Interferon-γ and tumor necrosis factor-α sensitize primarily resistant human endometrial stromal cells to Fas-mediated apoptosis

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Summary

The subtle interaction between the implanting embryo and the maternal endometrium plays a pivotal role during the process of implantation. Human endometrial stromal cells (ESCs) express Fas and the implanting trophoblast cells secrete Fas ligand (FASLG, FasL), suggesting a possible role for Fas-mediated signaling during early implantation. Here we show that ESCs are primarily resistant to Fas-mediated apoptosis independently of their state of hormonal differentiation. Pre-treatment of ESCs with interferon (IFN)-γ and tumor necrosis factor (TNF)-α sensitizes them to become apoptotic upon stimulation of Fas by an agonistic anti-Fas antibody. Incubation of ESCs with the early embryonic signal human chorionic gonadotropin (hCG, CGB) does not influence their reaction to Fas stimulation. The sensitizing effect of IFN-γ and TNF-α was accompanied by a significant upregulation of Fas and FLICE-inhibitory protein (FLIP, CFLAR) expression in ESCs. Additionally, we observed an activation of caspase 3, caspase 8 and caspase 9 upon apoptotic Fas triggering. In summary, we demonstrate that IFN-γ and TNF-α sensitize primarily apoptosis-resistant ESCs to Fas-mediated cell death. This might be due to an upregulation of Fas expression, and apoptosis seems to be mediated by active caspase 3, caspase 8 and caspase 9. The observed pro-apoptotic effect of IFN-γ and TNF-α on ESCs could play an important role in the modulation of early implantation.

Key words: Endometrium, Implantation, Apoptosis, Fas

Introduction

Implantation is the crucial step in the establishment of pregnancy. Repeated failure of implantation, recurrent miscarriage and different pregnancy-related complications, such as pre-eclampsia and intrauterine growth restriction, have been related to abnormalities in the implantation process (Norwitz, 2006). A successful implantation requires a subtle communication between the implanting embryo and the maternal endometrium (Herrler et al., 2003).

It has been shown that factors secreted by the invading trophoblast, such as human chorionic gonadotropin (hCG, CGB) and insulin-like growth factor (IGF)-II (IGF2), can modulate endometrial receptivity and the milieu at the implantation site (Giudice and Irwin, 1999; Licht et al., 1998; Fluhr et al., 2006). FasL (FASLG), typically inducing death-receptor-mediated apoptosis, is secreted by first-trimester trophoblast cells and has been assumed to play a pivotal role in promoting a state of immune privilege during implantation (Abrahams et al., 2004; Uckan et al., 1997). Fas, the receptor of FasL, is expressed in human endometrial stromal cells (ESCs), but there is no information about its role in implantation via Fas-mediated effects in these cells (Harada et al., 2004).

The interaction between FasL and Fas results in the trimerization of Fas, followed by the activation of caspase 8, and finally ends with apoptosis (Nagata, 1997). The amount of active caspase 8 determines whether a mitochondria-independent apoptosis pathway is activated or not and distinguishes between mitochondria-independent (type I) or mitochondria-dependent (type II) cells (Scaffidi et al., 1998).

The sensitivity of cells to Fas-mediated apoptosis can be regulated by different cytokines. Sensitizing effects have been shown, for example, for interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Aschkenazi et al., 2002; Igney and Krammer, 2002). Both cytokines belong to the pro-inflammatory T helper (Th)-1 family, members of which are thought to be harmful to implantation, and are secreted by decidual $CD56^{bright}$ natural killer (NK) cells, the predominant population of immune cells at the implantation site (Dosiou and Giudice, 2005). A direct inhibitor of death-receptor-mediated apoptosis is FLICE-inhibitory protein (FLIP, CFLAR), which structurally resembles caspase 8 but lacks proteolytic activity; FLIP acts downstream by inhibiting Fas apoptotic signaling (Scaffidi et al., 1999). Overexpression of FLIP renders cells resistant to Fas-mediated apoptosis, whereas its downregulation can sensitize cells to Fas signaling (Scaffidi et al., 1999).

Because human ESCs are exposed to FasL secreted by the trophoblast during early implantation, we investigated whether human ESCs become apoptotic upon stimulation of Fas. Moreover, we tested whether decidualized and undifferentiated ESCs react differently to stimulation by an activating anti-Fas monoclonal antibody. Furthermore, we wanted to examine the role of the early embryonal signal hCG and the decidual NK-cell-derived cytokines IFN-γ and TNF-α as possible modulators of apoptotic sensitivity of ESCs.

Results

ESCs are resistant to Fas-mediated apoptosis

Because FasL is secreted by the invading trophoblast during implantation, we tested whether stimulation of Fas in human ESCs induces apoptosis. Interestingly, treatment of ESCs with increasing concentrations of activating anti-Fas antibody (clone CH-11) did not result in a significant induction of apoptosis, whereas Jurkat cells became apoptotic under the same conditions (Fig. 1A,B). Treatment with mitomycin C was used as a positive control, clearly inducing apoptosis in ESCs (Fig. 1A). We further evaluated whether the observed resistance to apoptosis in ESCs is modulated after decidualization of the cells, thereby mimicking the differentiation during the luteal phase in vivo (Fluhr et al., 2006). As shown in Fig. 1C, ESCs were resistant to death-receptor-mediated apoptosis independently of their state of differentiation.

IFN-γ and TNF-α sensitize ESCs to Fas-mediated apoptosis

In a second step, we wanted to see whether the cytokines IFN-γ and TNF-α, typically secreted by decidual NK cells, as well as the early embryonal signal hCG have an influence on the sensitivity of ESCs to Fas-mediated apoptosis. Thus, we pre-treated ESCs with IFN-γ, TNF-α and hCG alone and in various combinations for 24 hours before stimulating the cells with an activating anti-Fas antibody. The treatment of undifferentiated as well as decidualized ESCs with IFN-γ and TNF-α in combination significantly increased sensitivity to Fas-mediated apoptosis (Fig. 2). The addition of hCG to these two cytokines did not significantly change their sensitizing effect on cells to Fas-mediated apoptosis. The treatment with IFN-γ, TNF-α and hCG alone or in combinations without Fas-stimulation did not induce apoptosis in a significant manner (data not shown).

Fas is expressed on ESCs but is not regulated during decidualization in vitro

The observed resistance of human ESCs to Fas-mediated apoptosis raised led us to investigate the expression level of Fas on these cells. We showed by flow cytometry that ESCs in vitro expressed Fas on their cell surface (Fig. 3A). The same result was observed in Jurkat cells (Fig. 3A), which are sensitive to Fas-mediated apoptosis. However, in contrast to Jurkat cells, the expression level (mean channel fluorescence intensity, MCFI) was markedly lower in ESCs (Fig. 3B). Because many receptors and other molecules on the cell surface are regulated in ESCs during decidualization, we tested whether the expression of Fas changes too. There was no significant difference between undifferentiated and decidualized ESCs, neither in *Fas* mRNA (Fig. 3C) nor in Fas protein (Fig. 3D,B).

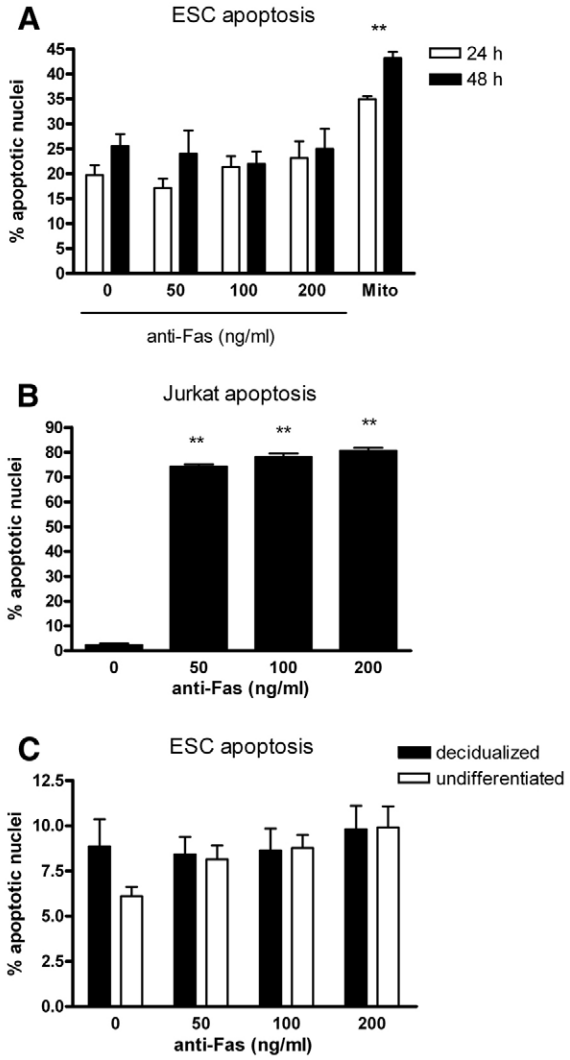

Fig. 1. Human ESCs are resistant to Fas-mediated apoptosis (in contrast to Jurkat cells) independently of their state of differentiation. (A) Human ESCs were incubated with increasing concentrations (0, 50, 100 and 200 ng/ml) of an activating anti-Fas antibody for 24 (white bars) and 48 (black bars) hours. Treatment of ESCs with 200 μg/ml mitomycin C (Mito) was used as a positive control. $^{**}P<0.01$ for both 24 and 48 hours mitomycin (Mito) treated versus the respective untreated control. (B) Jurkat cells were also incubated with increasing concentrations of an activating anti-Fas antibody for 24 hours. $^{**}P<0.01$ for treated cells versus untreated control. (C) Undifferentiated (white bars) and decidualized (black bars) human ESCs were incubated with increasing concentrations (0, 50, 100 and 200 ng/ml) of an activating anti-Fas antibody for 24 hours. The rate of apoptosis was measured by flow-cytometric detection of hypodiploid nuclei. Bars show the mean ± s.e.m.

Fas expression is upregulated in ESCs by IFN-γ and TNF-α

Because IFN-γ and TNF-α sensitized ESCs to Fas-mediated apoptosis, we tested the hypothesis that these two cytokines might upregulate Fas in ESCs. We incubated ESCs with IFN-γ, TNF-α and hCG and measured the levels of *Fas* mRNA and protein. Fig. 4 shows that the combined treatment with IFN-γ and TNF-α caused a significantly increased expression of both

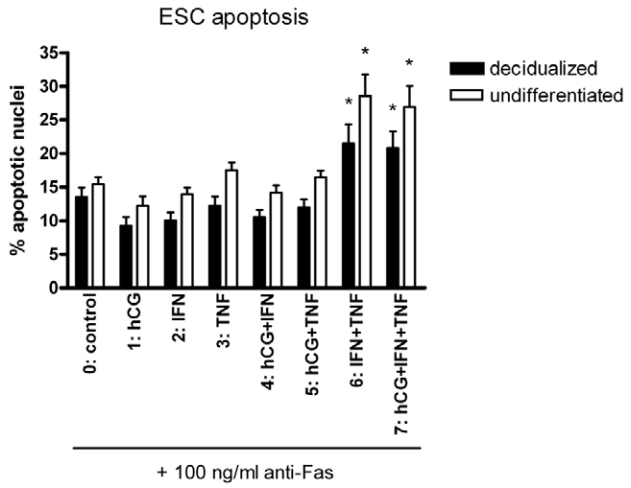

Fig. 2. IFN-γ and TNF-α sensitize human ESCs to Fas-mediated apoptosis. Decidualized (black bars) as well as undifferentiated (white bars) ESCs were treated with 50 ng/ml IFN-γ, 25 ng/ml TNF-α and 1 U/ml hCG alone and in the indicated combinations for 24 hours before stimulating the cells with 100 ng/ml of an activating anti-Fas antibody for another 24 hours. The rate of apoptosis was determined by flow-cytometric detection of hypodiploid nuclei (**P*<0.05 for both 6 and 7 versus 0-5). Bars show the mean ± s.e.m.

Fas mRNA (A) and Fas protein (Fig. 4B). All other treatments (TNF-α, IFN-γ or hCG alone as well as TNF-α + hCG or IFN-γ + hCG) had no significant influence on Fas levels in ESCs.

Caspase 8 is expressed in ESCs but is not regulated by IFN-γ, TNF-α or hCG

Because human ESCs are primarily resistant to Fas-mediated apoptosis, we further evaluated the expression of caspase 8 in these cells. As shown in Fig. 5A, caspase 8 was expressed in untreated ESCs in vitro. However, expression of caspase 8 in ESCs did not significantly change under any of the conditions shown (Fig. 5B).

Caspase 8, caspase 9 and caspase 3 are activated in ESCs upon apoptotic Fas stimulation

Using luminescence-based caspase-activity assays, we tested whether caspase 8, caspase 9 and caspase 3 are activated upon stimulation of Fas. ESCs pre-treated with IFN-γ and TNF-α for 24 hours were incubated with activating anti-Fas antibody for 1-24 hours and analyzed for caspase activity immediately thereafter. As shown in Fig. 6, there was a significant upregulation of caspase 8, caspase 9 and caspase 3 activity in ESCs, beginning after 12 hours of Fas stimulation. By contrast, cells stimulated with an activating anti-Fas antibody alone showed only weak caspase activity.

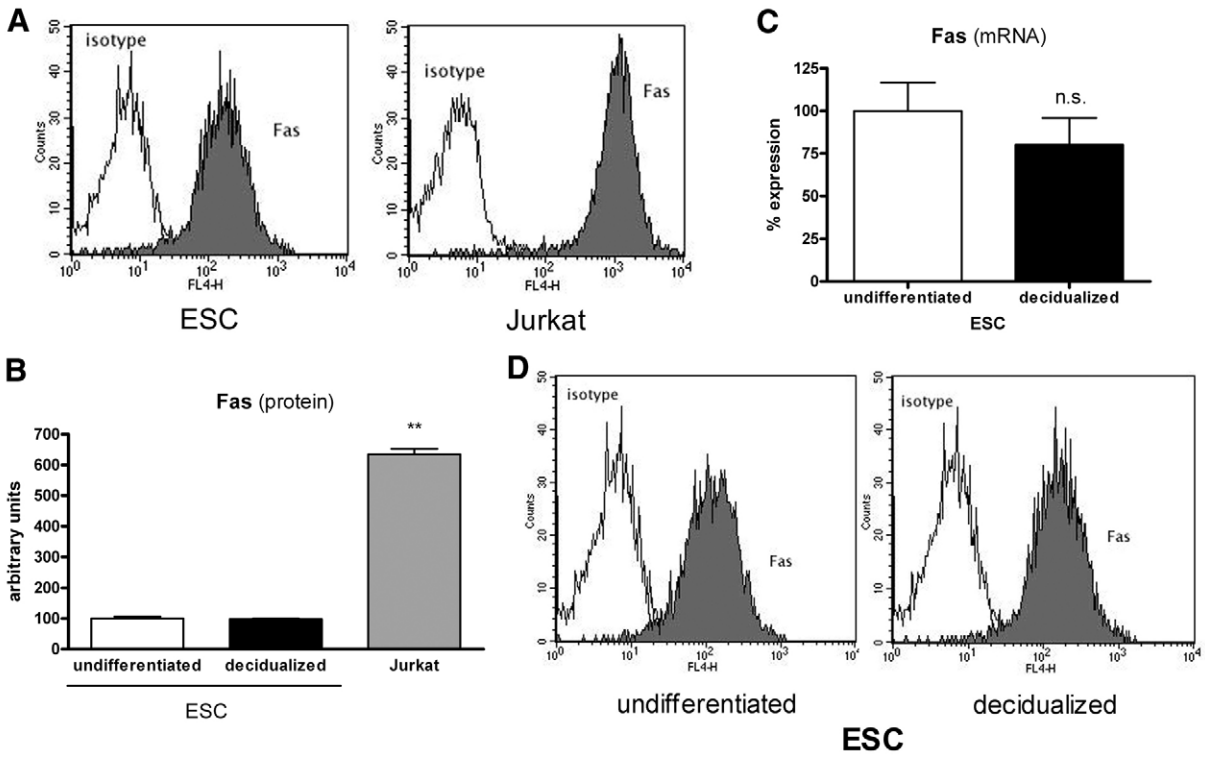

Fig. 3. Human ESCs express Fas on the cell surface at a lower level than do Jurkat cells. Fas expression does not change during decidualization in vitro. (A) Human ESCs and Jurkat cells were stained with a monoclonal anti-Fas antibody (filled histograms) and compared to cells stained with an IgG1 isotype control (white histograms) using flow cytometry. (B) The expression level of Fas as measured by MCFI. The signal derived from undifferentiated ESCs was set to 100 (***P*<0.01: Jurkat versus undifferentiated and decidualized ESCs). (C) *Fas* mRNA expression was measured by semiquantitative real-time RT-PCR to compare undifferentiated and decidualized ESCs (n.s., non significant). Bars show the mean ± s.e.m. (D) Fas protein level was determined by flow cytometry to compare undifferentiated and decidualized ESCs. Filled histograms, ESCs stained with a monoclonal anti-Fas antibody; white histograms, ESCs stained with an IgG1 isotype control.

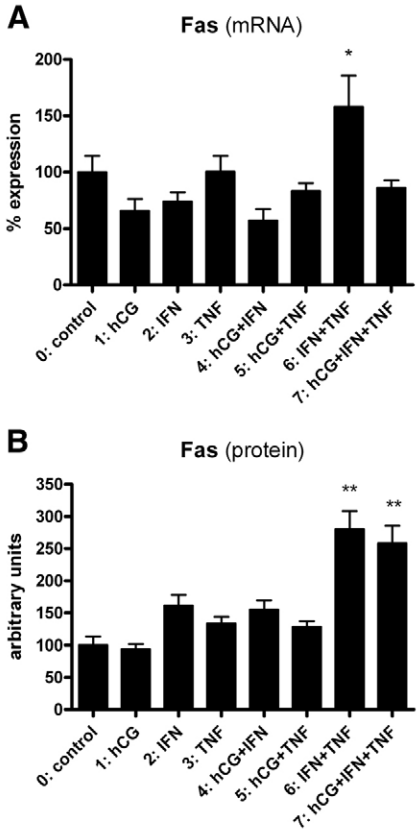

Fig. 4. IFN-γ and TNF-α cause an upregulation of Fas expression in human ESCs. Human ESCs were treated with 50 ng/ml IFN-γ, 25 ng/ml TNF-α and 1 U/ml hCG alone and in various combinations for 24 hours (mRNA) and 48 hours (protein) and were subsequently analyzed for the expression of Fas. (A) *Fas* mRNA level was determined by semiquantitative real-time RT-PCR (*P<0.05: 6 versus 0). (B) The amount of membrane-bound Fas was determined by flow cytometry (**P<0.01: each 6 and 7 versus 0-5). The expression level as measured by MCFI is shown. The signal derived from the untreated control was set to 100. Bars show the mean ± s.e.m.

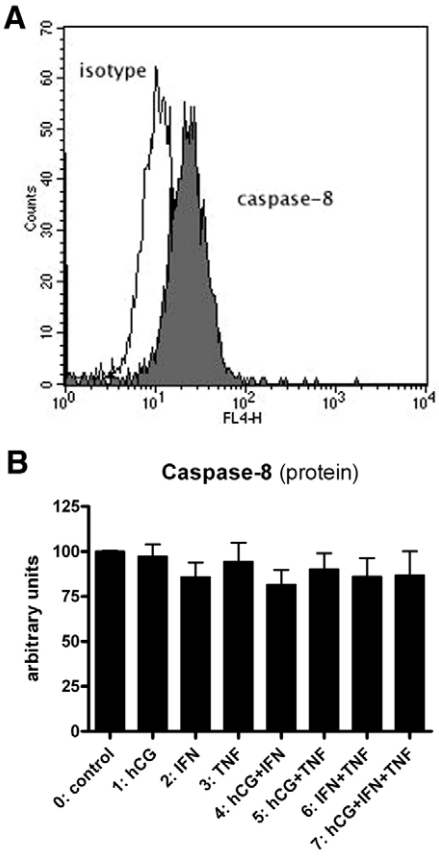

Fig. 5. Caspase 8 is expressed in human ESCs but is not regulated by IFN-γ, TNF-α or hCG. (A) ESCs were stained intracellularly to determine total caspase 8 levels and were analyzed by flow cytometry (filled histogram, caspase 8 staining; white histogram, isotype control). (B) ESCs were treated with 50 ng/ml IFN-γ, 25 ng/ml TNF-α and 1 U/ml hCG alone and in various combinations for 24 hours and were subsequently analyzed for the expression of caspase 8 by flow cytometry. The expression level as measured by MCFI is shown. The signal derived from the untreated control was set to 100. Bars show the mean ± s.e.m.

FLIP mRNA expression is upregulated in ESCs by IFN-γ and TNF-α

FLIP is known to prevent proteolytic activation of caspase 8 and thereby induces resistance to Fas-mediated apoptosis (Scaffidi et al., 1999). Therefore, we wanted to see whether FLIP is expressed in ESCs and whether it is modulated by IFN-γ, TNF-α and hCG. Fig. 7 shows that the combined treatment with IFN-γ and TNF-α caused a significantly increased expression of *FLIP* mRNA in ESCs. The addition of hCG to these two cytokines did not change the observed effect (Fig. 7). However, in the western blot, FLIP was barely detectable, showing only a weak band for the 58-kDa-long isoform of FLIP, not enabling us to perform a semiquantitative analysis (data not shown). However, in HeLa and Jurkat cells used as positive controls, we detected both isoforms (58 and 30 kDa), confirming the functionality of the applied assay (data not shown).

Discussion

In the present study, we examined the role of Fas in the induction of apoptosis at the implantation site. We demonstrated that ESCs are primarily resistant to Fas-mediated apoptosis, but can be sensitized by IFN-γ and TNF-α. Neither the state of differentiation of the cells nor the presence or absence of the early embryonal factor hCG had a significant influence on the reaction of the cells upon Fas stimulation. We also showed that human ESCs express Fas on their cell surface, and that IFN-γ and TNF-α cause a significant upregulation of this receptor. Furthermore, ESCs express caspase 8, and apoptosis of ESCs seems to be mediated by the activation of caspase 8, caspase 9 and caspase 3. The expression of FasL by the human first-trimester trophoblast (Abrahams et al., 2004) and the expression of Fas in the human endometrium (Harada et al., 2004) made us speculate about a role of the Fas-FasL system during early implantation. It has been shown that apoptosis in the epithelial layer of the endometrium seems to favor implantation and that epithelial apoptosis is in part mediated by the implanting embryo (Galan et al., 2000; von Rango et al., 1998). During the receptive phase of the endometrium ('window of implantation') there is an increasing number of apoptotic cells in the glandular epithelium. This

might be important for the establishment of an adequately prepared endometrium (von Rango et al., 1998). Furthermore, the adherence of the blastocyst to the maternal endometrium is accompanied by an apoptotic reaction of the epithelial cells (Galan et al., 2000). Taken together, apoptosis in the endometrial epithelium might be important for the trophoblast to cross the epithelial barrier and is in part mediated by the implanting embryo itself via the Fas-FasL system.

By contrast, nearly nothing is known about apoptosis in the endometrial stroma during implantation. We show here that human endometrial stromal cells do express Fas but are primarily resistant to death-receptor-mediated apoptosis, thereby sharing a typical property of some tumor and neuronal cells (Igney and Krammer, 2002). Our results on resistance to Fas are in accordance with observations by Tanaka and Umesaki, who showed that Fas does not mediate apoptotic signals in endometrial stromal cells (Tanaka and Umesaki, 2003). Furthermore, they saw a proliferative effect of Fas stimulation in combination with cAMP (Tanaka and Umesaki, 2003), suggesting non-apoptotic properties of the Fas-FasL system in ESCs. Fas signaling is mainly thought to be death inducing. However, there is increasing evidence that activation of Fas can also result in non-apoptotic responses, such as cell proliferation or induction of cytokines (Wajant et al., 2003). Mediators of inflammation and angiogenesis seem to be the main targets for non-apoptotic Fas signaling (Wajant et al., 2003). Regarding the observed resistance of ESCs to apoptotic Fas stimulation despite the presence of the Fas-FasL system at the embryo-maternal interface, it is intriguing to speculate about non-apoptotic effects mediated by the Fas-FasL system during the process of implantation.

Moreover, as reported recently, ESCs themselves produce immunoreactive FasL (Harada et al., 2004). Many factors, for example IL8, vascular endothelial growth factor (VEGF), chemokine ligand 2 (CCL2), various components of the extracellular matrix, the early embryonal signal hCG as well as the hormones progesterone and estradiol, have been shown to regulate FasL in human ESCs (Harada et al., 2004; Kayisli et al., 2003; Selam et al., 2001). So far, the expression of FasL by ESCs has been interpreted as a mechanism to regulate the population of immune cells present at the implantation site, thereby mediating immune tolerance in respect to the trophoblast. The observed resistance of ESCs to death-receptor-mediated apoptosis in our study might be necessary for these cells to protect themselves against a self-attack by secreted FasL. These findings suggest a Fas-counterattack model for ESCs, a phenomenon first described in some tumor cells (O'Connell et al., 1996). Thereby, the cells can resist Fas-

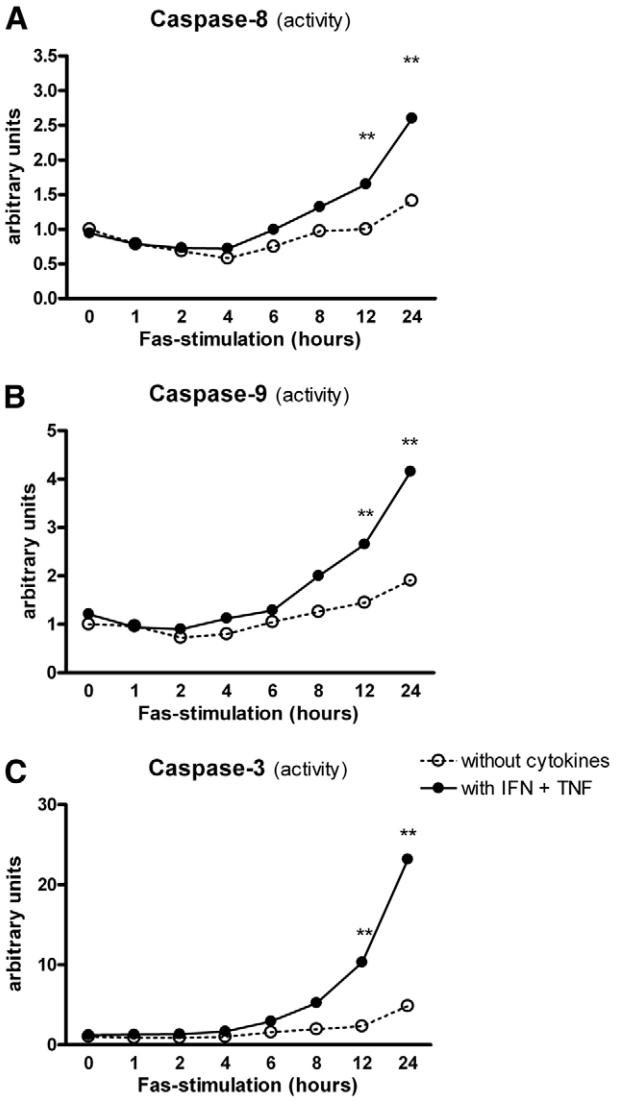

Fig. 6. Caspase 8, caspase 9 and caspase 3 are activated in human ESCs upon apoptotic Fas signaling. Human ESCs treated with 50 ng/ml IFN-γ and 25 ng/ml TNF-α for 24 hours (black dots) before stimulating the cells with 100 ng/ml of an activating anti-Fas antibody for the indicated time periods were compared to ESCs without pre-treatment with cytokines (white dots). The enzymatic activity of caspase 8 (A), caspase 9 (B) and caspase 3 (C) was measured using specific luminescent activity assays [**P<0.01: ESCs with cytokine pre-treatment versus cells without pre-treatment; values of control (0 hours) were set 1].

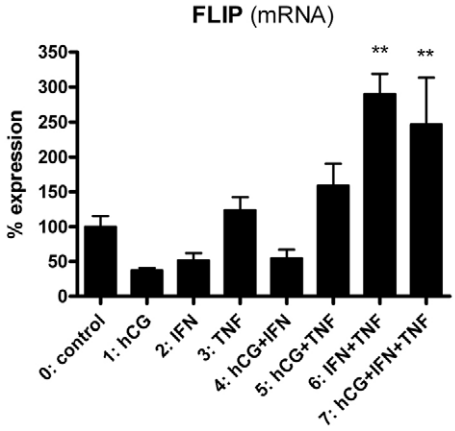

Fig. 7. *FLIP* mRNA expression is upregulated in ESCs by IFN-γ and TNF-α. Undifferentiated ESCs were treated with IFN-γ, TNF-α and hCG alone or in various combinations for 24 hours and the expression of *FLIP* mRNA was measured by semiquantitative real-time RT-PCR (**P<0.01: 6 and 7 each versus 0-5). Bars show the mean ± s.e.m.

mediated cytotoxicity but express the apoptotic signal FasL to regulate the surrounding milieu of immune cells.

Decidual CD56^{bright} CD16– NK cells are the predominant immune cell population in the human endometrium and play an important role in the processes of implantation and regulation of the endometrial milieu (Dosiou and Giudice, 2005). IFN-γ and TNF-α are cytokines of the pro-inflammatory Th1 family, and both are secreted by decidual CD56^{bright} CD16– NK cells, whereas peripheral CD56^{bright} CD16– and CD56^{dim} CD16+ NK cells do not express IFN-γ (Jokhi et al., 1994). In our study, only the combination of both cytokines (IFN-γ and TNF-α) sensitized ESCs to Fas-mediated apoptosis. This might reflect a characteristic property of decidual CD56^{bright} CD16– NK cells to modulate endometrial apoptosis by secreting these two cytokines. It has been shown that there are alterations in the CD56+ population of leukocytes in women with recurrent miscarriage (Laird et al., 2003). However, there is still a debate about the exact role of the different NK cell subpopulations in the endometrium and the early decidua during the process of implantation (Dosiou and Giudice, 2005; Jokhi et al., 1994; Laird et al., 2003). Whether apoptotic events in the endometrial stroma are necessary for successful implantation or rather hamper this process still remains open. Because contraceptive methods such as the levonorgestrel-releasing intrauterine system and the antiprogestin mifepristone (RU486) have been shown to induce apoptosis in endometrial cells, it is possible to speculate a role of endometrial apoptosis in disturbing implantation (Han and Sidell, 2003; Maruo et al., 2001). By contrast, HLA-G, the characteristic type of human lymphocyte antigen (HLA) in trophoblast cells, promotes IFN-γ and TNF-α production by decidual NK cells (van der Mer et al., 2007). Therefore, the role of endometrial apoptosis in the stromal compartment might be very localized and effective within a restricted time-frame.

HCG is one of the first embryonic signals sent to the maternal endometrium during early implantation (Lopata and Oliva, 1993). Direct effects of hCG on the trophoblast, the endometrium and endometrial vessels in vivo and in vitro have been shown previously (Fluhr et al., 2006; Licht et al., 1998; Licht et al., 2001; Fazleabas et al., 1999; Zygmunt et al., 2002; Zygmunt et al., 2005). In the study presented here, we did not see any effect of hCG on the sensitivity of human ESCs to Fas-mediated apoptosis. However, an indirect role of hCG in the regulation of endometrial apoptosis by the recruitment of NK cells at the implantation site can be postulated. An increased number of these cells would then provide a source for higher cytokine levels to promote apoptosis. Although Jasinska et al. recently reported an anti-apoptotic effect of hCG and of decidualization itself on ESCs treated with cytochalasin-D (Jasinska et al., 2006), this protecting effect was not seen for Fas-mediated apoptosis in our experimental setting. These different findings can be explained by a selective modulation of the intrinsic (activated by cytochalasin-D) and extrinsic (death-receptor-mediated) apoptotic pathways. Our observation that both decidualized and undifferentiated ESCs are resistant to apoptosis suggests that this might be a basic attribute of ESCs, independent of their hormone-driven differentiation in the luteal phase.

Several mechanisms have been described that make cells resistant to death-receptor-mediated apoptosis (Igney and Krammer, 2002). Our observations of a low expression level of Fas on ESCs and its upregulation by the two sensitizing cytokines IFN-γ and TNF-α suggest that the amount of cell-surface-expressed Fas is a determinant for sensitivity in this cell type. Interestingly, the response of trophoblast cells to Fas-mediated apoptosis is also in part regulated in this manner (Aschkenazi et al., 2002). However, we observed different results of Fas regulation at the mRNA and protein level. The addition of hCG to IFN-γ and TNF-α did not change the effect of the two cytokines on Fas protein expression. The missing response of *Fas* mRNA to combined treatment with all three substances might be due to a time-dependent regulatory effect of hCG: a possible early upregulation induced by IFN-γ and TNF-α might further be antagonized by hCG on the transcriptional level and consequently be visible on the protein level.

Caspase 8 is the key enzyme that transmits apoptotic Fas signals to the intracellular apoptosis pathway (Nagata, 1997). A lack of caspase 8 has been observed as a mechanism of cells to become resistant to Fas-mediated apoptosis (Igney and Krammer, 2002). In our experiments, we could clearly detect caspase 8 expression in ESCs and additionally its activation upon apoptotic Fas signaling after pre-treatment with IFN-γ and TNF-α. Furthermore, we observed an activation of caspase 9 and caspase 3 upon apoptotic Fas signaling in human ESCs. The observation of the occurrence of Fas-mediated apoptosis with a detectable activation of caspase 9 might be explained by the use of a mitochondria-dependent pathway (type II cells) (Scaffidi et al., 1998). This theory is further supported by data from Abe and co-workers, who recently showed an activation of the death-receptor-initiated and mitochondrial-mediated apoptotic pathway in the endometrial cell line HHUA (Abe et al., 2006). By contrast, there also might be a caspase-independent apoptotic pathway in human ESCs that is activated upon Fas signaling mediated by Fas death-associated protein 6 (DAXX) or receptor-interacting protein (RIP, RIPK5). DAXX is another Fas-interacting protein that has been shown to induce apoptosis (Curtin and Cotter, 2003). DAXX binds to the death domain of the Fas receptor and can activate the JNK (MAPK) kinase cascade independently of caspase 8 activation by recruiting and activating the upstream kinase ASK1 (MAP3K5) (Chang et al., 1998; Ko et al., 2001). Activation of JNK was found to accelerate Fas-mediated apoptosis in various cell lines (Curtin and Cotter, 2003). RIP constitutes the second enzyme, along with caspase 8, which is a direct downstream mediator of Fas-triggered cell death (Curtin and Cotter, 2003). It has been shown that RIP plays an essential role in Fas-ligand-induced caspase-independent apoptosis in T cells (Holler et al., 2000). Further investigations are in progress to clarify in detail the apoptotic pathway(s) activated and regulated in human ESCs.

Downstream of Fas-FasL interaction on the cell surface, FLIP is one of the first and central inhibitors of death-receptor-mediated apoptosis (Scaffidi et al., 1999). The long as well as the short isoform of FLIP can inhibit procaspase 8 processing and thereby interfere with Fas-mediated apoptosis (Scaffidi et al., 1999). We found FLIP to be expressed in ESCs and observed an upregulation of *FLIP* mRNA upon stimulation with IFN-γ and TNF-α, although this treatment sensitized the cells to Fas-mediated apoptosis. Because we could not detect

any significant amount of FLIP protein, the enhanced mRNA expression might be interpreted as a regulatory effect limited on the transcriptional level, which has no direct effect on protein expression.

In conclusion, we demonstrate that human ESCs are primarily resistant to Fas-mediated apoptosis but can be sensitized to it by the two decidual NK-cell-derived cytokines IFN-γ and TNF-α. Interestingly, hCG, one of the first signals of the embryo sent to the maternal endometrium, has no influence on the sensitivity of ESCs to Fas-mediated apoptosis. Our data suggest that modulation of apoptosis in endometrial stromal cells might play a role during early implantation. A better understanding of apoptotic and anti-apoptotic factors in the human endometrium can reveal new therapeutic targets for the development of strategies to improve implantation rates in infertile patients and to reduce the risk of miscarriage in patients suffering from habitual abortions.

Materials and Methods

Materials

An activating anti-Fas monoclonal antibody (clone CH-11) was obtained from MBL (Woburn, MA, USA). Human recombinant hCG, TNF-α and IFN-γ were obtained from Sigma (Taufkirchen, Germany), Biosource (Camarillo, CA, USA) and R&D Systems (Wiesbaden, Germany).

Cell culture

Endometrium was obtained from pre-menopausal women undergoing hysterectomy for benign reasons. Informed consent was given by each patient after ethical approval from the institutional ethical board of the University of Tübingen, Germany. Minced endometrial tissue was digested by incubation with collagenase (Biochrom, Berlin, Germany). The dispersed endometrial cells were separated by filtration through a 180 μm membrane and a 40 μm nylon sieve. ESCs were maintained in DMEM/F-12 without phenol red containing 10% charcoal-stripped FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). For all experiments, ESCs were passaged only once. The purity of ESCs cultures was tested by flow-cytometric analysis of intracellular vimentin expression. Jurkat and HeLa cells were cultured following standard procedures.

Decidualization in vitro

ESCs were cultured for 9 days in the presence of 1 μM progesterone and 30 nM 17β-estradiol (both from Sigma). Decidualization was demonstrated by measuring an increase of IGFBP1 and prolactin as described recently (Fluhr et al., 2006).

Measurement of apoptosis

For determination of apoptosis, cells were treated with the respective agents and the rate of apoptotic nuclei was measured by the method of Nicoletti et al. (Nicoletti et al., 1991). Apoptotic nuclei were prepared by lysing the cells in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100) containing 50 μg/ml propidiumiodide and subsequently analyzed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic. Analyses were performed on a FACSCalibur cytometer using Cell Quest Pro software (BD Biosciences, Heidelberg, Germany).

Semiquantitative real-time RT-PCR

Total RNA was isolated from ESCs using PeqGOLD Trifast (PeqLab, Erlangen, Germany) and reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Semiquantitative real-time PCR was performed to quantify mRNA levels of *Fas* and *FLIP* in relation to the housekeeping gene β-actin. cDNA samples were amplified using 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: Fas forward, 5′-GGACC-CTCCTACCTCTGGTTCTTA-3′; Fas reverse, 5′-TTGGAGTTGATGTCAGT-CACTTGG-3′; FLIP forward, 5′-GTTCTCCAAGCAGCAATCCAAA-3′; FLIP reverse, 5′-TCCCATTATGGAGCCTGAAGTT-3′; β-actin forward, 5′-CCTGG-CACCCAGCCAAT-3′; β-actin reverse, 5′-GCCGATCCACACGGAGTACT-3′. PCR amplification was performed in duplicate in an ABI Prism 7000 sequence detector (Applied Biosystems) using the following cycling program: 2 minutes at 50°C, 10 minutes at 95°C, followed by 15 seconds at 95°C and 1 minute at 60°C for a total of 40 cycles. PCR products were analyzed by thermal dissociation to verify that a single specific PCR product had been amplified. Relative expression levels of *Fas* and *FLIP* in relation to β-actin were determined using the mathematical model: ratio=$2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Flow cytometry

The expression of Fas on the surface of ESCs was determined by extracellular staining with an anti-Fas mAb (Bender MedSystems, Vienna, Austria) and an Alexa-Fluor-488-conjugated secondary antibody (Invitrogen). For intracellular staining of total caspase 8 (specific antibody from Cell Signaling Technology, Beverly, MA, USA), cells were permeabilized and fixed using FACS Permeabilizing Solution and CellFIX (both from BD Biosciences) following a standard protocol. Samples were analyzed on a FACSCalibur cytometer using Cell Quest Pro software (BD Biosciences). Each staining sample was accompanied by a parallel staining with an unspecific isotype control.

Measurement of caspase activity

The activity of caspase 8, caspase 9 and caspase 3 was analyzed using the Caspase-Glo assay system (Promega, Madison, WI, USA). After treating the cells with the respective agents, Caspase-Glo-8, -9, or -3 reagent was added and cells were incubated for 30 minutes. Luminescence was recorded using the FLUOstar OPTIMA system (BMG Labtech, Offenburg, Germany).

Western blot

Cells were lysed and total protein was resolved on 12% SDS-polyacrylamide gels for detection of FLIP. Proteins were transferred to a PVDF membrane (Millipore) and blocked with 5% milk powder in PBS/Tween. Blots were probed with anti-FLIP antibody (Cell Signaling Technology), followed by IRDye-680-conjugated secondary antibody (LI-COR, Lincoln, NE, USA) and visualized using the LI-COR Odyssey imaging system.

Statistics

Each experiment was performed in triplicate or quadruplicate on cell cultures derived from three to five different patients. For all experiments, ESCs from a single separation were used. Statistical-data analysis was made with one-way ANOVA, followed by Dunnett's and Bonferroni multiple comparison tests using GraphPad PRISM version 4 software (GraphPad, San Diego, CA, USA). The results are expressed as mean ± s.e.m.

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