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# Regulation of Fas ligand expression by vascular endothelial growth factor in endometrial stromal cells *in vitro*

**M.Berkkanoglu[1], O.Guzeloglu-Kayisli[1,2], U.A.Kayisli[1,3], B.F.Selam[1] and A.Arici[1,4]**

[1]Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06520, USA and Departments of [2]Medical Biology and Genetics and [3]Histology and Embryology, Akdeniz University School of Medicine, Antalya, Turkey, 07070

[4]To whom correspondence should be addressed. E-mail: aydin.arici@yale.edu

**When Fas ligand (FasL) interacts with the Fas receptor, it induces apoptosis through autocrine and/or paracrine signalling. Vascular endothelial growth factor (VEGF) is a potent mitogenic cytokine. VEGF plays a role during remodelling of the endometrium following menstruation. We hypothesized that, by regulating FasL expression, VEGF may play a role in endometrial stromal cell survival by decreasing autocrine apoptotic signalling. We aimed to determine the expression of FasL in cultured endometrial stromal cells and its modulation by VEGF. VEGF induced a decrease in both FasL-positive cell number and FasL intensity as determined by immunocytochemistry and western blot respectively ($P < 0.05$). These effects of VEGF were observed in a concentration-dependent manner (10–42%; $P < 0.05$). Anti-VEGF neutralizing antibody alone resulted in an increase in the FasL expression. When combined with VEGF, anti-VEGF reversed the VEGF-induced decrease in FasL level up to 100% ($P < 0.05$). In addition, western blot analysis showed that FasL expression in endometrial stromal cells demonstrated a cyclic change every 12 h during 48 h of incubation. These results suggest that down-regulation of FasL by VEGF may affect endometrial stromal cell survival in an autocrine or paracrine manner. The decrease in FasL level may be due to a stimulation of its degradation. Our results show that FasL in endometrial stromal cells in culture has a cyclic expression model, suggesting that there may be a regulation at the translation level.**

*Key words:* apoptosis/chemokines/cytokines/endometrium/growth factors

## Introduction

Apoptosis, programmed cell death, is an essential regulator of cell turnover in human endometrium. Regulation of apoptosis involves interaction of several genes that have stimulatory or inhibitory effects on cell death. Fas ligand (FasL), a mediator of apoptosis in differentiated cells and embryonic development, interacts with its receptor Fas and induces apoptosis through autocrine or paracrine signalling. FasL is a 37 kDa protein that belongs to the tumour necrosis factor (TNF) superfamily. Fas (CD95) is a 45 kDa type I membrane protein that belongs to the TNF/nerve growth factor receptor family (Suda *et al*., 1993; Nagata and Golstein, 1995). It has been previously demonstrated that endometrial stromal and glandular cells express both FasL and its receptor, Fas (Selam *et al*., 2001).

Vascular endothelial growth factor (VEGF) is a potent mitogenic cytokine. It increases cell survival and induces angiogenesis, which is an essential component of endometrial regeneration after menses in preparation for embryo implantation. VEGF is expressed in endometrial glandular, stromal and endothelial cells (Li *et al*., 1994; Sugino *et al*., 2002). Its expression is stimulated by estrogen and progesterone (Huang *et al*., 1998; Sugino *et al*., 2002). Besides being a potent angiogenic factor, VEGF may also have a direct proliferative or anti-apoptotic effect on stromal cells through autocrine or paracrine signalling. The effect of VEGF on apoptosis by way of the Fas/FasL system in human endometrial cells has thus far not been investigated. We hypothesized that by regulating FasL expression, VEGF may play a role in endometrial stromal cell survival by decreasing the pro-apoptotic signalling. To test our hypothesis, we studied the regulation of FasL expression and FasL-mediated apoptosis by VEGF in endometrial stromal cells in culture.

## Materials and methods

### *Tissue collection*

Endometrial tissue was obtained from human uteri after diagnostic laparoscopy or hysterectomy conducted for benign diseases. Informed consent in writing was obtained from each patient before surgery; consent forms and protocols were approved by the Human Investigation Committee of Yale University. The mean age of the patients was 37.2 (range 32–49) years. Tissues were placed in Hanks' balanced salt solution and transported to the laboratory for separation and culture of endometrial cells. Cells obtained from each patient were considered as separate experiments. Each experimental set-up was repeated on at least three occasions using cells obtained from different patients.

### *Isolation and culture of human endometrial stromal cells*

Endometrial stromal cells were separated and maintained in monolayer culture, as described previously (Arici *et al*., 1993; Selam *et al*., 2001). Human endometrial stromal cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Prior to experiments, cells were incubated with Phenol Red- and serum-free DMEM for 24 h. Cells were treated with VEGF (0.1–10 ng/ml; R&D Systems, USA) or vehicle (control), and with anti-VEGF neutralizing antibody (R&D Systems) alone or combined with VEGF for 24–72 h to evaluate concentration- and time-dependent effect of VEGF. Basal FasL expression was also studied by culturing endometrial stromal cells for 4–48 h sampled every 4 h.

### *FasL western blot analysis*

Protein extraction and western blot analysis were performed in cultured endometrial stromal cells as described previously (Selam *et al*., 2001). Incubation with mouse anti-human FasL monoclonal antibody (Transduction Laboratories, Inc., USA) diluted at 1:1000 was performed for 1 h at room temperature and thereafter washed with Tris-buffered saline [TBS-Tween-20

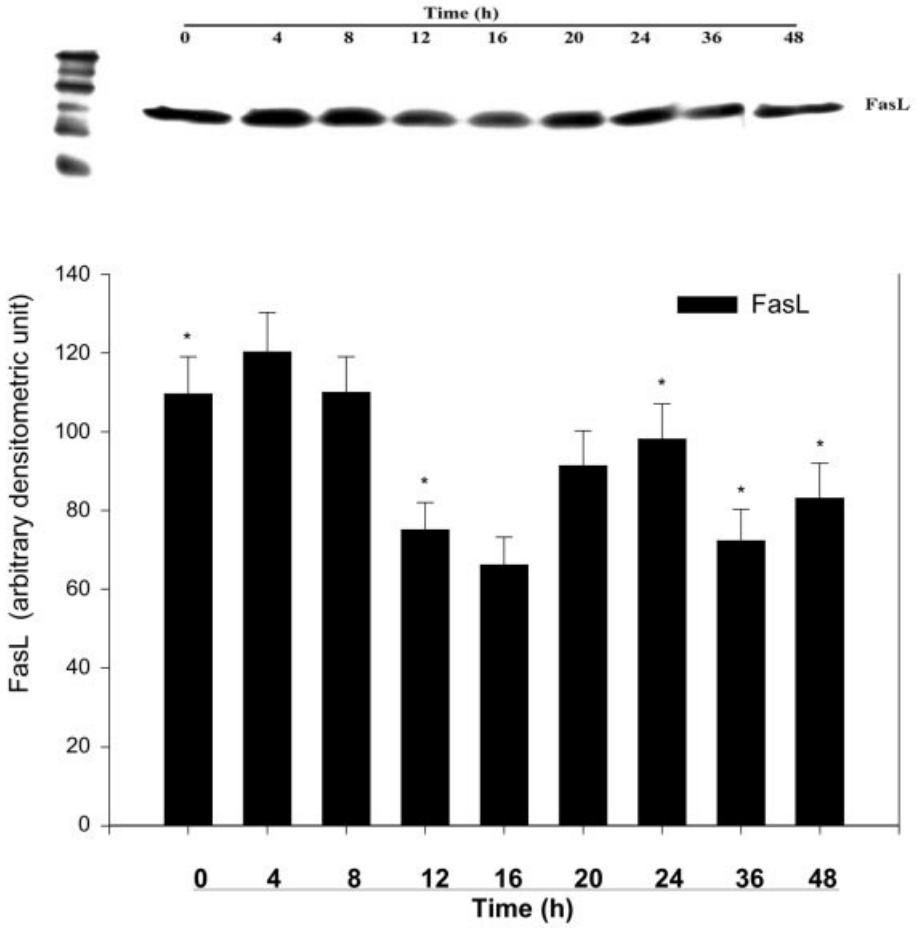


**Figure 1.** Western blot analysis of FasL expression in endometrial stromal cells in culture. The FasL expression in endometrial stromal cells demonstrated a cyclic change every 12 h during 48 h of incubation. There was a statistically significant decrease between 0 and 12 h and an increase between 12 and 24 h and again a decrease between 24 and 36 h, and an increase between 36 and 48 h ($P < 0.05$).

(TBS-T)]. The membrane was further incubated for 1 h with peroxidase-labelled anti-mouse IgG (Vector Laboratories, USA) diluted at 1:10 000. The immunoblot was developed using a chemiluminescent kit (NEN Life Science Products, USA). After stripping the membrane with western blot stripping buffer (Pierce, USA), the membrane was washed with TBS-T and blocked with 5% non-fat dry milk in TBS-T for 1 h. It was later incubated for 1 h with mouse anti-human glyceraldehyde phosphate dehydrogenase (GAPDH) monoclonal antibody (Santa Cruz Biotechnology, USA) diluted at 1: 30 000 to confirm equal loading of proteins in each lane. Thereafter, the same protocol with FasL immunoblot was carried out to develop the GAPDH bands. FasL expression was then normalized by dividing the arbitrary densitometry units for FasL by those for GAPDH for each band. Similar experiments were conducted on at least three different occasions with cells prepared from three different endometrial tissues

### *Immunocytochemistry*

Endometrial stromal cells were grown to preconfluence on 8-chamber slides (Falcon, USA). Following treatments, immunocytochemistry was performed as described previously (Selam *et al*., 2001). Immunocytochemical staining intensity was ranked from 0 (absent) to 3 (most intense). For each slide, an H-score was derived by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of the staining [H-score = $P_i(i + 1)$, where $i$ is the intensity scores and $P_i$ is the corresponding percentage of the cells]. In each slide, five different areas were evaluated under the microscope with ×50 original magnification, and two investigators blinded to treatments determined the percentage of the cells for each intensity within these areas. Average scoring of the investigators was used.

### *FasL RT–PCR*

Total RNA was extracted by Trizol Reagent (Gibco BRL) according to the manufacturer's instructions. Two micrograms of sample was reverse-transcribed in 20 µl of reaction mixture containing 10 mmol/l each of dATP, dCTP, dGTP and dTTP; 20 pmol oligo (dT); 40 IU/µl ribonuclease inhibitor, 10 IU/µl avian myeloblastosis virus–reverse transcriptase and buffer (95°C, 5 min; 42°C, 60 min: Perkin–Elmer model 9600 thermocycler, USA). RT–PCR was performed using primers specific for FasL as described previously (Selam *et al*., 2002; sense: 5′-ACACCTATGGAATTGTCCTGC-3′; antisense: 5′-GACCAGAGAGAGCTCAGATACG-3′) yielding 311 bp reaction product. Primers for actin were used to control for RT–PCR efficiency (sense: 5′-TTGCTGATCCACATCTGCTG-3′; antisense 5′-GACAGGATGCAGAAGGAGAT-3′) yielding a 120 bp fragment. PCR products and molecular weight markers were separated on agarose gels containing ethidium bromide (10 mg/ml) and visualized by UV light. The intensity of each band was normalized to its corresponding actin band to semiquantitatively compare values between samples.

### *Detection of apoptotic cells with DAPI staining*

Endometrial stromal cells were grown to preconfluence on 8-chamber slides. Following treatments, the cells were fixed with cold 4% paraformaldehyde for 20 min at +4°C and then washed twice with phosphate-buffered saline (PBS). They were stained with 2 pg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma) for 30 min. The slides were washed three times with PBS and examined under a fluorescence microscope.

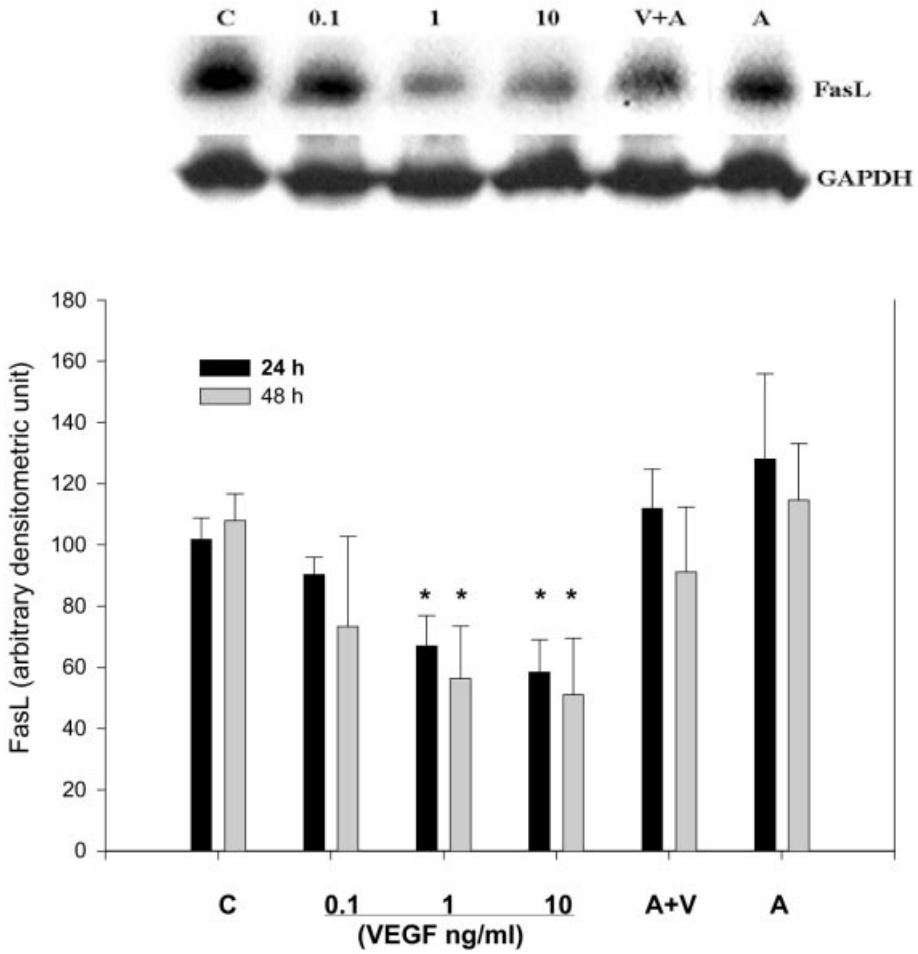


**Figure 2.** FasL protein expression in endometrial stromal cells: concentration- and time-dependent effect of vascular endothelial growth factor (VEGF). Cultured cells were treated with 0.1, 1 and 10 ng/ml VEGF for 24 and 48 h. Total protein was extracted and FasL protein expression was analysed by western blot ($n$ = 3). Bars represent mean ± SEM; $P < 0.05$ control versus 1 and 10 ng/ml VEGF. C: control; V+A: 1 ng/ml VEGF and 1 µg/ml anti-VEGF antibody; A: 1 µg/ml anti-VEGF antibody. The western blot for 24 h treatment is shown above the graph.

### *Cell proliferation assay*

Following treatments, cell proliferation was determined by a colorimetric assay using MTT [3-(4,5-dimethythiazol-2-yl-)-2,5-diphenyl tetrazolium bromide]. MTT is a water-soluble tetrazolium salt that yields a yellowish solution when prepared in media or salt solutions that lack Phenol Red. Dissolved MTT is converted to the coloured product formazan in active mitochondria, and then can be solubilized using acid–isopropanol mixture (1 N HCI: isopropanol 4:96 vol/vol). Optical density reading at 570 nm is directly proportional to the number of cells. The first row of each 96-well plate did not contain any cells and was used as a blank. Four hours before the end of each experiment, MTT solution was added into all wells (10 µl/100 µl medium per well), and plates were incubated at 37°C. At the end of the incubation period and after removal of the medium, acid–isopropanol mixture was added into each well (100 µl/well), and plates were read within 30 min with a multiwell plate reader (Thermomax; Molecular Devices Corp., USA). Data were expressed in optical density units. Experiments were conducted with replicates of 12 wells per treatment condition. Similar experiments were conducted on at least three different occasions with cells prepared from three different endometrial tissues.

### *Statistical analysis*

Data for densitometry of FasL protein, FasL mRNA, immunocytochemistry and DAPI scores were normally distributed (as determined by Kolmogorov–Smirnov test). Thus, analysis of variance (ANOVA) and *post hoc* Tukey test for pairwise multiple comparisons were used for statistical analysis. Data from MTT cell proliferation were not normally distributed. Therefore, they were analysed with non-parametric ANOVA by ranks (Kruskal–Wallis test). $P < 0.05$ was considered to be significant. Statistical calculations were performed using Sigmastat for Windows, version 2.0 (Jandel Scientific Corp., USA). Student's $t$-test was used for statistical analysis.

## Results

### *Endometrial stromal cells have cyclic FasL expression in culture*

To evaluate the basal FasL expression, endometrial stromal cells were cultured for 4–48 h and sampled every 4 h. The FasL expression in endometrial stromal cells demonstrated a cyclic change every 12 h during 48 h of incubation. A gradual decrease during the first 12 h was followed by a progressive increase during the next 12 h corresponding to a cyclic rhythm in FasL expression (Figure 1). The expression of the cyclic FasL was found to be statistically significant between 0 and 12 h and after every 12 h ($P < 0.05$). On the other hand, no significant change was detected between the subsequent time periods such as between 4 and 8 h.

### *Regulation of FasL protein expression by VEGF in endometrial cells in culture*

To investigate the regulation of FasL protein expression by VEGF, endometrial stromal cells were incubated with various concentrations of VEGF (0.1–10 ng/ml) or vehicle (control), and with anti-VEGF

neutralizing antibody alone (1 μg/ml) or combined with VEGF for 24–48 h. VEGF induced a decrease in the FasL expression in a concentration- and time-dependent manner. VEGF treatments, 0.1, 1 and 10 ng/ml, induced 10, 33 and 42% decrease in FasL protein levels in 24 h treatment group, and also 32, 49 and 53% decrease in 48 h treatment group respectively ($P < 0.05$ between control versus 1 and 10 ng/ml VEGF concentrations) (Figure 2). Anti-VEGF neutralizing antibody did not affect FasL expression, but when combined with VEGF, anti-VEGF reversed the VEGF-induced decrease in FasL level up to 100% for 24 h and 81% for 48 h ($P < 0.05$).

Endometrial stromal cells plated on chamber slides were incubated with various concentrations of VEGF (1 and 10 ng/ml) or vehicle (control) for 24 and 48 h, and cells were analysed by immunocytochemistry. Similar to western blot results, VEGF induced a decrease in both FasL-positive cell number and FasL intensity in endometrial stromal cells, compared with vehicle ($P < 0.05$) (Figure 3, top panel).

### *Regulation of FasL mRNA expression by VEGF in endometrial cells in culture*

To evaluate whether the down-regulatory effect of VEGF on FasL protein expression was secondary to an increase in FasL mRNA levels, we measured FasL mRNA levels by RT–PCR analysis. Endometrial stromal cells were incubated with various concentrations of VEGF (1–10 ng/ml) for 2 and 4 h. VEGF did not induce any concentration- or time-dependent alterations in FasL mRNA expression in human endometrial stromal cells (Figure 4).

### *Effect of VEGF on endometrial cell apoptosis*

Endometrial stromal cells plated on tissue chamber slides were incubated with different concentrations of VEGF (0.1–10 ng/ml) for 24 and 48 h, and cells were analysed by DAPI staining for apoptosis. VEGF treatments at 0.1, 1 and 10 ng/ml concentrations, resulted in 42, 61 and 59% decrease at 24 h, and 52, 48 and 67% decrease at 48 h respectively, in the ratio of apoptotic cells, compared with untreated cells ($P < 0.05$ control versus 1 and 10 ng/ml VEGF at 24 h, and control versus all VEGF concentrations at 48 h) (Figure 3, bottom panel and Figure 5).

### *Effect of VEGF on endometrial cell proliferation*

Effect of VEGF on endometrial stromal cell proliferation was assessed using the MTT colorimetric assay. Endometrial stromal cells were treated with various concentrations of VEGF (0.1–10 ng/ml) for 24 h. All VEGF concentrations induced 22–33% increase in endometrial stromal cell proliferation ($P < 0.05$ between control and all VEGF concentrations) (Figure 6).

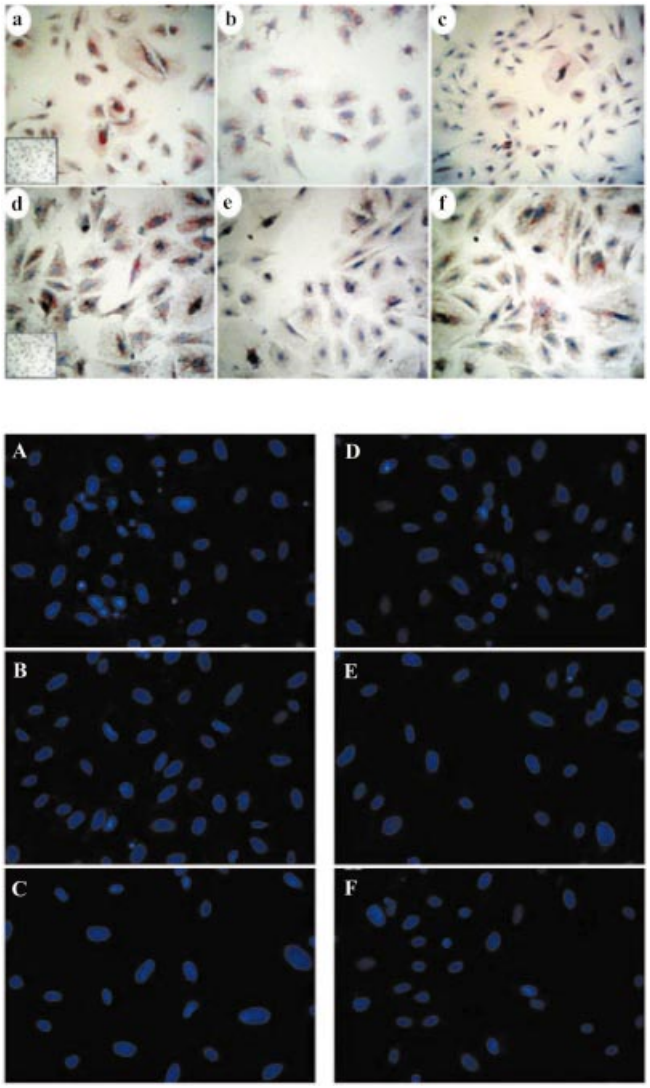


**Figure 3.** Top panel: FasL expression in endometrial stromal cells in culture. Endometrial stromal cells plated in chamber slides were incubated with vehicle only (control) or vascular endothelial growth factor (VEGF) (1, 10 ng/ml) for 24 and 48 h. Untreated cells (control) (**a**), cells treated with VEGF (1 ng/ml) (**b**) and treated with VEGF (10 ng/ml) (**c**) for 24 h. Untreated cells (control) (**d**), cells treated with VEGF (1 ng/ml) (**e**) and treated with VEGF (10 ng/ml) (**f**) for 48 h. Normal mouse IgG was used instead of primary antibody (insert **a** and **d**). Bottom panel: 4,6-diamidino-2-phenylindole staining for the assessment of apoptosis in endometrial stromal cells in culture. Endometrial stromal cells plated in chamber slides were incubated with vehicle only (control) or different concentrations of VEGF (0.1, 1, 10 ng/ml) for 24 and 48 h. Untreated cells (control) (**A**), treated with VEGF (1 ng/ml) (**B**), and treated with VEGF (10 ng/ml) (**C**) for 24 h. Untreated cells (control) (**D**), treated with VEGF (1 ng/ml) (**E**), treated with VEGF (10 ng/ml) (**F**) for 48 h ($P < 0.05$ between control versus 1 and 10 ng/ml VEGF for 24 h, and between control and all VEGF concentrations for 48 h).

## Discussion

Apoptosis is an essential regulator of cell turnover in human endometrium. The Fas–FasL interaction is one of the crucial events in the induction of apoptosis (Itoh *et al*., 1991; Suda *et al*., 1993). We have previously demonstrated that endometrial stromal and glandular cells express both FasL and its receptor, Fas (Selam *et al*., 2001). FasL expression in endometrial stromal cells is regulated by many factors including steroid hormones, interleukin-8 and macrophage-derived growth factors (Garcia-Velasco *et al*., 1999; Selam *et al*., 2001, 2002).

Cytokines and growth factors are proteins with paracrine and autocrine effects on cell growth and differentiation. VEGF is a major angiogenic protein that has the potential to play a dynamic role in endometrial remodelling. It has been demonstrated that VEGF mRNA and protein are localized in both glandular epithelium and stroma (Charnock-Jones *et al*., 1993; Shifren *et al*., 1996). Furthermore, it was found that both membrane receptors of VEGF, KDR and flt1, and soluble form of flt1 mRNA were detected in isolated endometrial epithelial and endometrial stromal cells (Krussel *et al*., 1999). In another study, strong KDR immunostaining was observed in glandular epithelial cells and in decidualized stromal cells, whereas flt-1 immunostaining was negligible (Sugino *et al*., 2002). In addition, the same study showed that the expression of VEGF and KDR mRNA was increased significantly by estrogen and medroxyprogesterone acetate

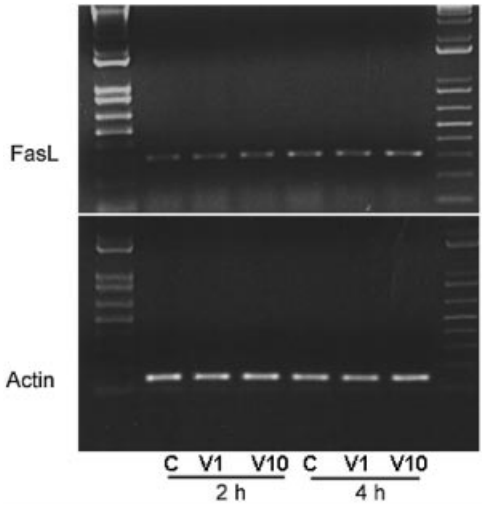


**Figure 4.** Effect of vascular endothelial growth factor (VEGF) on FasL mRNA expression in endometrial stromal cells in culture. Cells were treated with VEGF (1–10 ng/ml) for 2 and 4 h. Total RNA was extracted, and FasL mRNA expressions were analysed by RT–PCR. VEGF did not induce any concentration- or time-dependent alterations in FasL mRNA expression.

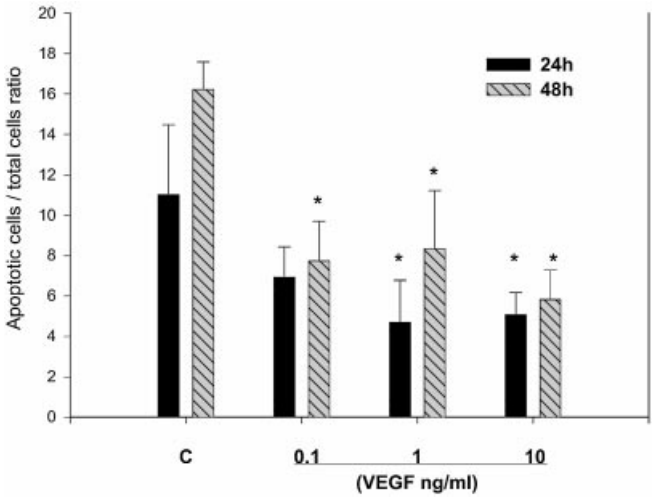


**Figure 5.** 4,6-Diamidino-2-phenylindole staining for the assessment of apoptosis in endometrial stromal cells in culture. Endometrial stromal cells plated in chamber slides were incubated with vehicle only (control) or different concentrations of vascular endothelial growth factor (VEGF) (0.1, 1, 10 ng/ml) for 24 and 48 h. Bars represent mean ± SEM; $P < 0.05$ between control versus 1 and 10 ng/ml VEGF for 24 h, and between control and all VEGF concentrations for 48 h.

during decidualization. VEGF mRNA expression in endometrium increases during the late secretory phase and reaches its maximum levels at menses (Charnock-Jones *et al*., 1993; Shifren *et al*., 1996). This may lead to endometrial shedding. However, in the presence of pregnancy the predecidualized stromal cells need to survive, especially during decidualization when the expression of KDR receptor increases significantly. Thus VEGF–KDR interaction may regulate the cell survival by affecting FasL level. In other words, the increase in VEGF during mid- to late-secretory phase may be involved in the prolongation of the lifespan of endometrial cells for successful pregnancy by decreasing FasL expression in endometrial stromal cells.

Hypoxia can induce VEGF in human endometrial stromal cells, which supports a role for this mitogen in peri-menstrual endometrium

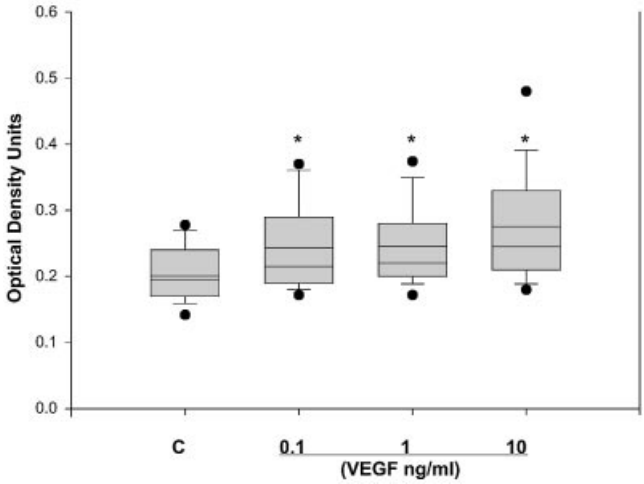


**Figure 6.** Effect of vascular endothelial growth factor (VEGF) on endometrial stromal cell proliferation analysed by MTT [3-(4,5-dimethythiazol-2-yl-)-2,5-diphenyl tetrazolium bromide] colorimetric assay. VEGF-treated groups had significantly increased cell proliferation rate compared to the control group ($P < 0.05$).

(Popovici *et al*., 1999). In addition, the expression of VEGF is regulated by estradiol and progesterone in cultured endometrial stromal cells (Shifren *et al*., 1996). VEGF is important for the regulation of endometrial angiogenesis. However, its presence is also important for other cell types in the endometrium. The current study demonstrates that VEGF decreases FasL expression of endometrial stromal cells and results in a decrease in apoptosis and a modest increase in proliferation in endometrial stromal cells. This observation suggests that the down-regulation of FasL by VEGF may affect endometrial stromal cell survival in an autocrine or paracrine manner, which in turn may play a role for successful pregnancy or for remodelling of the endometrium following menstruation.

Since the PCR results showed no change in FasL mRNA levels, the decrease in FasL levels is not due to alterations in its transcription but may be due to stimulation of its degradation or to its conversion to a soluble form. Furthermore, we also assessed whether the medium of the stromal cell cultures either not treated or treated with VEGF might contain soluble FasL (data not shown) and no soluble FasL was found in the medium as in our previous study (Garcia-Velasco *et al*., 2002). Other possible mechanisms could be either stimulation of FasL degradation or modifications at post-transcriptional level. FasL expression in endometrial stromal cells is cyclic, suggesting there may be a regulation at its transcription or translation levels, or conversion of FasL to its soluble form.

In conclusion, our results show that endometrial stromal cells in culture have a cyclic FasL expression and that VEGF regulates FasL expression at the protein level either by affecting its translation or degradation. This, in turn, may take part in endometrial remodelling by decreasing endometrial apoptosis and increasing cell proliferation.

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