Full Length Research Paper

Fas ligand (FasL) and Fas-associated death domain (FADD) were elevated in decidual stromal and glandular epithelial cells in spontaneous early miscarriage women

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Examine the Fas ligand (FasL) and Fas-associated death domain (FADD) expression of decidual tissues in first trimester human pregnancies and spontaneous early miscarriage (SEM), in order to find a possible involvement in the pathogenic etiology of SEM. In this study, we included 35 women with SEM and 35 women with healthy pregnancy. The protein expressions of FasL and FADD of decidual tissues were examined using immunohistochemistry and immunofluorescence. The decidual stromal cells, glandular epithelial cells, and vessel endothelial cells in the first trimester pregnancies expressed FasL and FADD proteins. SEM was accompanied by increased FasL and FADD protein expression in decidual stromal cells and glandular epithelial cells, especially women with at least one miscarriage prior (P < 0.01). Stronger FasL and FADD staining in the decidual stromal cells and glandular epithelial cells suggest increased expression of FasL and FADD in SEM. These data suggest that there is a different regulation of the FasL system in three types of decidual cells in early human pregnancies. Disturbance in expression of FasL and FADD in decidual of early pregnancies seems to be associated with SEM.

Key words: Fas ligand, Fas-associated death domain and spontaneous early miscarriage.

INTRODUCTION

The etiology of approximately 40% of the spontaneous early miscarriage (SEM) is not fully understood. Immunological mechanisms have been proposed to explain at least some of these cases of SEM. Dysregulation of maternal immune tolerance to the trophoblast is thought to cause some cases of early fetal demise. Different mechanisms have evolved to help make this possible.

FasL is expressed by NK cells, activated T cells and within immune privileged sites, such as the eye and brain (Bechmann et al., 1999). Fas is a member of the tumour necrosis factor (TNF) receptor family and is expressed by a wide variety of cell types. Upon cross-linking by either FasL or an agonistic anti-Fas monoclonal antibody (mAb), Fas intracellularly recruits Fas-associated death domain (FADD) and procaspase-8 forming the death-inducing signalling complex, after which the caspase pathway can be activated, resulting in apoptosis and cell death (Krammer, 2000). Th1 cytokines, IFN-γ and TNF-α, promote Fas expression on trophoblast, thus making them susceptible to Fas/FasL-mediated apoptosis by activated maternal lymphocytes, whereas Th2 cytokines increase the resistance of trophoblast cells to Fas-mediated apoptosis (Aschkenazi et al., 2002). The intrauterine Fas/Fas ligand (FasL) system has been considered primarily as a mechanism utilized by trophoblasts to escape maternal immune attack (Kauma

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et al., 1999). In contrast to normal pregnancy, miscarried deciduals contain leukocytes that are positive for FasL and extravillous trophoblasts, which show increased expression of Fas and increased rates of apoptosis (Minas et al., 2007). Excessive apoptosis in the embryo, regardless of its nature, if uncompensated, ultimately leads to maldevelopment or embryonic death. Apoptosis of activated maternal immune cells occurs in the human decidual mainly through the Fas/Fas ligand (FasL) system (Jerzak and Bischof, 2002). FasL, a member of the tumor necrosis factor superfamily, is known to trigger apoptosis of target cells through its interaction with Fas, expressed by most cell types (Bohana-Kashtan and Civin, 2004). Fas/FasL interaction triggers the formation of trimeric Fas complexes followed by clustering and recruitment of the adapter protein, the serine-phosphorylated adapter Fas-associated death domain (FADD) to form a death-inducing signaling complex, which subsequently activates the caspase system, eventually leading to target cell apoptosis (Crnici et al., 2005). Most studies were performed on placental tissue/villous trophoblasts. Trophoblast expression of FasL has been suggested as a protective mechanism against maternal leukocyte-induced apoptosis Hammer and Dohrm, (2000). Reduced and enhanced FasL expression has been reported in SEM (Guvendag et al., 2008; Kaponis et al., 2008; Choi et al., 2003). Only one previous study has focused on changes in decidual FasL expression in association with disease (Minas et al., 2007). Minas et al. (2007) observed expression of FasL in decidual leukocytes in cases with SEM but did not find detectable amounts of FasL in placenta decidual cells from termination of unwanted pregnancy or SEM. Moreover, the authors did not discriminate other types of cells expressed FasL within decidual.

The closest interaction between fetal and maternal cells occurs in decidual and thus this tissue will probably provide the most sensitive detection of changes in FasL expression. It is important for us to understand the regulatory mechanisms of the expression of FasL in decidual cells if we are to investigate the possible involvement in the pathogenic etiology of SEM. Thus, in the present study, we examined the hypothesis that SEM in humans may be associated with aberrant expression of the FasL at the decidual cells. Concomitantly, decidual FADD expression levels were assessed to learn whether disease is associated with changes.

SUBJECTS AND METHODS

Subjects

Thirty-five pregnant women with unexplained vaginal bleeding at 7 to 10 weeks of gestation, who had referred to First Affiliated Hospital of Xi’an Jiaotong University Medical School, Shaanxi Province, China, between October 2007 and December 2008, after confirmed pregnancy loss by ultrasound, were enrolled in the SEM group. 13 of 35 women had at least one miscarriage prior. The diagnosis of healthy and failing pregnancies was based on transvaginal ultrasound. Women with intact gestational sac or absence of fetal heartbeat matched for gestational age, or a gestational sac containing embryo remnants, growing less than 2 mm over a five-day period or less than 3 mm over a seven-day period, were diagnosed for pregnancy loss. The average age of these women was 29.0 ± 3.4 years and average gestational age was 59.4 ± 5.7 days. All of the women examined had a regular menstrual cycle, and their gestational age was confirmed by ultrasound examination, based on the last menstrual period. In all of the women studied, chromosomal abnormalities, uterine anomalies, thyroid dysfunction, autoimmune disorders, hypertension, diabetes mellitus, infection with rubella, toxoplasma, cytomegalovirus and herpes virus, and taken hormone medication in the recent three months, were excluded. 35 women without abnormal gynecologic history at 7 to 10 weeks of viable gestation who wanted to have a therapeutic abortion were included in the control group. Their average age was 27.6 ± 3.6 years and average gestational age was 57.5 ± 4.6 days. All of the women included were in good general health and gave written informed consent before participation. The study was approved by the Department of Science and Technology of the First Affiliated Hospital of Xi’an Jiaotong University Medical School.

Assessment of decidual FasL and FADD by SP Immunohistochemistry

Decidual samples were collected by curettage from women with SEM (35) and early viable pregnancies (35) undergoing artificial abortion. All the tissue samples were washed with 0.9% sterilized sodium chloride as soon as the decidual had been removed from uterus and fixed in 4% buffered paraformaldehyde for 24 h at room temperature. The fixed tissue samples were then carried through a series of changes to infuse them with paraffin for sectioning. After the procedure to infuse the tissue samples was completed, the tissue was embedded in solid paraffin blocks. Serial 4-μm-thick sections were sliced of a tissue microslicer (Leica RMZ135, Germany) and mounted on slides with Poly-L-Lysin adhesive. At least one paraffin section from each case was stained with haematoxylin and eosin (H and E) to allow morphological assessment.

The paraffin sections were analyzed with a SP immunohistochemistry kit. Briefly, after deparaffinization sections were pretreated in 0.01 M citrate buffer solution (pH 6.0) for antigen retrieval prior to antibody incubation, sections were then incubated with 0.3% H2O2 to block endogenous peroxidase activity. Negative controls were sections incubated with normal goat serum solution, and without primary antibody. For the detection of FasL and FADD sections were incubated with 50 μl of rabbit anti-human FasL antibody (Catalog No.BA0049, Boster Biological Technology, Ltd., China) or rabbit anti-human FADD antibody (Catalog No. Sc5559, Santa Cruz Biotechnology, USA) diluted at 1:100 for overnight at 4°C after SEM led in the buffer containing normal bovine serum albumin for 20 min at room temperature. Hereafter sections were incubated with biotin-goat anti-rabbit IgG (catalog No. BA1003) for 30 min at 37°C and stained with streptavidin-HRP for 30 min at 37°C. Sections were then incubated in DAB reagent, counterstained with hematoxylin, coverslipped using Protexx mounting media (DAB-Stock Stain box; Boster Biological Technology, Ltd., China), and observed under a microscope coupled to an image analysis workstation (Q550CW, Leica Co., Germany). The image software was used to semi-automatically estimate the volume fraction of immunopositive cells within the tissue sample. Five different fields of every section were observed randomly. The average of greyscale for five fields was used in the final analysis. The option allows the visualization of the selected gray level values within the region of interest between 0, which
represents pure white, and 255 pure black, respectively.

Co-localization of decidual FasL with FADD by double immunofluorescence

The fluorescence intensity corresponding to the value of FasL and FADD was detected by double immunofluorescence employing a confocal laser scanning microscopy (CLSM) (TCS SP2, Leica Co., Germany), in accordance with the manufacturer’s instructions. Briefly, after deparaffinization the 4-μm-thick sections were immersed in a buffer containing 30% H2O2 and distilled water (30% H2O2: distilled water = 1:10) for 10 min at room temperature. Then rinsing in distilled water three times (5 min each), the sections were sealed in a buffer containing normal bovine serum albumin at a concentration of 1 mg/ml for 20 min at 37°C, followed by incubation with 50 μl of mice anti-human FasL antibody diluted at 1:100 for overnight at 4°C. After being washed repeatedly with 0.02 mol/L pH7.4 phosphat (PBS) buffer, the fluorescein isothiocyanate (FITC)-goat anti-mice IgG antibody (catalog No. BA1105, Boster Biological Technology, Ltd., China) was applied for overnight at 4°C. They were then rinsed four times in PBS buffer, PH 7.4, for 5 min each time and incubated with 50 μl of rabbit anti-human anti-FADD antibody for overnight at 4°C. After incubation with primary antibody, slides were incubated with carboxyamine 3 (Cy3)-sheep anti-rabbit IgG antibody (catalog No. BA1032, Boster Biological Technology, Ltd., China) for overnight at 4°C and then mounted on glass slides with the aid of glycerol buffer. Goat pre-immune serum was used as negative control. The FasL and FADD fluorescence distribution was observed using the CLSM. To further exclude operator bias, observations were performed on coded samples in a blinded fashion. The fluorescence excitation was provided by a 488/543 nm argon laser beam and emission was 535/700 nm for FITC and Cy3. Five fields were scanned for each sample by CLSM. The images were analyzed using the software Image Plus (Leica, Germany). The mean fluorescence intensity of positive cells was analyzed within the cell population.

Statistical analysis

SPSS-PC+ for Windows v11.0 was used. Intensity of fluorescence and greyscale value were expressed as the mean ±SD. Statistical analysis was performed using student’s t-test. A value of p < 0.05 was considered significant.

RESULTS

Seventy women were included in the study (35 SEM and 35 controls). The women with spontaneous miscarriage and women with healthy pregnancies did not differ significantly with respect to their average age and average gestational days.

Detection of FasL and FADD in human decidual by immunohistochemistry

The expression of FasL proteins was detected in the cytoplasm and nuclei of stromal cells, glandular epithelial cells and vessel endothelial cells in decidual at early pregnancies. Despite the detection of FasL in all three types of decidual cells, the expression intensity of FasL was different in SEM and early healthy pregnancies. The expression intensity of decidual stromal cells and decidual glandular epithelial cells was significantly increased in women with SEM compared with early healthy pregnancies (Figure 1 and Table 1). Especially, women, having at least one miscarriage prior, had significant stronger expression of FasL in decidual stromal cells and decidual glandular epithelial cells than women with the first miscarriage (Greyscale values: 176.59 ± 1.52, t = 7.625, p < 0.001; 165.31 ± 2.02, t = 2.161, p < 0.05). In contrast, no significant difference for the expression intensity of FasL was observed in decidual vessel endothelial cells between SEM and early healthy pregnancies (Table 1).

Similarly, a significantly higher expression of FADD in decidual stromal cells and decidual glandular epithelial cells in SEM was observed than that in early healthy pregnancies (Figure 2, Table 1). Especially in the SEM group, expression of FADD in women having at least one miscarriage prior was significantly higher than those with the first miscarriage in decidual stromal cells and decidual glandular epithelial cells (Greyscale values: 164.57 ± 2.33 and 167.50 ± 4.50, t = 2.176, p < 0.05; 161.31 ± 2.66 and 165.54 ± 1.08, t = 6.637, p < 0.001). A weak expression intensity was observed in the cytoplasm of decidual vessel endothelial cells from the SEM group, but had no statistical significance, compared with early healthy pregnancies (Table 1).

Detection of FasL and FADD in human decidual by double immunofluorescence

The intensity of the immunofluorescence representing FasL and FADD in decidual cells was determined by double immunofluorescence employing a CLSM. A similar localization and expression intensity of FasL and FADD was also observed in three types of decidual cells by CLSM (Figure 3, Table 2). The combined identification of FasL-expressing and FADD-expressing cells revealed that decidual cells showed a consistency in the expression levels of FasL and FADD proteins, that is, FasL and FADD proteins are co-expressed in decidual stromal cells and decidual glandular epithelial cells of early pregnancies, and had the higher expressive level in SEM than in early healthy pregnancies.

DISCUSSION

The feto-maternal interface is an interweave of tissues comprised of various types of immune and non-immune cells, where complex immune phenomena take place. Apoptosis within the maternal decidual may be important in the establishment of immune privilege in the pregnant uterus to protect fetal cells from killing by maternal cells
Figure 1. Significantly higher staining intensity of FasL of decidual stromal cells and decidual glandular epithelial cells in the h SEM group than in the control group by immunohistochemistry. A) Control; B) SEM. SP×400.

Table 1. Comparison of FasL and FADD expression in decidual cells between SEM and controls. Greyscale values are given as mean ± SEM (n = 70).

<table>
<thead>
<tr>
<th>Decidual cells</th>
<th>SEM (n = 35)</th>
<th>Control (n = 35) value</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal cells</td>
<td>175.33 ± 2.09</td>
<td>177.04 ± 2.15</td>
<td>3.384</td>
<td>0.001</td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>164.72 ± 2.19</td>
<td>166.20 ± 2.26</td>
<td>2.778</td>
<td>0.007</td>
</tr>
<tr>
<td>Vessel endothelial cells</td>
<td>178.72 ± 1.70</td>
<td>179.43 ± 1.63</td>
<td>1.763</td>
<td>0.082</td>
</tr>
<tr>
<td>FADD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal cells</td>
<td>166.41 ± 4.06</td>
<td>169.32 ± 4.57</td>
<td>2.819</td>
<td>0.006</td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>163.97 ± 2.74</td>
<td>165.52 ± 1.85</td>
<td>2.770</td>
<td>0.007</td>
</tr>
<tr>
<td>Vessel endothelial cells</td>
<td>167.85 ± 2.92</td>
<td>168.45 ± 2.18</td>
<td>0.972</td>
<td>0.335</td>
</tr>
</tbody>
</table>

Figure 2. Significantly higher staining intensity of FADD of decidual stromal cells and decidual glandular epithelial cells in the h SEM group than in the control group by immunohistochemistry. A) Control; B) SEM. SP×400.
However, it has not been demonstrated how apoptosis-related genes are expressed in decidual cells of early pregnancies. In this study, we have addressed the expression of FasL and FADD in the human decidual. We have found that FasL protein is expressed by first trimester decidual cells. This observation is in accordance with a previous report (Qiu et al., 2005). We also found that the higher expression level of FasL protein is shown in decidual stromal cells and glandular epithelial cells from women with SEM than those from healthy controls, especially women with at least a previous SEM, which suggest a possible association between decidual FasL protein overexpression and SEM. In a previous report, Choi et al. (2003) found that the higher expression the level of FasL is shown in chorionic villi from recurrent pregnancy loss patients than those from normal controls and suggests that the abnormal expression of apoptosis-related genes is one of primary reasons to be involved in recurrent pregnancy loss. Kaponis et al. (2008) also reported that the expression of Fas and FasL in coelomic fluid in missed abortion was higher than in healthy pregnancies at the same gestational age.

The major function of the Fas–FasL interaction is the induction of apoptosis in activated cells carrying Fas (Makrigiannakis et al., 2008). It has been shown that the FasL–Fas interaction between decidual cells expressing FasL and Fas-bearing leukocytes leads to apoptosis of activated leukocytes. The FasL system represents one of the main apoptotic pathways controlling cell proliferation and tissue remodeling (Song et al., 2000). Aberration of apoptosis during the early stages of pregnancy can result in pregnancy loss or embryonic maldevelopment (Savion et al., 2002). The FADD is a key adaptor protein and it plays an essential role in the apoptotic signaling of the Fas death pathway. Even though sufficient amounts of normal Fas and FasL exist on the cell surface, a deficiency of FADD fails to transduce an apoptotic signal to caspase-8 and further downstream. If the transduction of downstream apoptosis signal of FADD is blocked, cell

**Table 2.** Comparison of FasL and FADD fluorescence intensity in decidual cells between SEM and controls. Values are given as mean ± SEM (n = 70).

<table>
<thead>
<tr>
<th></th>
<th>SEM (n = 35)</th>
<th>Control (n = 35)</th>
<th>t</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FasL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal cells</td>
<td>120.50 ± 3.82</td>
<td>112.48 ± 3.04</td>
<td>-9.722</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>131.11 ± 1.71</td>
<td>128.13 ± 1.86</td>
<td>-6.973</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vessel endothelial cells</td>
<td>89.01 ± 2.63</td>
<td>87.80 ± 2.83</td>
<td>-1.850</td>
<td>0.069</td>
</tr>
<tr>
<td><strong>FADD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal cells</td>
<td>120.98 ± 3.81</td>
<td>114.07 ± 3.84</td>
<td>-7.544</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>132.28 ± 2.47</td>
<td>129.47 ± 1.47</td>
<td>-5.792</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vessel endothelial cells</td>
<td>90.94 ± 4.05</td>
<td>89.21 ± 3.26</td>
<td>-1.967</td>
<td>0.053</td>
</tr>
</tbody>
</table>
damage will be decreased, thus allograft survival will be prolonged. Studies with gene-targeted mice and transgenic mice expressing a dominant-negative mutant of FADD demonstrated that FADD-mediated activation of the proteolytic activity of caspase-8 is essential for FAS-induced apoptosis in many cell types both in vitro and in vivo (Strasser et al., 2009). In an earlier study, Hu et al. (2004) reported that inhibiting the function of FADD will lead to blocking downstream apoptosis signal, which protects pancreatic beta cells from destruction by Fas-FasL pathway in vitro. The overexpression of FADD induces apoptosis in the cells regardless of Fas expression on the cell surface (Komata et al., 2001). We demonstrated that the amount of FADD protein was markedly increased in decidual stromal and glandular epithelial cells from women with SEM, and the upregulation of FADD is an important event for FasL-induced apoptosis in decidual. This suggests that the co-expression of FasL and FADD in decidual stromal and glandular epithelial cells triggers the activation of the downstream signaling of the death receptor pathway.

In this study, we provide experimental evidence which links spontaneous abortion in humans with expression of FasL and FADD in the first trimester decidual stromal and glandular epithelial cells. Aberration of the FasL-mediated apoptosis may represent one of the execution step necessary for pregnancy loss in spontaneous abortion. Disturbance in expression of FasL and FADD in decidual of early pregnancies seems to be associated with early pregnancy loss. Progress towards improving early pregnancy success rates is one of the most enticing aims of reproductive medicine. It cannot be excluded that influence on this process may also allow introducing new treatment modalities.

ACKNOWLEDGEMENT

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