Accumulation of IL-17-Positive Cells in Decidua of Inevitable Abortion Cases

Akitoshi Nakashima1*, Mika Ito1*, Tomoko Shima1, Nguyen Duy Bac2, Takao Hidaka1, Shigeru Saito1

1Department of Obstetrics and Gynecology, Faculty of Medicine, University of Toyama, Toyama, Japan; 2Department of Anatomy and Deputy Head of Department of Genomics and Cytogenetics, Vietnam Military Medical University, Ha Noi, Vietnam

Introduction

CD4+ helper T cells are classified as T-helper (Th) 1 cells or Th2 cells according to their patterns of cytokine production.1 Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, and they are presumed to cause spontaneous abortion.2,3 Although conflicting data have also been reported,4,5 a novel family of CD4+Th cells was detected, which was characterized by IL-17 production and named ‘Th17’.6,7 IL-17, a pro-inflammatory cytokine, induces the expression of many mediators of inflammation. So far, experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis are believed to be Th1 response-related diseases, but recent data have shown that Th17 cells play a central role in the pathogenesis of these diseases.8 Interestingly, the differentiation and functions of Th17 cells and regulatory T (Treg) cells occur in opposite directions. The differentiation of Th17 cells is initiated by transforming growth factor (TGF)-β1 and IL-6, which activate signal transducer and activator of transcription 3 (Stat3) and induce the expression of the transcription factor retinoic acid-related orphan receptor gamma t (RORγt). On the other hand, the presence of TGF-β1 but not IL-6

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Correspondence
Shigeru Saito, MD, Professor and Chairman, Department of Obstetrics and Gynecology, University of Toyama, 2630 Sugitani, Toyama-shi, Toyama, 930-0194, Japan.
E-mail: s30saito@med.u-toyama.ac.jp

*A.N. and M.I. contributed equally to the study.

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Problem

Th17 cells, a new subset of helper T cells, have been focused on as a producer pro-inflammatory cytokines. It is, however, still unknown how Th17 cells affect pregnancy outcome. We investigated the expression of IL-17-producing cells in human spontaneous abortion.

Method of study

IL-17 expression was analyzed in decidual tissues among normal pregnancy, missed abortion, and inevitable abortion cases by immunohistochemistry and flow cytometry.

Results

IL-17+ cells were accumulated in decidua and were detected in decidual CD4+ T cells and few decidual CD8+ T cells in spontaneous abortion cases. The number of decidual IL-17+ cells in inevitable abortion cases involving active genital bleeding was significantly higher than that in normal pregnancy cases (P < 0.05). On the other hand, there were no significant differences in the numbers of decidual IL-17+ cells between missed abortion cases and normal pregnancy subjects. Furthermore, the number of IL-17+ cells was positively correlated with the number of neutrophils in spontaneous abortion cases.

Conclusion

IL-17+ cells might be involved in the induction of inflammation in the late stage of abortion, but not in the early stage of abortion.
induces the expression of Foxp3, resulting in Treg induction.\textsuperscript{9} It is well known that Treg cells play very important roles in the maintenance of allogeneic pregnancy,\textsuperscript{10} and decreased numbers of Treg cells and decreased expression of Foxp3 mRNA are observed in the decidua and endometrium in abortion\textsuperscript{11} and implantation failure.\textsuperscript{12} An elevation in IL-17 was also observed in an acute renal rejection model.\textsuperscript{13} Thus, the balance between Th17 and Treg might be correlated with successful pregnancy. In addition, IL-17 has a function in recruitment and activation for neutrophils.\textsuperscript{14} As an inflammation is involved in inducing abortion, Th17 may play a role in the pathogenesis of abortion. In this study, we examined Th17 cells in the decidua of spontaneous abortion cases in humans.

Materials and methods

Tissue Collection
All samples for this study were approved by the University of Toyama Ethics Committee, and informed consent was obtained from all patients. Ten specimens from cases of elective termination of pregnancy (maternal age median: 28 years, range: 24–37 years; gestational age median: 8 weeks, range: 6–10 weeks) were obtained. These specimens were treated as normal pregnant subjects. Gestational age was calculated from the last menstrual period and confirmed by ultrasound measurements of crown-rump length. Seventeen samples from first-trimester spontaneous abortion cases (maternal age median: 30 years, range: 17–38 years; gestational age median: 7 weeks, range: 4–9 weeks) were collected. Anembryonic pregnancies or fetal death was confirmed by ultrasonography. These samples were divided into two groups: missed abortion and inevitable abortion. A missed abortion was defined as a nonviable pregnancy without vaginal bleeding, uterine cramping, or cervical dilatation. An inevitable abortion was defined when there was active vaginal bleeding and an open external cervical os. All samples were collected by vaginal curettage. In inevitable abortion, curettage was carried out within 12 hr of diagnosis. Both groups were subjected to the same exclusionary criteria: women receiving any medication or with autoimmune diseases or other systemic or local diseases were excluded. Clinical details were recorded for each woman (Table I). The tissue samples were fixed in formalin and embedded in paraffin blocks for histological examination and immunohistochemical staining.

Immunohistochemistry
Five-micron sections from formalin-fixed, paraffin-embedded human choric tissues were deparaffinized in xylene and rehydrated in graded alcohols, before being subjected to antigen retrieval by immersion in 1% sodium citraconic acid in aqueous solution (Nissin EM, Tokyo, Japan) and irradiated with standard microwave equipment (maximum 500 W; Sharp, Tokyo, Japan) for 15 min. After the tissue samples had been cooled down to 37°C at room temperature, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. After non-specific staining had been prevented by soaking the sections in 10% rabbit serum, they were incubated with anti-human CD3 mouse mAb (1:100; Novocastra, Newcastle, UK) or goat polyclonal anti-human IL-17 (1:100; R&D, Minneapolis, MN, USA), before being intermittently irradiated (4 s irradiation, 3 s rest) using specialized microwave equipment (MI33; Azumaya, Tokyo, Japan) for 15 min to improve the immunostaining and then incubated for 30 min at room temperature.\textsuperscript{15,16} Further processing of the sections for detection was performed using the dextran-polymer method (Dako, Glostrup, Denmark) and diaminobenzidine (DAB; Sigma, Poole, UK). After being washed, the sections were counterstained with Mayer’s hematoxylin, washed in water, and successively immersed in graded ethanol solutions and xylene before coverslipping. In the control sections, the primary antibody was replaced by control non-immune goat IgG (Vector Laboratories, Burlingame, CA, USA). Specific IL-17 staining was confirmed by recombinant IL-17 treatment. All
samples were processed under the same conditions. When counting the number of IL-17-positive cells in the IL-17 staining tissues samples, at least three high-power fields were chosen randomly on each sample. Additionally, the number of neutrophils, which have a lobulated nucleus, was counted in the same fields as used for the IL-17 counting in the hematoxylin–eosin-stained samples.

Flow Cytometry

Decidual tissues from missed abortion cases were used for flow cytometry because the samples from the inevitable abortion cases had degenerated. Decidual mononuclear cells (leukocytes) were purified by the Ficoll–Hypaque method after homogenization and filtration through a 32-μm nylon mesh. Decidual tissues were not enzymatically digested so as to prevent the possibility that enzymatic treatment would affect the fluorescence intensity of surface antigens. Decidual mononuclear leukocytes were stimulated with phorbol myristate acetate (PMA, 10 ng/mL; Sigma Chemical Co., Deisenhofen, Germany) and 1 μg/mL of ionomycin (Sigma Chemical Co.) in the presence of 10 μg/mL of brefeldin A (Sigma Chemical Co.) for 4 hr at 37°C in an atmosphere containing 5% CO2. These cells were stained for 20 min at room temperature with FITC-conjugated mAb to CD4 or CD8 (BD PharmingenTM, San Diego, CA, USA). The cells were then washed and fixed in 4% formaldehyde/PBS for 5 min at room temperature, before being treated with permeabilizing solution buffer (BD Bioscience, San Jose, CA, USA) for 10 min at room temperature. They were then stained with PE-conjugated anti-IL-17 (eBioscience, San Diego, CA, USA) for 30 min on ice. After being washed, the cells were analyzed on a FACS Calibur flow cytometer using the CellQuest software (BD Bioscience). We counted 50,000 cells in each sample. A gate was set on the lymphocytes using characteristic forward scatter (FSC) and side scatter (SSC) parameters. The analyses of CD4 and CD8 staining were performed using the obtained decidual mononuclear cells. An isotype-matched PE-conjugated mouse IgG1 antibody (eBioscience) was used as a control.

Statistical Analysis

Background data are presented as the median value and the range. P-values < 0.05 were considered significant. The frequency of IL-17-positive cells was analyzed with Mann–Whitney U-test. Spearman rank correlation coefficient was used to determine associations between the numbers of IL-17-positive cells and neutrophils.

Results

Accumulation of IL-17-Positive Cells in Decidua from Spontaneous Abortions

We first examined IL-17 expression in abortive samples obtained from spontaneous abortion cases by immunohistochemistry. Numerous IL-17 antibody-reacted cells were detected in the spontaneous abortive decidual samples (Fig. 1b). Almost all the cells had a round shape and were located in the stroma or blood vessels, suggesting that they were leukocytes (Fig. 1b, arrowheads and arrows). Subsequently, when CD3 staining was performed with serial sections of spontaneous abortive samples, many CD3+ T cells, which had infiltrated into the stroma, were detected in the same area, suggesting that the IL-17+ cells were T cells (Fig. 1a). On the other hand, IL-17+ cells were rare in the decidua of the elective termination samples, in which T cells were recognized. These results suggested that the number of IL-17+ cells is increased in spontaneous abortion, which causes T-cell infiltration.

IL-17-Producing Cells in Decidual CD4+ T Cells and CD8+ T Cells

We next examined whether T cells produce IL-17 in decidual lymphocytes by flow cytometry. The main population of IL-17-producing cells was CD4+ T cells, on the other hand, very few CD8+ T cells produced IL-17 in the spontaneous abortion cases (Fig. 2), suggesting that the decidual IL-17+ cells were CD4+ Th17 cells. The main population of decidual lymphocytes was CD56bright NK cells, which belong to CD4− and CD8− cell population. IL-17+ cells were very rare in the CD4+ cell population, suggesting that CD56bright NK cells do not produce IL-17.

Increase in the Number of Decidual IL-17-Positive Cells in Inevitable Abortion Cases

We next focused on the localization of IL-17+ cells in spontaneous abortion cases. IL-17+ cells were distributed over the entire region of the decidua, the
cell column in the decidua basalis, as well as the decidua parietalis (Fig. 3a–c). Around the cell column, IL-17+ cells were detected not only in the blood vessels (Fig. 3b, arrows) but also in the stroma (Fig. 3b, arrowheads), suggesting that IL-17+ cells might infiltrate from blood vessels and into the stroma. However, these cells were absent in the villous trophoblastic layer (Fig. 3d). Additionally, we found differences in the number of IL-17+ cells in the decidua among spontaneous abortion samples. Therefore, we divided the spontaneous abortion samples into two groups: inevitable abortion and missed abortion according to the presence or absence of symptoms, such as genital bleeding and lower abdominal pain. Subsequently, we compared the number of IL-17+ cells among the three groups: normal pregnancy, missed abortion, and inevitable abortion. The median values and the ranges of IL-17+ cell numbers were 0 (0–21), 0 (0–25), and 7 (0–34) in normal pregnant women, missed abortion cases, and inevitable abortion cases, respectively (Fig. 4). Interestingly, the number of IL-17+ cells in the inevitable abortion cases was significantly higher than that in the normal pregnancy cases (Fig. 4, \( P < 0.05 \)). These data showed that the number of IL-17+ cells was significantly increased in the inevitable abortion cases but was not changed in the missed abortion cases.

Coexistence of the IL-17-Positive Cells and the Neutrophils in the Inevitable Abortion Cases

As IL-17 is a pro-inflammatory cytokine that plays an important role in neutrophil infiltration, we next examined the correlation between the number of IL-17+ cells and the number of neutrophils in the inevitable abortion cases. After counting the numbers of IL-17+ cells and neutrophils around the IL-17+ cells in high-power fields, the correlation between the numbers of IL-17+ cells and neutrophils was analyzed in the spontaneous abortion cases. The index of correlation was 0.89, and a significant positive correlation was observed between the number of IL-17+ cells and the number of neutrophils in the spontaneous abortion cases (Fig. 5, \( P < 0.0001 \)). On the other hand, few neutrophils were detected in the
Fig. 3 Distribution of IL-17+ cells in inevitable abortion cases of 7-week gestation: Our immunohistochemical study showed the IL-17 expression in the decidua basalis (a), decidua parietalis (c), and villous (d) of inevitable abortion cases. Panel (b) shows the region outlined by a black line in panel (a). In the control sections, the primary antibody was replaced by control non-immune goat IgG (e). The expression of IL-17 was detected in the decidual stroma (arrowheads) and blood vessels (arrows), but not in chorionic villi. COL, cell column. The cell column was localized on the left side of panel (b).

Fig. 4 Comparison of the numbers of IL-17+ cells in deciduas: The numbers of IL-17+ cells in deciduas from normal pregnancy, missed abortion, and inevitable abortion cases. The bars indicate the median values. *P < 0.05.

Fig. 5 Correlation between the numbers of IL-17+ cells and neutrophils: A scatter graph was constructed between the numbers of IL-17+ cells (X-axis) and neutrophils (Y-axis) in spontaneous abortion cases. The coefficient of correlation (r) is shown on the upper side of the graph. The line indicates the regression line.
normal pregnant subjects. These results showed that the coexistence of IL-17-positive cells and neutrophils was detected in the late stage of spontaneous abortion.

Discussion

The etiology of spontaneous abortion varies, including chromosomal aberrations, anatomic anomalies, endocrine disorders, infections, reproductive anti-phospholipid syndrome, and immunologic abnormalities. Predominant Th1 type immunity might induce abortion; however, recent studies have revealed the specific functions of Th17 cells beyond their previously described effects on Th1 and Th2 immunity, including specific roles in host defense against certain pathogens and in autoimmunity.

This study demonstrated that the number of decidual IL-17+ cells was increased in inevitable abortion cases involving active genital bleeding, but not in missed abortion cases without symptoms. The main population of these IL-17+ cells was CD4+ T cells, suggesting that decidual IL-17+ cells are Th17 cells. Interestingly, Th17 cells coexisted with neutrophils in the inevitable abortion patients. Recent data that IL-17 plays important roles in the induction of neutrophil-mediated protective immune responses against extracellular bacteria and fungal pathogens support our findings. Th17 cells also play an important role in the induction of inflammation.

In the obstetrics and gynecologic field, it has been reported that IL-17 stimulates IL-8 production in endometriotic stromal cells and amniotic mesenchymal cells in chorioamnionitis. IL-17 also enhances TNF-α-induced IL-8 secretion by amniotic mesenchymal cells. Thus, (TNF)-α and IL-17 might cooperatively augment IL-8 secretion, resulting in neutrophil accumulation at the decidua in inevitable abortion. In this study, the number of IL-17+ cells did not increase in the missed abortion cases without clinical symptoms. Our recent study showed that the number of circulating Th17 cells did not change during pregnancy and that the proportion of Th17 cells in the decidua was significantly higher than that in the peripheral blood. These findings suggest that IL-17 plays a role in the maintenance of pregnancy during the early pregnant period. Indeed, it has been reported that IL-17 augments extravillous trophoblast invasion. However, in the late stage of spontaneous abortion, excessive IL-17 expression may induce neutrophil accumulation, resulting in tissue degeneration or the onset of clinical symptoms. Thus, IL-17 expression level may be involved in a successful pregnancy.

Three major populations in the decidual leukocytes have been identified: uterine natural killer cells, macrophages, and T lymphocytes. Our previous report showed that the number of granulysin+ decidual NK cells was increased in the decidua basalis in spontaneous abortion cases and that these NK cells induced apoptosis in extravillous trophoblasts. This study showed that IL-17+ cells were distributed over the entire region of the decidua, decidua basalis, and the decidua parietalis, in the inevitable abortion cases, but IL-17+ cells did not increase in the missed abortion cases, suggesting that IL-17 expression is not the cause of such abortions but rather is the result of inflammation caused by tissue degeneration or infection. In regard to the IL-17 expression in decidual leukocytes, we have already reported that decidual CD56bright NK cells did not produce IL-17. IL-17 expression was identified in not only CD4+ T cells but also monocytes; however, our previous study showed no IL-17 expression in CD14+ cells in decidual leukocytes. And the population of IL-17+ cells in monocyte area detected by forward and SSCs in flow cytometry was only 0.14%. There are two types of macrophages in the decidua. CD14+CD68+ macrophages predominate in decidua, while CD14+CD68− macrophages are found in superficial myometrium, and the biological significance of these two macrophage populations is unclear. CD4 is also expressed on macrophage, but the staining intensity is rather weaker than that on T cells. In this study, IL-17 expression was detected in CD4bright cell population, suggesting that the main population of IL-17-producing cells is CD4+ T cells, and IL-17-producing CD14+ macrophage is very few (0.14%) in the decidua.

In conclusion, decidual IL-17+ cells were increased in the inevitable abortion cases involving active genital bleeding, but not in missed abortion cases without clinical symptoms. Furthermore, the number of IL-17+ cells was significantly positively correlated with the number of neutrophils, suggesting that IL-17+ cells might be involved in the inflammation in the late stage of abortion, but not in the early stage of abortion. Further studies are needed for understanding the role of Th17 cells in unexplained cases of recurrent pregnancy loss with normal fetal chromosomal content.
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