Non-apoptotic Fas-induced regulation of cytokines in undifferentiated and decidualized human endometrial stromal cells depends on caspase-activity

Herbert Fluhr*,†, Henriette Wenig†, Julia Spratte, Stephanie Heidrich, Jens Ehrhardt, and Marek Zygmunt

Department of Obstetrics and Gynecology, University of Greifswald, Sauerbruchstr., 17475 Greifswald, Germany

*Correspondence address. Tel: +49-3834-866500; Fax: +49-3834-866501; E-mail: herbert.fluhr@uni-greifswald.de

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abstract:

Fas has originally been described as a member of the death-receptor family, mediating apoptosis upon stimulation by Fas-ligand (FasL). However, Fas expressing human endometrial stromal cells (ESCs) are resistant to Fas-mediated apoptosis. Since the implanting embryo secretes FasL, we examined whether Fas mediates non-apoptotic effects in human ESCs in vitro. ESCs were isolated from hysterectomy specimens, decidualized using progesterone and 17β-estradiol and incubated with an activating anti-Fas antibody, recombinant FasL, a caspase-inhibitor. Leukemia inhibitory factor (LIF), interleukin (IL)-11, -6, -8, monocyte chemoattractant protein (MCP)-1 and RANTES (Regulated on Activation Normal T cell Expressed and Secreted) were measured using ELISA and real-time RT–PCR. Viability of ESCs was determined using an MTT assay. Caspase-activity was measured by luminescent assays. Activation of nuclear factor (NF)-κB was detected by in-cell western and transcription factor assays. LIF and IL-11 in undifferentiated and IL-8 in decidualized ESCs were up-regulated by non-apoptotic Fas-signaling. In contrast, IL-6, MCP-1 and RANTES were not regulated by Fas. Caspases were activated upon Fas-stimulation and the Fas-mediated effects on LIF, IL-11 and -8 were reversed by caspase-inhibition. The transcription factor NF-κB was not activated in ESCs after stimulation of Fas. These results suggest a differential regulatory role of caspase-dependent Fas-signaling at the feto-maternal interface during early implantation. Remarkably, this typical death machinery mediates non-apoptotic effects in the human endometrium rather than inducing apoptosis.

Key words: apoptosis / caspases / cytokines / endometrium / Fas

Introduction

Endometrial cytokines and chemokines regulate the complex events of embryo implantation and are, at least in part, directly modulated by early embryonal signals, such as human chorionic gonadotrophin, interleukin (IL)-1β or insulin-like growth factor (IGF)-II (Herrler et al., 2003; Dimitriadis et al., 2005; Licht et al., 2007). In addition, the apoptotic molecule tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) regulates IL-6, -8, leukemia inhibitory factor (LIF) and monocyte chemoattractant protein (MCP)-1 in human endometrial stromal cells (ESCs) in a non-apoptotic manner (Fluhr et al., 2009). Members of the IL-6 family are known to play an important role at the feto-maternal interface (Dimitriadis et al., 2005; Strowitzki et al., 2006). Disturbance of their expression or action may be involved in failure of implantation or abnormal placentation (Dimitriadis et al., 2005). The chemokines IL-8, MCP-1 and RANTES (Regulated on Activation Normal T cell Expressed and Secreted) are produced by the human endometrium and have an influence on endometrial leukocyte recruitment (Garcia-Velasco and Arici, 1999).

Fas is a member of the death-receptor family. Fas activation by Fas-ligand (FasL) typically triggers apoptosis in susceptible cells by activation of caspases (Nagata, 1997; Scaffidi et al., 1998). The Fas/FasL-system is one of the major effectors of cytotoxic T cells and...
natural killer cells and seems to play an important role in the maintenance of the immune privilege in specialized organs like the eye, testis or placenta. Fas is expressed by human ESCs and Fasl is secreted by the embryo at the feto-maternal interface (Uckan et al., 1997; Abraham et al., 2004; Harada et al., 2004). As recently shown, human ESCs are usually resistant to Fas-mediated apoptosis (Fluh et al., 2007b).

Additionally, evidence has emerged that the Fas/FasL-system is involved in non-apoptotic signaling in the absence of its classical death-inducing function (Wajant et al., 2003). It has been shown that Fas induces the expression of cytokines, stimulates proliferation and activates signaling pathways like the transcription factor nuclear factor kappa B (NF-kB) in different cell types (Rensing-Ehl et al., 1995; Ahn et al., 2001; Choi et al., 2001; Wajant et al., 2003). Thereby Fas-mediated signaling appears to be involved in the non-apoptotic regulation of various cellular processes, including inflammation and chemotraction. However, the role of caspases in non-apoptotic Fas-mediated signaling is still controversial (Choi et al., 2002).

On the basis of these observations we investigated whether undifferentiated and decidualized ESCs show non-apoptotic responses upon Fas-stimulation. Therefore, possible Fas-mediated effects on cytokines/chemokines as well as the role of caspases and the transcription factor NF-kB were studied.

Materials and Methods

Tissue collection and cell culture

Endometrial tissue samples were obtained after written informed consent from premenopausal women undergoing hysterectomy for benign reasons. The study protocol was approved by the institutional ethical board of the University of Greifswald, Greifswald, Germany. ESCs were isolated, cultured and characterized as described previously (Fluh et al., 2007a). Briefly, minced endometrial tissue was digested by incubation with collagenase (Biochrom, Berlin, Germany) and the dispersed endometrial cells were separated by filtration. ESCs were maintained in DMEM/F-12 cell culture medium without phenol red (Gibco/Invitrogen, Karlsruhe, Germany) containing 10% charcoal-stripped fetal bovine serum (Biochrom) and 50 µg/ml gentamycin (Ratiopharm, Ulm, Germany). The purity of ESC cultures was proven by immunofluorescent staining of vimentin. Jurkat cells (ATCC, Manassas, VA, USA) were maintained following standard procedures.

Decidualization in vitro and experimental conditions

ESCs were decidualized in vitro by incubating the cells with 1 µM progesterone and 30 nM 17β-estradiol (both from Sigma, Taufkirchen, Germany) for 9 days. Decidualization was proved by measuring a significantly increased secretion of insulin-like growth factor-binding protein (IGFBP)-1 and prolactin as described previously (Fluh et al., 2007a). At Day 9, decidualized as well as undifferentiated ESCs were incubated with the following agents: activating anti-Fas antibody (clone CH-11; MBL, Woburn, MA, USA), isotype control mouse IgM (MBL), human recombinant FasL together with a cross-linking enhancer (Alexis/ Axxora, San Diego, CA, USA), Z-VAD-FMK or Z-FA-FMK (BD Biosciences, Heidelberg, Germany), and human recombinant TNF-α (Biosource, Camarillo, CA, USA). The different combinations, dosages and incubation times of the mentioned agents are indicated in detail in the corresponding figures.

Cell viability assay

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was used to measure the viability of ESCs and Jurkat cells. Cells were incubated for 6 h in the presence of 2.5 mg/ml MTT and thereafter 10% sodium dodecyl sulfate (Sigma) was added. After an incubation overnight at 37°C, absorbance (550 nm) was determined using the FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany).

Enzyme-linked immunosorbent assays

Cell culture supernatants were collected and analyzed for LIF, IL-11, -6, -8, MCP-1 and RANTES using commercially available ELISA kits (Bender Medsystems, Vienna, Austria; R&D Systems, Wiesbaden, Germany). The sensitivities of the assays were 1.13 pg/ml (LIF), 1.6 pg/ml (IL-6), 2 pg/ml (IL-8), 15.6 pg/ml (IL-11), 15.6 pg/ml (MCP-1) and 2 pg/ml (RANTES), respectively. There was no significant cross-reactivity or interference. Intra- and inter-assay variabilities were lower than 5%. All assays were performed according to the manufacturer’s instructions. Absorbance was measured using the FLUOstar OPTIMA system.

Caspase-activity assays

The activity of caspases in ESCs was detected using the Caspase-Glo® assay system (Promega, Madison, WI, USA). Caspase-Glo® substrates IETD, LEHD or DEVD were added to the cell cultures followed by an incubation for 60 min. Luminescence was recorded using the FLUOstar OPTIMA system.

Real-time reverse transcriptase–polymerase chain reaction

Total ribonucleic acid (RNA) was isolated from ESCs using PeqGOLD TrFixFast® (PeqLab, Erlangen, Germany) and reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Semiquantitative real-time polymerase chain reaction (PCR) was performed to quantify mRNA levels of LIF, IL-11 and -8 in relation to the housekeeping gene β-actin. cDNA samples were amplified using the Power SYBR® Green PCR-Master Mix (Applied Biosystems) and the respective forward and reverse primers. The primers (Invitrogen) were designed using Primer Express® Primer Design Software v2.0 (Applied Biosystems) with the resulting amplicons having an intron-overlapping sequence. The sequences of the primers used were:

- LIF forward 5′-GCAATGCCCTTTATATCTTCT-3′,
- LIF reverse 5′-CCACATAGCTGTCCAGGTGT-3′,
- IL-11 forward 5′-TCTCTCCTGCGCCAGACCG-3′,
- IL-11 reverse 5′-AATCCAGGTGTTGCTCCC-3′,
- IL-8 forward 5′-TCTTGGACCTTCTCCTGATT-3′,
- IL-8 reverse 5′-TTAGCCTCTCTGCCAAACTC-3′,
- β-actin forward 5′-CTTGCGACCCAGACACAT-3′,
- β-actin reverse 5′-GCCGATCCACACGGAGTACT-3′.

PCR amplification was performed in duplicate in an ABI Prism™ 7300 sequence detector (Applied Biosystems) using a standard cycling program (Fluh et al., 2007a). PCR products were analyzed by thermal dissociation to verify that a single-specific PCR product had been amplified. Relative expression levels of LIF, IL-11 and -8 in relation to β-actin were determined using the mathematical model:

\[ \text{ratio} = 2^{\frac{-\Delta \Delta C_T}{\text{Livak and Schmittgen, 2001}}} \]

In-cell western assay

To quantify the levels of phosphorylated (Ser536) NF-kB p65 in ESCs we used an in-cell western assay, based on immunofluorescent staining performed in a 96-well microplate format. The cells were fixed with 3.7%
formaldehyde and permeabilized with ice-cold methanol (both from Sigma). Blocking with Odyssey® Blocking Buffer (LI-COR®, Lincoln, NE, USA) was followed by the incubation with an anti-phospho (Ser563) NF-κB p65 antibody (#3033, Cell Signaling Technology, Danvers, MA, USA) and an IRDye® 800CW conjugated secondary antibody (LI-COR®). Normalization of each sample was performed by parallel DNA-staining using DRAQ5™ (Biotostatus Limited, Shepshed, UK). The assays were visualized and analyzed using the LI-COR® Odyssey imaging system (LI-COR®).

Nuclear transcription factor assays

Nuclear extracts were isolated from ESCs using the Nuclear Extract Kit and quantified using the ProStain™ kit following the manufacturer’s instructions (both kits from Active Motif, Rixensart, Belgium). Activation and nuclear translocation of NF-κB were measured by the use of the TransAM™ Chemi NF-κB p65 and p50 kits (Active Motif) according to the manufacturer’s protocol. These assays measure the level of active NF-κB p65/p50 in nuclear extracts by its binding to an oligonucleotide containing the NF-κB consensus-binding site (5′-GGGACTTTCC-3′) attached to a 96-well microplate. The specificity of the assays was proved by the use of an excess of oligonucleotides containing either a wild-type or mutated consensus-binding site. Luminescence was recorded using the FLUOstar OPTIMA system.

Statistics

Each experiment was performed in triplicates or quadruplicates on cell cultures derived from three to five different patients. Statistical analysis was carried out with one-way analysis of variance, followed by Dunnett’s and Bonferroni multiple comparison tests or unpaired Mann–Whitney t-tests using GraphPad PRISM version 5 software (GraphPad, San Diego, CA, USA). The results are expressed as mean ± SEM. Differences were considered to be significant if \( p < 0.05 \).

Results

Cytokine secretion in ESCs is selectively regulated upon non-apoptotic Fas-stimulation

To test whether Fas might mediate non-apoptotic signals in human ESCs, undifferentiated as well as decidualized cells were incubated with an activating anti-Fas antibody for 48 h and selected cytokines/chemokines were measured. The selected molecules were all within the detection range of the ELISAs used. As summarized in Table 1, Fas-stimulation enhanced the secretion of LIF and IL-11 in undifferentiated ESCs and IL-8 in decidualized ESCs, whereas IL-6, MCP-1 and RANTES were not altered.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Undifferentiated ESCs</th>
<th>Decidualized ESCs</th>
</tr>
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<tbody>
<tr>
<td>LIF</td>
<td>Stimulation(^*)</td>
<td>No effect</td>
</tr>
<tr>
<td>IL-11</td>
<td>Stimulation(^*)</td>
<td>No effect</td>
</tr>
<tr>
<td>IL-6</td>
<td>No effect</td>
<td>No effect</td>
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<tr>
<td>IL-8</td>
<td>No effect</td>
<td>Stimulation(^*)</td>
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<tr>
<td>MCP-1</td>
<td>No effect</td>
<td>No effect</td>
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<tr>
<td>RANTES</td>
<td>No effect</td>
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\(^*\)See Fig. 1.

The observed effects of the activating anti-Fas antibody were dose-dependent and human recombinant FasL revealed similar effects (Fig. 1A–C). The specificity of the effects was proved by incubating the cells with an unspecific isotype control antibody (mouse IgM), which had no significant impact (Fig. 1A–C).

Caspases are activated and necessary for non-apoptotic Fas-signaling in ESCs

As caspases are the essential mediators of apoptotic Fas-signaling, we characterized the activation of caspases in ESCs under the influence of non-apoptotic Fas-stimulation. Therefore, we measured their enzymatic activity using the three different caspase-substrates IETD, LEHD and DEVD. Stimulation of ESCs with the activating anti-Fas antibody led to a time-dependent induction of caspase-activity in comparison to untreated cells (Fig. 2A–C).

We further evaluated whether caspases are necessary for the observed non-apoptotic Fas-effects on cytokines in ESCs. The general caspase-inhibitor Z-VDAD-FMK completely abolished the Fas-mediated up-regulation of LIF and IL-11 secretion in undifferentiated ESCs (Fig. 2D and E) and of IL-8 secretion in decidualized ESCs (Fig. 2F). The specificity of this inhibitory effect was proved by the application of the negative control Z-FA-FMK, having no significant impact on the Fas-mediated effects (Fig. 2D–F). Caspase-inhibition had no significant impact on the basal secretion level of the mentioned cytokines in ESCs (Fig. 2D–F).

Non-apoptotic Fas-effects on cytokines in ESCs are mediated on the transcriptional level

We further evaluated whether the observed effects of Fas-stimulation on the cytokine secretion level are accompanied by changes of the mRNA expression. Incubation of undifferentiated ESCs with activating anti-Fas antibody caused a rapid and significant up-regulation of LIF mRNA (Fig. 3A). Similar results were seen for IL-11, as Fas-stimulation significantly enhanced IL-11 mRNA levels during the first 12 h of incubation (Fig. 3B). In decidualized ESCs, the activating anti-Fas antibody rapidly stimulated IL-8 mRNA with significantly elevated levels during the first 24 h of stimulation and a peak after 12 h (Fig. 3C).

The transcription factor NF-κB is not activated in ESCs upon non-apoptotic Fas-stimulation

On the basis of the observed effects on the transcriptional level we wanted to see whether NF-κB is induced in ESCs under the influence of Fas-stimulation. Neither in undifferentiated nor in decidualized ESCs could we detect any phosphorylation of NF-κB p65 under the influence of Fas-stimulation (Fig. 4A and B). Moreover, using nuclear transcription factor assays, no induction of NF-κB p65 or p50 was seen.
upon Fas-stimulation (Fig. 4C and D). The functionality of the assays and the presence of inducible NF-κB in ESCs were proved by incubating the cells with TNF-α (Fig. 4A–D).

**Discussion**

In the present study, we observed non-apoptotic caspase-dependent effects of Fas-stimulation in human ESCs, which are primarily resistant to Fas-mediated apoptosis (Fluhr et al., 2007b).

Various cytokines are expressed in the human endometrium and most of them show specific cycle-dependent expression patterns due to their temporarily defined functions in endometrial differentiation and receptivity (Dimitriadis et al., 2005; Strowitzki et al., 2006). LIF is known to play an important role during implantation, which has been discovered in LIF knock-out mice being infertile due to defective implantation (Stewart, 1994). In humans, LIF gene mutations have been described in infertile women (Giess et al., 1999). Women suffering from idiopathic infertility show lower...
intrauterine LIF levels when compared with controls (Laird et al., 1997). The observed Fas-induced up-regulation of LIF in ESCs might thus be a missing link in the chain of events rendering the endome-
trium receptive. During the decidualization of the endometrial stroma in the second half of the cycle, IL-11 has been shown to be an important autocrine promoter of progesterone-induced differen-
tiation (Dimitriadis et al., 2002). Since the endometrium itself has been reported to express FasL an additional autocrine impact of the

Figure 2 Non-apoptotic stimulation of Fas in ESCs activates caspases and the Fas-mediated stimulation of LIF, IL-11 and IL-8 can be reversed by caspase-inhibition. (A–C) Undifferentiated ESCs were either left untreated or stimulated with 200 ng/ml activating anti-Fas antibody for 12 and 24 h. The enzymatic activity of caspases was measured by luminescent activity assays using the substrates IETD (A), LEHD (B) and DEVD (C). (D–F) Undiffer-
entiated (for LIF and IL-11) and decidualized (for IL-8) ESCs were incubated for 30 min with 20 μM of the caspase-inhibitor Z-VAD-FMK or the control Z-FA-FMK, followed by the addition of 200 ng/ml activating anti-Fas antibody for another 48 h. For comparison, ESCs were also incubated with anti-Fas, the inhibitor or its control alone. Thereafter, the levels of LIF (D), IL-11 (E) and IL-8 (F) in the cell culture supernatant were determined by ELISAs. Results are standardized relative to the values of untreated cells, which are set to one arbitrary unit (A–C) or 100% (D–F). Bars represent mean ± SEM; *p < 0.05 compared with untreated cells. #p < 0.05 compared with anti-Fas-stimulated cells.
endometrial Fas/FasL-system on the process of decidualization might be postulated (Harada et al., 2004). In our experiments, Fas-stimulation induced a dose-dependent up-regulation of IL-8 in decidualized ESCs. IL-8 belongs to the family of chemokines which are known to regulate leukocyte migration and to be involved in endometrial differentiation and implantation (Dimitriadis et al., 2005). The Fas-induced up-regulation of IL-8 might favor implantation, since IL-8 is also known to stimulate migration of villous cytotrophoblast cells (Hirota et al., 2009). In addition, IL-8 has regulatory effects on the survival of endometrial cells and modulates collagenase activity in the endometrial stroma (Gazvani et al., 2002; Mulayim et al., 2004).

Interestingly, Fas-stimulation had no significant effects on IL-6, MCP-1 and RANTES, which are also known to play a role in endometrial differentiation and implantation, especially in respect to endometrial immunology.

The observation of selective effects of the Fas/FasL-system on distinct cytokines suggests a coordinated function of this signaling pathway rather than an uncoordinated inflammatory scenario. Since the invading trophoblast secretes functional FasL (Uckan et al., 1997; Abrahams et al., 2004), the observed regulatory effects may reflect a possibility of the embryo to modulate the maternal endometrium and its own implantation. Interestingly, we have recently demonstrated that TRAIL, another member of the death-receptor ligands, also induces non-apoptotic effects in human ESCs rather than the induction of apoptosis (Fluhr et al., 2009). At the feto-maternal interface death-receptor ligands like TRAIL and FasL might therefore play a role as regulatory molecules without mediating cell death.

Caspases are a family of cysteine proteases, which have primarily been described as executioner enzymes of apoptotic cell death (Nagata 1997). Recent investigations suggest that caspases also function as regulatory molecules in cell-fate determination and immunity (Kuranaga and Miura, 2007). In early pregnancy, the initiator caspase-8 has been shown to play a role in syncytiotrophoblast formation and the differentiation of macrophages (Kuranaga and Miura, 2007). Furthermore, the effector caspase-3 is involved in osteogenic differentiation and the differentiation of keratinocytes (Kuranaga and Miura, 2007). In our experiments, we observed that the Fas-induced up-regulation of LIF, IL-11 and IL-8 can be blocked by the inhibition of caspases, further underlining the concept of caspases as non-lethal regulators.

The transcription factor NF-κB can be activated by a variety of stimuli involving inflammatory cytokines (e.g. TNF-α), UV light or bacterial and viral products (Hayden and Ghosh, 2008). Some studies have described Fas-mediated stimulation of NF-κB. For example, Fas-stimulation of fibroblasts causes NF-κB activation (Rensing-Ehl et al., 1995; Ahn et al., 2001). In epidermal keratinocytes and colon cancer cells stimulation of Fas also leads to an induction of NF-κB (Manos and Jones 2001; Farley et al., 2006). However, in our experiments, we did not detect the induction of NF-κB in human ESCs and one might therefore speculate about other transcription factors
being responsible for the Fas-mediated effects on the transcriptional level.

In conclusion, we demonstrate for the first time caspase-dependent non-apoptotic Fas-mediated effects in human ESCs. These observations further underline the concept of the Fas/FasL-system as a signaling pathway with apoptotic as well as non-apoptotic features. At the feto-maternal interface, the Fas/FasL-system seems to enable the implanting embryo to modulate the endometrial milieu. The subtle balance between apoptotic and non-apoptotic effects might be essential for the success of early implantation. A better understanding of the physiological and pathophysiological impact of death-receptor-mediated apoptotic versus non-apoptotic signals in the human endometrium might reveal new therapeutic targets for women suffering from repeated implantation failure and habitual miscarriages.

**Authors’ roles**

H.F. designed this study, analyzed the data and wrote the manuscript. H.W. and J.S. performed experiments, analyzed the results and revised the manuscript. S.H. and J.E. assisted with experiments. M.Z. supervised this study and contributed to the writing of the manuscript.

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**Figure 4** Non-apoptotic stimulation of Fas does not induce NF-κB in ESCs. Undifferentiated (A, C, D) and decidualized (B) ESCs were incubated with 200 ng/ml activating anti-Fas antibody or 50 ng/ml TNF-α for the indicated times. The induction of NF-κB was measured by the phosphorylation (Ser536) of NF-κB p65 using an in-cell western assay (A, B) and the nuclear translocation and activation of NF-κB p65 and p50 using transcription factor assays (C, D). The specificity of the transcription factor assays was proved by the use of an excess of oligonucleotides containing either a wild-type (wt) or mutated (mu) consensus-binding site. Results are standardized relative to the values of untreated ESCs (A, B) or TNF-α-stimulated ESCs (C, D), which are set to one arbitrary unit. Bars represent mean ± SEM; *P < 0.05 compared with untreated ESCs; #P < 0.05 compared with TNF-α-stimulated ESCs (co).