Dendritic cells are equally distributed in intrauterine and tubal ectopic pregnancies

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Objective: To examine different stages of dendritic cells (DCs) in intrauterine (IUPs) and viable tubal (VTPs) pregnancies to further elucidate mechanisms of fetomaternal tolerance and extravillous trophoblast invasion.

Design: Experimental study on patient-controlled material.

Setting: University hospital.

Patient(s): Seven women with normal IUPs and ten with VTPs in the first trimester.

Intervention(s): Suction curettage in IUP, laparoscopy in VTP.

Main Outcome Measure(s): Immunohistochemistry for cytokeratin-7 (trophoblast), CD83 (mature DCs), DEC205 (activated but not fully mature DCs), DC-SIGN (immature macrophage-like DCs), and CD14 (macrophages) alone and in double staining.

Result(s): The numbers of CD83$^+$ and DEC205$^+$ cells were similarly low in IUP and VTP (0.83 and 0.44 cells/mm^2; 2.28 and 2.96 cells/mm^2). The number of DC-SIGN$^+$ cells was higher, though without significant differences among the entities examined (57.5 and 47.4 cells/mm^2). About two-thirds of DC-SIGN$^+$ cells were also CD14$^+$ in IUP and VTP.

Conclusion(s): The almost equal distribution of CD83$^+$, DEC205$^+$, and DC-SIGN$^+$ cells in IUP and VTP suggests analogue control mechanisms in intrauterine and extrauterine DC differentiation and a comparable role of these DCs for the development of fetomaternal tolerance. (Fertil Steril® 2011;95:28–32. ©2011 by American Society for Reproductive Medicine.)

Key Words: Ectopic pregnancy, intrauterine pregnancy, fetomaternal tolerance, dendritic cells, CD83, DEC205, DC-SIGN

Dendritic cells (DCs), which are found in all mucosal and epidermal surfaces of the female reproductive tract, are potent antigen-presenting cells. On the one hand, they are able to induce immunologic reactions against exogenous antigens (1). On the other hand, they induce tolerance to self antigens (2), which is especially important in pregnancy.

These contradictory functions seem to be dependent (at least in part) on the stage of differentiation: whereas immature DCs are supposed to promote T-cell tolerance, mature DCs induce T-cell immunity (3). During differentiation from immature to mature state, DCs change their surface antigens (4): whereas immature states are characterized by DC-SIGN (CD209) (5), maturing DCs exhibit first DEC205 (CD205) (6) and then CD83 expression (7–11).

In early intrauterine pregnancy (IUP), high numbers of immature decidual DC-SIGN$^+$ DCs (12) are detected. For the induction of fetomaternal tolerance by promoting T-cell tolerance (3), they are supposed to interact with natural killer (NK) cells, the dominant leukocyte subpopulation around implantation (11). In contrast, few mature CD83$^+$ cells have been found (13–16). The number is increased only in the case of pregnancy failure (16). DEC205$^+$ cells, which are characterized as immature but activated DCs, were found in relatively low numbers in first-trimester deciduas (15).

Until now, in tubal pregnancies—where NK cells are almost absent —only the presence of mature CD83$^+$ cells has been shown, their number being similar to IUP (17). However, nothing is known about immature DCs in tubal pregnancies, which are hypothesized to be predominantly important for fetomaternal tolerance induction (3).

Furthermore, no differentiation has yet been made between a viable tubal pregnancy (VTP) prone to rupture and a dissolving tubal abortion. However, we have recently shown that maternal leukocyte patterns are different in both entities (18, 19). Therefore, it can be suggested that DC pattern may differ as well.

We examined and compared the different maturation stages of DCs in VTPs and viable IUPs. It was the aim of the study to gain insight into their site-specific distribution and to draw conclusions about potential mechanisms of fetomaternal tolerance in early intra- and extrauterine pregnancy.

TABLE 1

Immunohistochemistry.

Antibody	Pretreatment	Peroxidase block	Serum block (from kit)	Incubation specific (first) antibody	Incubation secondary antibody
Anti-CD83, clone HB15e; Serotec	Pepsin, 1 packet/500 mL, 0.2 N HCl, 20 min, RT	0.3% H_2O_2 and methanol, 30 min, RT, dark	Histostain Plus kit, 20 min, RT	1:300 in PBS/1.5% BSA, overnight, 4°C	Histostain Plus kit, 20 min, RT
Anti–DC-SIGN, clone 120507; R&D Systems	High-pressure cooker, citric acid, pH 6, 3 min, 124°C	0.3% H_2O_2 and methanol, 30 min, RT, dark	Histostain Plus kit, 20 min, RT	1:300 in PBS/1.5% BSA, 1 h, RT	Histostain Plus kit, 40 min, RT
Anti-DEC205, clone 11A10; Novocastra	High-pressure cooker, citric acid, pH 6, 3 min, 124°C	0.3% H_2O_2 and methanol, 30 min, RT, dark	Histostain Plus kit, 20 min, RT	1:80 in PBS/1.5% BSA, 1 h, RT	Histostain Plus kit, 40 min, RT
Anti-CD14, clone 7; Novocastra	None	0.3% H_2O_2 and methanol, 30 min, RT, dark	Histostain kit, 20 min, RT	1:50 in PBS/1.5% BSA, 1 h, RT	Histostain kit, 40 min, RT
Anti–cytokeratin-7, clone OV-TL 12/30; Dako	None	0.3% H_2O_2 and methanol, 30 min, RT, dark	Histostain kit, 20 min, RT	1:200 in PBS/1.5% BSA, 1 h, RT	Histostain kit, 40 min, RT

Note: BSA = bovine serum albumin; PBS = phosphate-buffered saline solution; RT = room temperature.

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MATERIALS AND METHODS
Materials

This study was approved by the Ethics Committee of the School of Medicine, University of Aachen. Each patient gave informed consent to the investigation. Seven specimens of normal IUP and ten of VTP with similar mean gestational ages (7.1 and 6.4 postmenstrual weeks) were examined.

Decidual tissues were collected from legal terminations for social reasons of normal healthy pregnancies in collaboration with the Center for Contraception, Sexuality, and Abortion (CASA), Maastricht. Fertile women without hormone treatment within the last 3 months before pregnancy and without spontaneous abortions but with at least one normal delivery were included in this study. These patients' median age was 29 years (range 24–41 years). Gestational age was determined by the duration of amennorhea, analysis of individual cycle data, and the crown-rump length (CRL) determined sonographically before operation.

Pregnancy was terminated by vacuum aspiration. Fragments of decidua basalis (with trophoblast) and parietalis (without trophoblast) were obtained. Decidua parietalis and basalis were identified by histologic examination and cytokeratin staining of the trophoblast as previously described (21).

Tubal tissue was obtained from laparoscopic salpingectomy for viable ectopic pregnancy. Patients were proved to be pregnant by ß-hCG serum test and sonographic control immediately before surgery. These patients' median age was 34 years (range 28–41 years). All patients had a regular menstrual cycle and at least one normal delivery before. All patients except two were nonsmokers. Gestational age was determined by the duration of amenorrhea and by sonographic measurement of the chorionic cavity and/or the CRL if present.

Viability of intrauterine pregnancies was confirmed before the procedure by fetal heart action, and viability of tubal pregnancies either by fetal heart action (five cases) or by the intense vascularization around the gestational sac by color Doppler sonography. Furthermore, specimens were assessed immunohistochemically for increased trophoblast proliferation, vascularization, and invasion (18, 20). Only specimens from VTPs were included; tissues showing signs of necrosis were excluded from the study. The implantation site was usually found in the center of the tubal thickening. Therefore, this area was selected for the study.

Tissue samples (maximal 5 × 5 × 3 mm) were fixed in an at least 1:30 volume of 3.7% buffered formalin for 4 hours at room temperature on a shaker to assure uniform fixation. This was in agreement with the recommendations of Dako for small tissue specimens (10 × 10 × 3 mm, 6–24 hours' fixation time). Then the samples were dehydrated, cleared in xylene, and embedded in paraffin.

Methods

Monoclonal antibodies Single and double immunohistochemical staining was performed in all intrauterine and tubal specimens with five antibodies suitable for antigen detection in formalin-fixed paraffin-embedded tissue (Table 1): cytokeratin-7 (epithelial and trophoblast cells), CD83, DEC205 (CD205), DC-SIGN (CD209), and CD14 (macrophages).

Pretreatment for immunohistochemistry Paraffin sections (4–6 μm) were mounted on 3-aminopropyltriethoxysilane-coated glass slides, deparaffinized in xylene, and rehydrated. For antigen retrieval, see Table 1. After cooling for ~30 minutes, the sections were placed in 0.3% H_2O_2/methyl alcohol for 30 minutes to block endogenous peroxidase activity.

Single labeling Immunostaining was performed with the Histostain Plus kit (CD83, DC-SIGN, DEC205, cytokeratin-7; Zymed Laboratories, San Francisco, CA) as described earlier (21). All washing steps were performed with phosphate-buffered saline solution (PBS)/0.1% bovine serum albumin (BSA).

Briefly, nonspecific binding sites were blocked with the blocking serum provided by the kit (Table 1). Incubation with the primary antibodies diluted in PBS/1.5% BSA was performed as summarized in Table 1. After washing, sections were covered with the secondary antibody provided by the kit (30 minutes at room temperature). After another washing, step sections were incubated for 20 minutes at room temperature with streptavidin-peroxidase conjugate (Histostain Plus kit). Antibody binding was detected using the AEC kit (red staining; Zymed, San Francisco, CA) according to the manufacturer's protocol under microscopic control (5–15 minutes). Finally, slides were washed in tap water, counterstained with hematoxylin for 30 seconds, and mounted in glycerol gelatin.

For negative control, primary antibodies were replaced by normal mouse IgG (same subtype as the first antibody) at the same protein concentration as the specific antibody. For positive control, sections from lymph nodes (CD83 and DC-SIGN) or palatine tonsil (CD205) were stained in parallel.

FIGURE 1

Immunohistochemical staining (*red*) for (**A, B**) DC-SIGN, (**C, D**) DEC205, and (**E, F**) CD83 within the implantation zone of (**A, C, E**) intrauterine pregnancy (IUP) and (**B, D, F**) viable tubal pregnancy (VTP). Inserts with higher magnification show (**A, C, E**) dendritic morphology, (**A, C, E**) control staining, and (**B, D, F**) cytokeratin-7 staining.
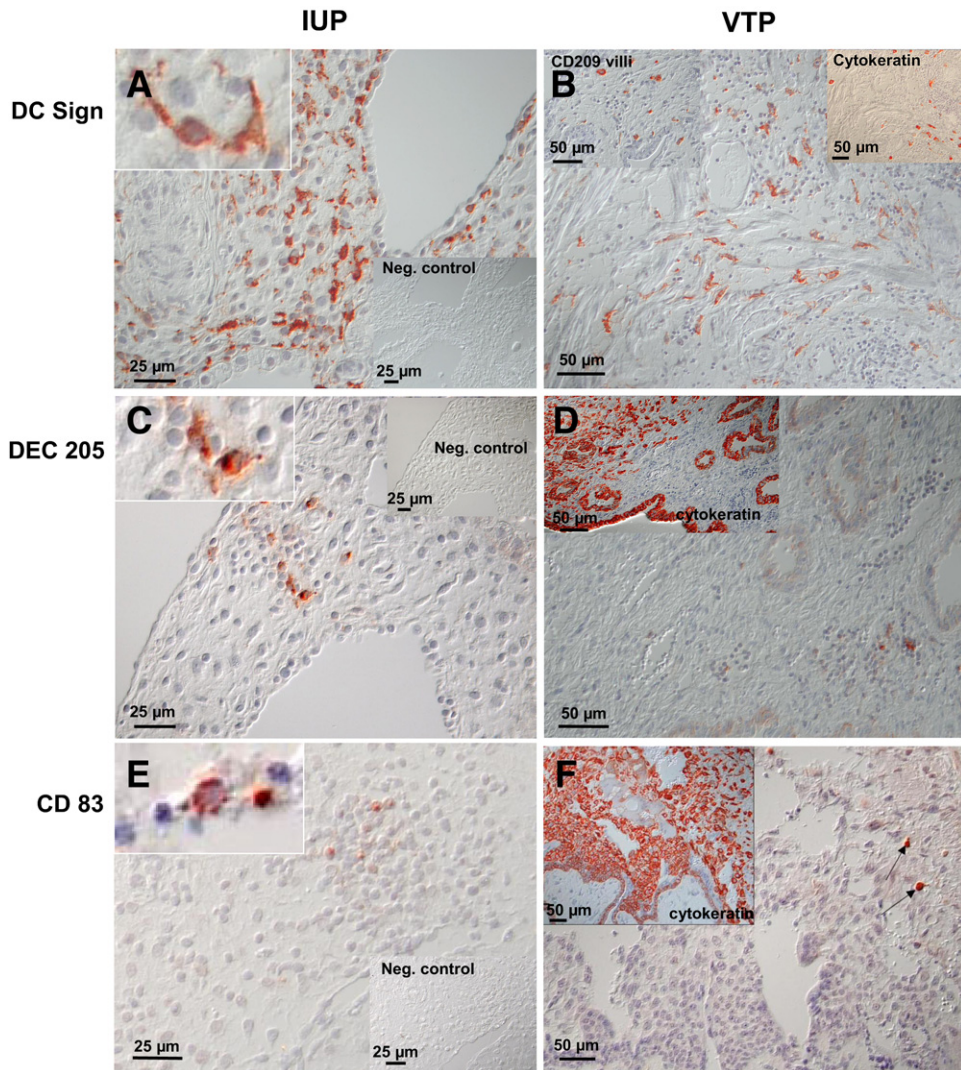
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Double labeling (DC-SIGN and CD14) After pretreatment for DC-SIGN staining (see the preceding section), slides were treated for 20 minutes with the blocking solution provided by the Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Afterward, sections were incubated with the anti-CD14 antibody (Table 1). Sections were then washed in PBS and incubated with the kit's secondary antibody for 30 minutes at room temperature. After washing in PBS, the slides were placed in streptavidin-peroxidase conjugate from the kit for 30 minutes. Slides were washed again in PBS, and SG Substrate (Vector) was used as chromogen. The blue/gray color reaction developed within 12 minutes. Slides were thoroughly washed in PBS, and then the second part of the double immunohistochemical labeling started with the application of the blocking solution of the Histostain Plus kit followed by incubation with the DC-SIGN antibody. All incubation steps were the same as for the single-labeling procedure (Table 1). Slides were not counterstained.

Quantification and statistical analysis Immunostaining for cytokeratin allowed discrimination between the implantation site and the mucosa distant from it. The numbers of DCs at the implantation sites of IUP and VTP were compared.

Because the tubal and decidual implantation sites are similar in composition of the tissue (lamina propria of tubal or uterine wall containing connective tissue, glandular and surface epithelium, trophoblast cells, and lymphocytes) and cell density, this allows the normalization of the lymphocyte counts on the tissue area.

Dendritic cells were counted within an area of 0.34 mm^2 (in case of CD83, DEC205) or 0.085 mm^2 (in case of DC-SIGN, CD14) using high-power magnification fields ($\times 400$ and $\times 200$). Two different slides from each block were evaluated for each marker. Five high-power fields of the invaded decidua/tubal wall were randomly chosen and examined in each section by two independent investigators (S.S. and B.K.). In addition, the tissue slides were presented to an investigator (C.K.) not familiar with the study. This third
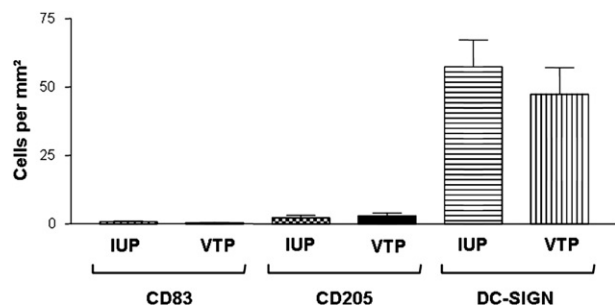
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investigator counted tissue areas selected by the two investigators familiar with the study as well as tissue areas selected by herself. The results were analyzed by calculating the mean and standard error of the mean for the number of DCs identified. Differences in cell numbers regarding the two different entities were tested using the Student two-tailed t test.

All calculations were made with the use of GraphPad Prism, version 3.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Extravillous trophoblast (EVT) cells were identified via cytokeratin cytoplasmic staining. This allowed differentiation of the implantation sites by the presence of invading trophoblast cells from the tissue distant from the implantation sites.

CD83$^+$, DEC205$^+$, and DC-SIGN$^+$ DCs were identified by clear membrane-associated immunostaining for surface markers with minimal background staining. In addition, their typical morphologic appearance was assessed: whereas CD83$^+$ cells were characterized by their compact form with few membrane processes, DEC205$^+$ cells were bigger and seen to have frequent cytoplasmic processes,

exhibiting a typical dendritic morphology (Figs. 1C and 1E). DC-SIGN$^+$ cells also showed a typical dendritic form with numerous membrane processes (Fig. 1A).

In IUP as well as VTP, CD83$^+$ cells were rarely found. If present, they were situated in small groups of about three cells or singularly in the stroma, mostly near spiral arteries or decidual glands, within clusters of leukocytes or at the border to the cell columns of the trophoblast (Figs. 1E and 1F).

DEC205$^+$ cells were similarly arranged as CD83$^+$ cells. They also were mostly seen in leukocyte clusters and often at the border of vessels as well as in close neighborhood to EVT cells (Figs. 1C and 1D).

In IUP and VTP, DC-SIGN$^+$ cells were detected within the stroma, often around vessels or decidual glands or, in VTP, also among EVT cells and within the placental villi (Figs. 1A and 1B).

Mature CD83$^+$ DCs were scarcely seen (0.83 cells/mm^2 in IUP, 0.44 cells/mm^2 in VTP). The mean number of activated, not fully mature DEC205$^+$ DCs was slightly higher (2.28 cells/mm^2 in IUP, 2.96 cells/mm^2 in VTP). In contrast, we observed a large number of immature DC-SIGN$^+$ DCs in IUP (57.5 cells/mm^2) and VTP (47.4 cells/mm^2). The differences between IUP and VTP were not statistically significant for all three markers (Fig. 2). This was confirmed by the results of the third investigator. When counting the same tissue areas as the two investigators familiar with the study, the interobserver variability was 5%–10%. When selecting different areas, the third investigator's numbers were slightly higher but reached the same relative result, i.e., no statistically significant difference between IUP and VTP and a gradient from a relatively high number of immature to a low number of mature DCs.

Double immunohistochemical labeling for DC-SIGN and CD14 allowed further characterization of DC-SIGN$^+$ cells (Fig. 3). Most DC-SIGN$^+$ cells exhibited double-staining for CD14, and few appeared to be CD14$^-$. The percentage of CD14$^+$ DC-SIGN$^+$ cells showed no significant difference between IUP and VTP (66.94% and 84.63%, respectively).

DISCUSSION

In this study, distribution of mature and immature DCs in VTPs without signs of abortion or pregnancy rejection was studied and compared with normal IUPs for the first time. A gradient from a large

FIGURE 3

Double immunohistochemical labeling for DC-SIGN (*red*) and CD14 (*blue*) in (**A**) intrauterine pregnancy (IUP) and (**B**) viable tubal pregnancy (VTP).
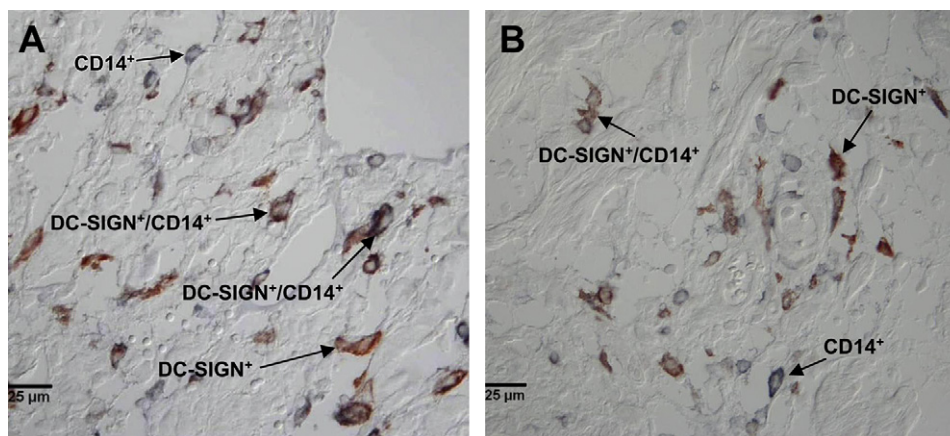
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number of immature DC-SIGN$^+$ dendritic cells through an intermediate though relatively low number of activated but not fully mature DEC205$^+$ DCs to a very low number of mature CD83$^+$ DCs was demonstrated. The cell numbers and histologic patterns were similar in IUP and VTP.

In accordance with Kämmerer et al. (14) and Rieger et al. (12), the present data show that there is little DC maturation in early IUP; the same is obviously true for extrauterine pregnancy. Although our results need cautious interpretation because of small sample size, the similar findings in intrauterine and extrauterine pregnancy suggest that DCs are involved in the fetomaternal dialogue of both entities in a similar manner. The different function of DCs (tolerance induction versus antigen rejection) is dependent on their developmental stage: In early IUP and VTP, immature DCs, which presumably induce tolerance, are the most abundant population.

Decidual DC-SIGN$^+$ cells are described as pregnancy-specific CD14$^+$ subpopulations of macrophages in IUP (14, 15, 22, 23). The same is obviously true for VTP: The present results show that most of DC-SIGN$^+$ cells in VTP are CD14$^+$ as well. Because macrophages and DCs are able to convert into one another until a certain stage of their maturation process (24), these CD14$^+$ DC-SIGN$^+$ cells may represent a temporary differentiation cell type, as assumed for IUP (14). The small amount of CD14$^-$ DC-SIGN$^+$ cells in VTP may represent a further stage in differentiation toward mature DCs.

Compared with the results of our study, the number of DC-SIGN$^+$ cells detected by Soilleux et al. (22) in IUP is similar, whereas Rieger et al. (12) found more than twice as many cells. This may be due to methodology: Whereas we performed our experiments on paraffin sections, Rieger et al. used frozen tissue.

In contrast to IUP, DC-SIGN$^+$ cells in VTP could be found among EVT cells and within the placental villi. Obviously, DC-SIGN$^+$ cells get into contact to placental villi earlier, possibly because of the lacking decidua.

In accordance with Gardner and Moffett (15), we detected few DEC205$^+$ cells in IUP, their number being higher than the CD83$^+$ cells and lower than the DC SIGN$^+$ cells. This confirms the hypothesis of Gardner and Moffett (15) that DEC205 is an intermediate stage of an activated but not mature DC. The close neighborhood of DEC205$^+$ and EVT cells supports the hypothesis of uptake and presentation of apoptotic EVT peptides by DEC205$^+$ cells.

Regarding CD83$^+$ DCs, our data are in accordance with Rieger et al. (12), who also found very few CD83$^+$ cells. The particular distribution of CD83$^+$ cells within clusters of leukocytes suggests an immunologic cooperation of DCs with other leukocytes. In IUP, Kämmerer et al. (13) and Askelund et al. (16) assumed that these leukocytes are CD3$^+$ T cells. Therefore, CD83$^+$ cells may present antigens to CD3$^+$ T cells.

Because the presence and maturation of DCs are similar in IUP and VTP, the differences in the invasive behavior of the EVTs (20) may result from different interaction partners available for DCs: Whereas NK cells may modulate interaction of DCs with T cells in IUP, thereby limiting trophoblast invasion, NK cells are almost absent in VTP. Therefore, only a direct interaction with T cells or macrophages seems to be possible. Further analysis of DC interaction with T cells, NK cells, macrophages, and EVTs may help us to understand the role of DCs in IUP and VTP.

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