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Apoptosis occurs in implantation site of the rhesus monkey during early stage of pregnancy☆

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Abstract

The exact role of apoptosis that occurs in human placenta during the early stage of pregnancy remains unknown because of the difficulty in obtaining the intact implantation site. In this study, we used rhesus monkey as an animal model to examine apoptosis occurring in the implantation site at various stages of early pregnancy. It was shown that Fas and FasL mRNA and protein were localized in both the chorionic villi and glandular epithelium from day 15 to day 30 of pregnancy. Fas and FasL protein were also expressed in the epithelial plaque on day 15 of pregnancy. In situ 3′-end-labeling results showed that glandular epithelial cells underwent extensive apoptosis with obvious morphological degradation during the early stage of pregnancy. It was found that the cells that were 3′-end-labeled in the chorionic villi and anchoring villi were mainly localized in cytotrophoblast and cytotrophoblast column. It is therefore suggested that in primates apoptosis, which may be involved in the regulation of proliferation of trophoblast villi and degradation of epithelial plaque, as well as remodeling of the glands in the maternal decidua, may play an important role during the early stage of implantation and placentation.

Keywords: Apoptosis; Implantation site; Fas; FasL

1. Introduction

Apoptosis (programmed cell death) that preserves homeostasis and occurs during normal tissue turnover [1] is different from necrosis. The cells undergoing apoptosis have characteristic structural change in the nucleus, such as DNA fragmentation, and in the cytoplasm, such as formation of apoptotic bodies [2,3]. Apoptosis is a normal physiological process [2,3] regulated by a set of related genes, such as Fas, FasL, bcl-2 and Bax [4]. Fas is a type II membrane protein related to the tumor necrosis factor receptor family [5]; FasL can either be membrane bound (42–48 kDa form) or cleaved by metalloproteinase to release the extracellular portion as a soluble form with 26 kDa in size [6]. Fas/FasL interaction and Fas activation can induce cell apoptosis [7].

Placental apoptosis during placentation has been shown to be a normal physiological phenomenon by various methods [8–10]. FasL expressed in the trophoblast cells can induce apoptosis in activated peripheral lymphocytes, which may provide a local mechanism for maternal immuno-tolerance to the fetus [11–16]. Several lines of evidence suggest that in addition to its vital functions for protecting the fetus from attack by the maternal immune system, apoptosis also plays an important role in maintaining homeostasis of the endometrium during tissue remodeling of decidualization [17] and implantation [18,19] in mammals. Although there were many studies to focus on the role of apoptosis in the process of placentation, most of them used experimental animals as models. Considerable species differences were present in the processes of implantation and placentation. Because of the difficulty in obtaining the intact implantation site, the exact function of apoptosis in human during the early stage of implantation is poorly understood.

To better understand the function of apoptosis during the early stage of human placentation, we used the rhesus monkey as an animal model, which is more similar to human in the process of implantation and placentation than that of other experimental animals, and examined the expression of Fas and FasL mRNA and protein at the implantation sites by using immunohistochemistry and in situ hybridization. Terminal deoxynucleotitdyl transferase-mediated ddUTP nick

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end labeling (TUNEL) was also conducted to morphologically define the apoptotic cells.

2. Materials and methods

2.1. Reagents

Polyclonal antibodies against human Fas and FasL were purchased from Santa Cruz, California (USA); Fas and FasL cDNA used in this study was kindly provided by Professor Shigekazu Nagata at Osaka University, Japan. Digoxigenenin (DIG)-RNA labeling kit, blocking reagent, alkaline phosphate conjugated anti-DIG antibody, dig-ddUTP, 4-nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Beijing, People's Republic of China). Proteinase K was purchased from Merck-Schuchardt (Beijing, People's Republic of China). Restriction enzyme and TdT were purchased from Promega Co., Ltd., (Beijing, People's Republic of China). Diethyl pyrocarbonate (DEPC), ficoll, dextrasulphate were purchased from Sigma (Beijing, People's Republic of China).

2.2. Animals

Female rhesus monkeys, 5–7 years old, were obtained from the Primate Research Center in Fujian Research Institute of Parenthood and Family Planning. The use of the monkeys for the experiments was approved by both the Academic Committee of Zoology Institute of Chinese Academy of Sciences and the Academic Committee of the Primate Research Center in Fujian Research Institute of Parenthood and Family Planning. Menstrual cycles of the monkeys were monitored, and the animals were permitted to mate over a period of 3 days at the anticipated time of ovulation; the second day of mating was designated day 0 of pregnancy. The presence of a conceptus was confirmed by ultrasound examination. The animals were divided into several groups. At various days from day 15 to day 30 of pregnancy, the uteri with the implantation sites were removed and fixed in fixative [4% formaldehyde in phosphate-buffered saline (PBS) pH 7.4], and further processed for paraffin embedding. The operations on the animals were carried out under standard anesthesia conditions (before surgery 10–15 mg/kg Ketamine hydrocholide supplemented with 0.02 mg/kg atropine sulfate and sodium pentobarbital 8–12 mg/kg were given).

2.3. In situ hybridization

The plasmids that contain the cDNA fragment of Fas and FasL were linearized with corresponding restriction enzyme and were transcripted with corresponding RNA polymerases in vitro. Transcription was performed by using an in vitro transcription system, and cRNA was labeled with digoxigenin by using a DIG-RNA labeling kit purchased from Boehringer Mannheim. The validation of the labeled probe was evaluated with Dot Blot analysis. The labeled probe was linked with nylon membrane with ultraviolet radiation and incubated with AP-conjugated anti-DIG antibody then incubated with the substrate (BCIP and NBT). If the probe was properly labeled, a blue signal was detected.

Paraffin embedded sections were deparaffinized in fresh xylene (2 × 10 min), xylene:100% alcohol (1:1, 5 min), 100% alcohol (5 min), 95% alcohol (5 min), 90% alcohol (5 min), 80% alcohol (5 min), 70% alcohol (5 min). Then the slides were washed in DEPC-treated PBS (3 × 5 min) and permeablized with proteinase K (20 μg/mL) in Tris-EDTA buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) for 20 min. The sections were washed in PBS (2 × 5 min), and post-fixed with 4% paraformaldehyde in PBS (4°C, 10 min). The slides were washed with PBS (2 × 5 min) and DEPC-treated H_2O (5 min). The sections were dehydrated with serial alcohol and air-dried. The slides were incubated in prehybridization buffer [2 × standard sodium citrate buffer (SSC), 50% deionized formamade, room temperature (RT), 2–4 h]. After prehybridization, the hybridization solution were applied onto the slides and covered with paraffin film. Hybridization solution were made by mixing DIG-labeled cRNA probes (20–30 ng per slide) with 100 μL hybridization buffer (2 × SSC, 50% deionized-formamide, 10 mM Tris-HCl, 250 μg/mL yeast tRNA, 0.5% sodium dodecyl sulfate, 1 × Denhardt, 10 mM dithiothreitol, 10% dextrasulphate). The sections were incubated 16–20 h at 48°C. At the end of hybridization, the paraffin film was removed by incubating the slides in 4 × SSC. Subsequently, the slides were washed with 2 × SSC (2 × 15 min, RT), 1 × SSC × 15 min, 42°C × SSC (2 × 15 min, 42°C). Sections were washed by shaking for 10 min with buffer 1 (100 mM Tris-HCl, 150 mM NaCl); sections were covered with blocking reagent solution containing 1% blocking reagent for 1 h. Sections were incubated in a humid chamber with blocking solution containing anti-DIG alkaline phosphatase antibody Fab fragment at a dilution of 1:200. Sections were washed by shaking in buffer 1 (3 × 10 min) and incubated with buffer three (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5–10 min. Sections were covered with color generating solution [1 ml buffer three, 4.5 μL NBT solution (75 mg NBT/mL 70% dimethyl formamiade), 3.5 μL BCIP solution (50 mg BCIP/mL 100% dimethyl formamide)], and sections were incubated in a humid chamber for 2–7 h in the dark. When the color development was optimal, the reaction were stopped by incubating the slides in buffer one.

2.4. Immunohistochemistry

The paraffin embedded sections (6 μm in thickness) were deparaffinized as described previously for in situ hybridization. To unmask the antigens on the tissue, sections

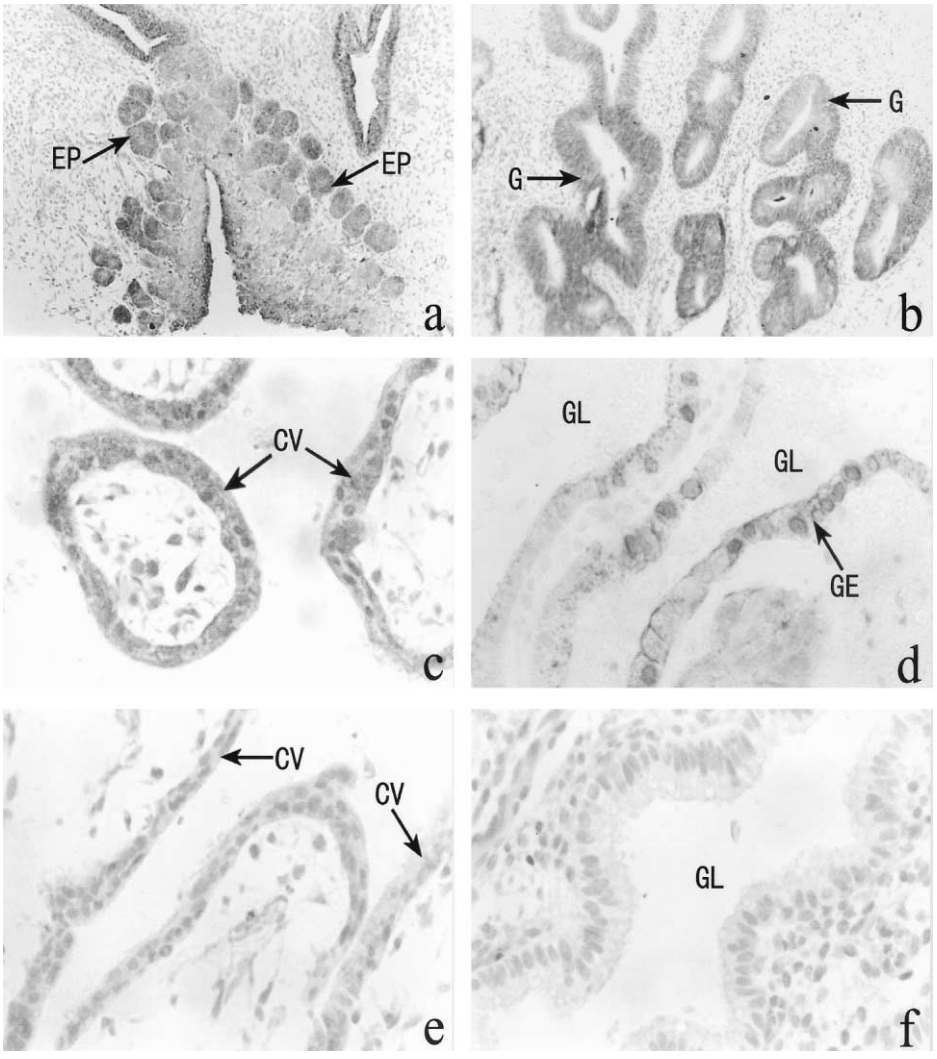

Fig. 1. Immunolocalization of Fas protein at the implantation site of the rhesus monkey during the early stage of pregnancy. (a) The epithelial plaque adjacent to the implantation site contains Fas protein (on day 15) ×100. (b) Fas protein in the glandular epithelium (on day 15) ×100. (c) The trophoblast villi labeled by anti-Fas antibody (on day 26) ×400. (d) The glandular epithelial cells contains Fas protein (on day 26) ×400. (e) and (f) Negative control ×400. EP = epithelial plaque; G = gland; CV = chorionic villi; GE = glandular epithelium; GL = glandular lumen.

were immersed in 10 mM citric acid buffer and boiled in a microwave oven at 92–98°C for 10 min. Endogenous peroxidase was quenched by incubating the sections with 3% H_2O_2 in 60% methanol for 10 min at room temperature, and the slides were washed with PBS (3 × 5 min). Then the sections were blocked with 5% normal goat serum (20 min, RT), and further incubated with primary antibodies specific for Fas and FasL (1:100 diluted with PBS) at RT for 1 h. Primary antibodies were replaced by normal rabbit IgG in negative control. The sections were washed with PBS (3 × 5 min), and the slides were incubated with biotin-conjugated second antibodies (1:200 in dilution) for 45 min. After washing with PBS (3 × 5 min), the section were incubated for 45 min with a mixture of reagent A (Avidin) and B (Biotin) prepared 30 min in advance with PBS (1:100 for each reagent). Sections were washed thoroughly with PBS and incubated with 3,3′-diaminobenzidine substrate solution for 2–7 min. The nuclei of the tissue were counterstained using haematoxylin.

2.5. TUNEL

DNA fragmentation in histologic sections was done by using a nonradioactive detection method [20] with slight modification. Namely, sections (6 μm) were mounted on coated slides, deparaffinized, and hydrated. The slides were incubated with proteinase (20 μg/mL) for 15 min at 37°C and washed in PBS. Then DNA 3′-end-labeling with digoxigenenin-dideoxyuridine triphosphate (dig-ddUTP) was preformed after incubation of the slides for 10 min in terminal transferase buffer (200 mM potassium cacodylate, 25 mM Tris, 0.25 mg/mL bovine serum albumin, and 5 mM $CoCl_2$, pH 6.6) at room temperature. Terminal transferase (1 U/μL), dig-ddUTP (1 μM), and dideoxy ATP (49 μM)

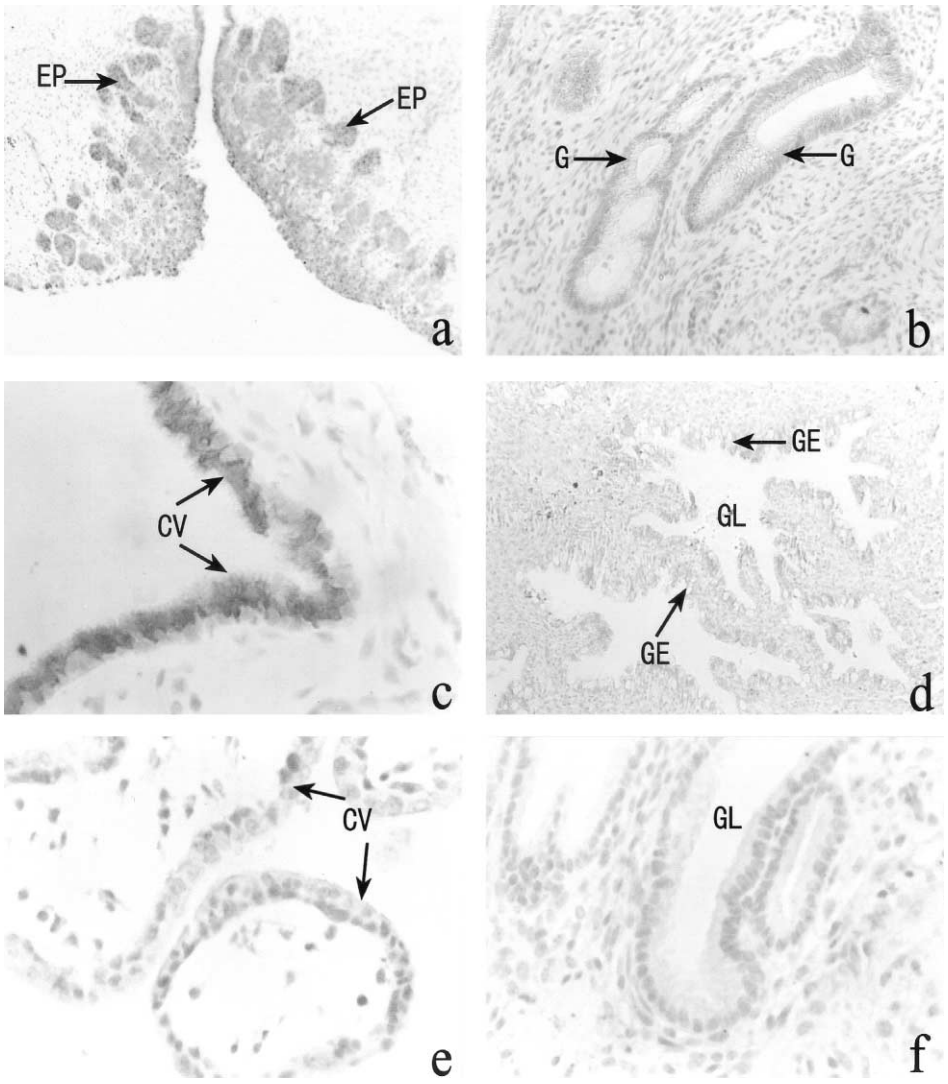

Fig. 2. Immunolocalization of FasL protein at the implantation site of the rhesus monkey during the early pregnancy. (a) The epithelial plaque adjacent to the implantation site contains FasL protein (on day 15) ×100. (b) FasL protein in the glandular epithelium (on day 15) ×100. (c) The chorionic villi express FasL protein (on day 26) ×400. (d) The glandular epithelial cells contains FasL protein (on day 30) ×100. (e) and (f) Negative control ×400. EP = epithelial plaque; G = gland; CV = chorionic villi; GE = glandular epithelium; GL = glandular lumen.

were added in fresh buffer and incubated at 37°C in a humidified chamber for 1 h. After three washes in Tris buffer, the slides were incubated with a blocking buffer (100 mM Tris, 150 mM NaCl, pH 7.5, and 2% wt/vol blocking reagent) for 30 min at room temperature before addition of antidigoxigenin antibody conjugated to alkaline phosphatase. After incubation with the antibody (1:10,000 in 2% wt/vol blocking reagent, 100 mM Tris, and 150 mM NaCl, pH 7.5) at room temperature for 2 h in a humidified chamber, the slides were washed three times in Tris buffer and finally equilibrated in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) before addition of enzyme substrates (337.5 μg/mL nitrobblue tetrazolium and 175 μg/ml 5-bromo-4-choro-3-inodyl-phosphate) for alkaline phosphatase. After 60 min, the color reaction was terminated with 10 mM Tris, 1 mM EDTA, pH 8, and the slides were counter-stained with eosin. Blue staining represented positive reaction.

2.6. Data analysis

Every experimental group contained three monkeys, and the data were obtained from at least three independent experiments; one of the representative figures from at least three similar results are shown.

3. Results

3.1. Localization of Fas, FasL antigen at implantation site of the rhesus monkey

To study the expression of Fas and FasL antigen in the monkey implantation site, immunohitochemistry using anti-

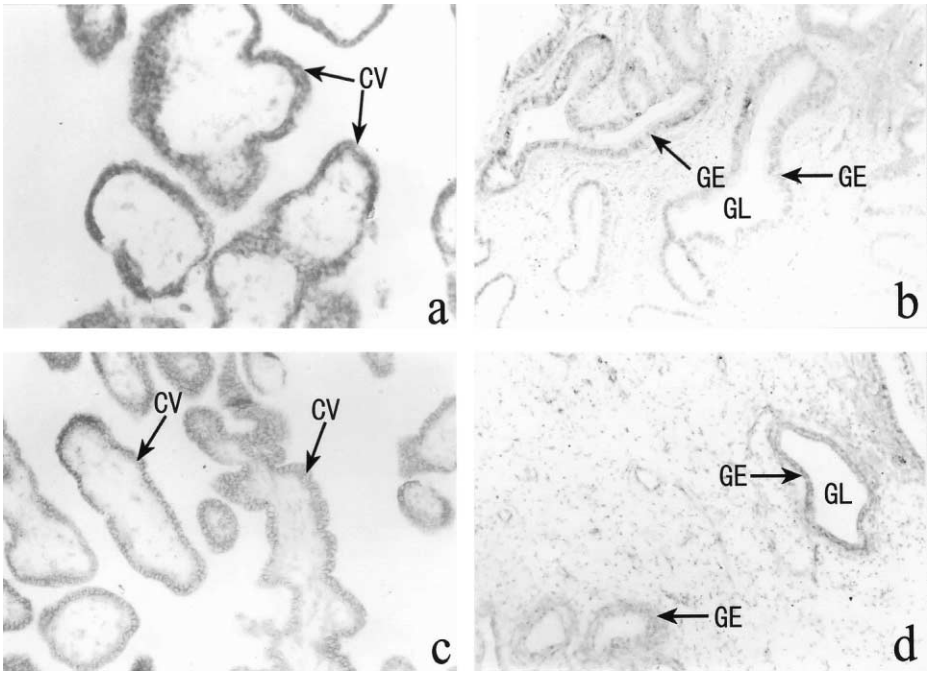

Fig. 3. In situ hybridization of Fas and FasL mRNA at the implantation site of the monkey during the early stage of pregnancy. (a) and (c) Specific binding for Fas and FasL cRNA probe (arrow) on day 26. (b) and (d) The glandular epithelium contains Fas and FasL mRNA (arrow) on day 26. CV = chorionic villi; GE = glandular epithelium; GL = glandular lumen. (Magnification ×200.)

Fas and anti-FasL polyclonal antibody was carried out. The results showed that the syncytiotrophoblast (ST) and cytotrophblast (CT) were strongly labeled by anti-Fas antibody (Fig. 1c). The localization of FasL protein, however, was different from that of the Fas protein, which was mainly detected in the ST, as shown in Fig. 2c. Fas and FasL antigen were also localized in the glandular epithelium from day 15 to day 30, but their expression in the glandular epithelium was gradually decreased (Figs. 1b and d and 2b and d). During this period, the morphological change of the gland was also obvious. On day 15 the glands were regular, and the glandular lumen was small (Figs. 1b and 2b). As the implantation proceeded, the glands became irregular, and the lumen became greatly enlarged (Fig. 1d). The glandular epithelium degenerated and contained many vesicle (arrow, Fig. 2d). On day 15 of pregnancy, epithelial plaque at the implantation site strongly expressed Fas and FasL antigen (Figs. 1a and 2a).

3.2. Localization of Fas and FasL mRNA at the monkey implantation site

In situ hybridization showed that the localization of Fas mRNA was correlated well with the expression of its protein (Fig. 3a and b), mainly detected in the chorionic villi and glandular epithelium. FasL mRNA was detected not only in ST, but also in CT (Fig. 3c). The FasL mRNA was also expressed in the glandular epithelium, just like the expression of its protein (Fig. 3d).

3.3. In situ 3′-end-labeling identify the apoptotic cells at the implantation site

To identify the cells involved in the apoptotic fragmentation of DNA in the chorionic villi and deciduas during the early stage of pregnancy, we labeled DNA at 3′-ends in histologic sections of the villous and decidual tissues. In deciduas, the labeled cells mainly localized in the glandular epithelium (Fig. 4a-d). In the chorionic villi, the labeled cells were detected locally in the cytotrophoblast (Fig. 4e and f). Some cytotrophoblast column cells of the anchoring villi were also labeled (Fig. 4g).

3.4. Immunocytochemical identification of epithelial plaque at the implantation site of rhesus monkey

Immunohistochemistry using anti-cytokeratin antibody showed that the epithelial plaque was labeled by this antibody on day 15. This result indicated that these cells were of epithelial origin (Fig. 5a).

4. Discussion

Abnormalities of maternal immune tolerance to the fetus have been implicated in several disease processes of pregnancy, including recurrent spontaneous abortion [21] and preeclampsia [22]. The basic mechanisms involved in maternal immune tolerance to the fetal semi-allograft, however, are poorly understood. Recent studies have demon-

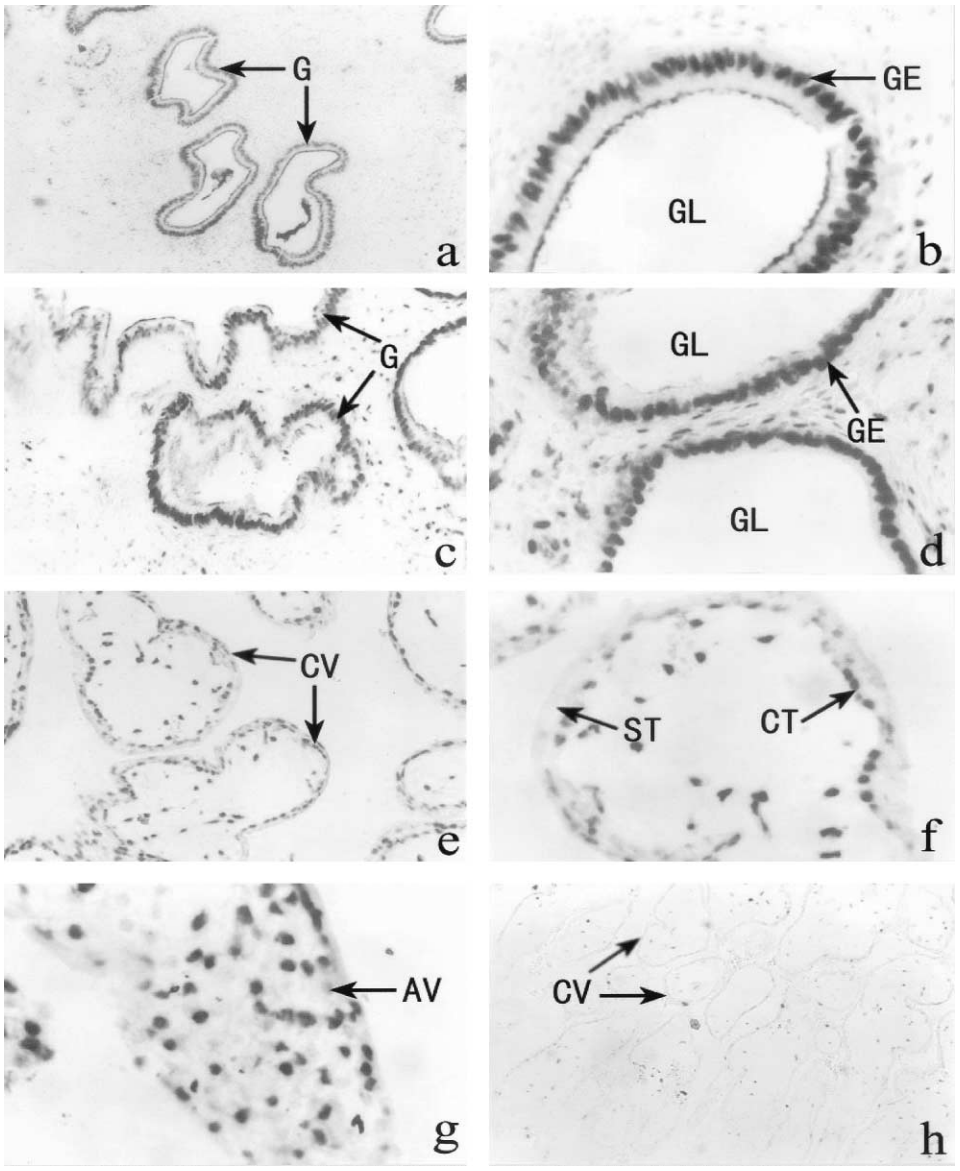

Fig. 4. In situ 3′-end-labeling of the apoptotic cells in the section of implantation site of the rhesus monkey during the early stage of pregnancy. Incorporation of dig-ddUTP is represented by blue coloration, and such staining is the result of the apoptotic cleavage of DNA. (a) The glandular epithelium (arrow) on day 15 ×100. (b) The higher magnification of image a, arrow shows the apoptotic epithelial cells ×400. (c) The glandular epithelium (arrow) on day 20 ×100. (d) The higher magnification of image c, arrow shows the apoptotic epithelial cells ×400. (e) Placental villi (arrow) on day 26 of pregnancy ×100. (f) Higher magnification of image e, arrow shows the apoptotic cells in CT×400. (g) The apoptotic cells (arrow) located in the cytotrophoblast cell column of the anchoring villi ×400. (h) Negative control ×100. G = gland; GE = glandular epithelium; GL = glandular lumen; CV = chorionic villi; ST = syncytiotrophoblast; CT = cytotrophoblast; AV = anchoring villi.

strated that the immune privileged nature of the testis and anterior chamber of the eye is caused by the expression of FasL in these tissues. When T cells are activated by infection or foreign antigens, they express Fas. Activation of T cell in the testis or anterior chamber of eye results in apoptosis of these cells secondary to the local production of FasL [23,24]. The immune protective effect of FasL has been exploited in transplantation biology by transfecting myoblasts with FasL complementary DNA [25]. Cotransplantation of these FasL-expressing myoblasts with pancreatic islet cells into donor mice of a different strain resulted in functional islet cells that were not rejected by the host's immune system. These findings suggest that Fas/FasL could be a very elegant mechanism for local immuno-tolerance. Some other reports also suggest that FasL expressed in the trophoblast cells can induce apoptosis in the activated peripheral lymphocytes, which may provide a local mechanism for maternal immuno-tolerance to the fetus [11,12].

The results in the present study showed that the monkey placental villi expressed FasL mRNA and protein. Therefore, it is reasonable to consider that the FasL expressed in the rhesus monkey chorionic villi may be involved in the mechanism of maternal immune tolerance. We also found in this study that Fas mRNA and protein localized in the

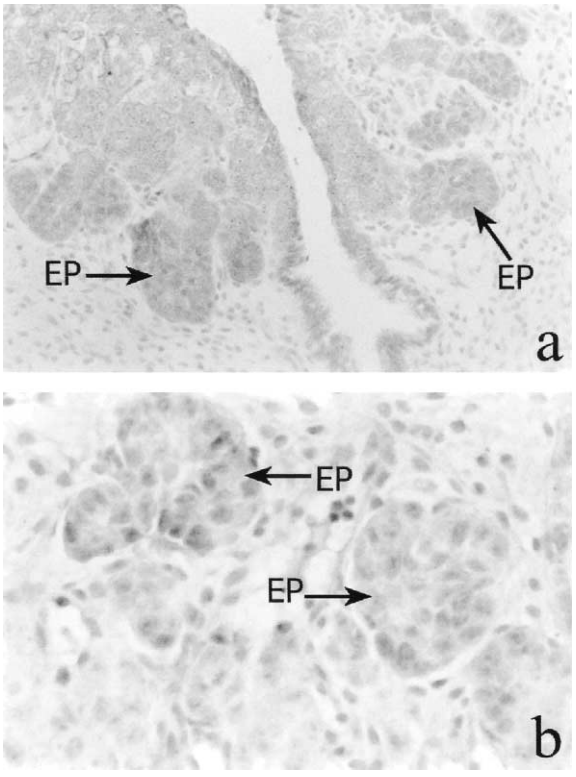

Fig. 5. Immunocytochemical identification of epithelial plaque at the implantation site of the rhesus monkey on day 15 of pregnancy. (a) The epithelial plaque was labeled by anti-cytokeratin antibody ×200. (b) Negative control ×400. EP = epithelial plaque.

chorionic villi and many such cells were undergoing apoptosis. It is more likely that apoptosis occurring in this part of the placenta may be part of the mechanism controlling placental growth as suggested by Huppertz et al. [13]. The Fas/FasL system may act as part of the apoptotic cascade responsible for the villous trophoblast turnover. This is because the cytotrophoblast cells contain very strong proliferative ability, and their proliferation must be locally well controlled, otherwise it would result in disease.

The epithelial plaque comes from the rapid proliferation of maternal uterine epithelial and glandular cells. In normal physiological conditions, the physical contact between fetal trophectoderm and uterine epithelium may trigger this reaction. However, the epithelial plaque has been shown to be short-lived, and its true function in the implantation process remains unclear. But the timing of the emergence as well as the degeneration and localization of the epithelial plaque convinced many scientists that it must play an important role in implantation. The epithelial plaque was strongly labeled by anti-FasL and anti-Fas antibodies in the deciduas at the implantation site on day 15; this result indicated that epithelial plaque cells undergo apoptosis. The epithelial plaque was shot-lived and disappeared on day 17 of pregnancy. So apoptosis may be involved in the degradation of the epithelial plaque.

The Fas and FasL mRNA and protein were also detected in the glandular epithelium in this study. The TUNEL experiment indicated that the nuclei of glandular epithelial cells in the decidual tissue were strongly labeled from day 15 to day 30. The morphological degeneration of glands was also obvious during this period. These results suggest that the glandular epithelium undergoes extensive remodeling and degeneration through apoptosis, and the function of glands might be changed during the early stage of pregnancy. We also observed blood cells in the glandular lumen, which may have originated either from inter villous space or from maternal arteries (unpublished data). Thus, the degenerated glands might be involved in placental circulation. This hypothesis, however, will need further experiments to verify.

In summary, apoptosis plays an important role during the early stage of implantation and placentation in the rhesus monkey. It not only provides an elegant mechanism for local maternal immuno-tolerance to the fetal semi-allograft during pregnancy, but is also involved in the regulation of proliferation of trophoblast cells and degradation of epithelial plaque as well as remodeling of the gland in the maternal deciduas.

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