeclamptic cases, suppressed EVT invasion by inhibiting autophagy. The sENG-inhibited EVT invasion was recovered by TGFB1 treatment in a dose-dependent manner. A high dose of sENG inhibited the vascular construction by EVT cells and human umbilical vein endothelial cells (HUVECs), meanwhile a low dose of sENG inhibited the replacement of HUVECs by EVT cells. A protein selectively degraded by autophagy, SQSTM1, accumulated in EVT cells in preeclamptic placental biopsy samples showing impaired autophagy. This is the first report showing that impaired autophagy in EVT contributes to the pathophysiology of preeclampsia.

Introduction

Preeclampsia, characterized by hypertension and proteinuria after 20 weeks of gestation, complicates 3–5% of all pregnancies worldwide, and is the leading cause of maternal, fetal and neonatal mortality. Preeclampsia remains a major cause of maternal mortality (15–46%) and is responsible for a 5-fold increase in perinatal mortality.[1,2] The World Health Organization has recognized the importance of preeclampsia by launching a global program to conquer preeclampsia-eclampsia.[3] Additionally, women with a history of preeclampsia and their offspring are at greater risk of developing cardiovascular disease later in life.[4]

The current hypothesis regarding the etiology of preeclampsia is focused on inadequate trophoblast invasion and placentation.[2] Trophoblast stem cells differentiate into two cell types, villous trophoblasts and extravillous trophoblasts (EVTs) in humans. Invading trophoblasts called interstitial EVTs migrate into the decidualized endometrium and endovascular EVTs migrate along the lumina of spiral arterioles.[5] The invasion of EVTs into spiral arteries starts earlier in pregnancy and the endovascular trophoblastic cells aggregate in the lumen of the vessel forming the "trophoblastic plug," to allow the growth of the embryo and placenta in a low-oxygen environment in the first stage of pregnancy.[6] This EVT-invasion anchors the fetus to the mother and creates the large-diameter and low-resistant vessels that carry blood to the

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