Effect of early luteal phase administration of mifepristone (RU486) on leukaemia inhibitory factor, transforming growth factor β and vascular endothelial growth factor in the implantation stage endometrium of the rhesus monkey

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Abstract

A single, low-dose administration of a potent anti-progesterone such as mifepristone (RU486) in the early luteal phase results in inhibition of blastocyst implantation in primates. The aim of the present study was to examine the status of leukaemia inhibitory factor (LIF), transforming growth factor β (TGF β) and vascular endothelial growth factor (VEGF) in day 6 gestational endometrium of rhesus monkeys with or without exposure to a single dose (2 mg/kg body weight, s.c.) of mifepristone on day 2 after ovulation. Densitometric analyses of immunoblots of endometrial spent media revealed an increase ($P < 0.01$) in TGF β pan (TGF β 1, 2, 3 and 5) and a decrease ($P < 0.01$) in VEGF secretion from RU486-exposed endometrial samples compared with control samples. Secretory profiles for LIF, TGF β 1 and TGF β 1 LAP (latency associated peptide) remained unchanged in the two treatment groups. Morphometric analyses of immunohistochemical staining showed altered cell-specific distribution. TGF β 1 ($P < 0.01$) and TGF β pan ($P < 0.02$) were higher, while VEGF declined ($P < 0.05$) in endometrial glands of RU486-exposed endometria compared with control tissue samples. Stromal cell staining patterns for all experimental cytokines studied remained unchanged. In blood vessels, VEGF was found to be low ($P < 0.05$), while LIF ($P < 0.05$)

and TGF β 1 ($P < 0.01$) were higher in mifepristone-exposed endometrial samples compared with control tissue samples. Increased TGF β secretion together with elevated levels of TGF β in glandular epithelia and in blood vessels with no apparent change in stromal levels of TGF β or in levels of TGF β LAP in any endometrial compartment in the two treatment groups suggest an altered paracrine involvement of this cytokine and an enhanced activation of latent TGF β in endometrium following mifepristone treatment. Higher levels of TGF β in gland cells may result in dysregulated growth control and degenerative morphology. Also, higher levels of LIF and TGF β together with lower levels of VEGF in the vascular compartment in mifepristone-exposed endometrium suggest that endometrial vascular physiology is a target of this anti-progestin during the peri-implantation stage. It is thus plausible that LIF, TGF β and VEGF in the glandular and vascular compartments of implantation stage endometrium play important roles in rendering the endometrium receptive, and that early luteal phase treatment with an anti-progestin such as mifepristone affects the involvement of these cytokines resulting in endometrial contraception.

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Introduction

Mifepristone (RU486) is a potent anti-progesterone. A single dose application of this anti-progestin agent on day 2 after ovulation can inhibit blastocyst implantation, with no notable changes in menstrual cyclicity in women and in monkeys (Gemzell-Danielsson *et al.* 1993, Ghosh & Sengupta 1993). It has been shown that luteal phase administration of mifepristone to women and to monkeys induced desynchronization of endometrium, repressed glandular secretory differentiation and vascular maturation (Li *et al.* 1988, Johannisson *et al.* 1989, Gemzell-Danielsson

et al. 1994, Ghosh *et al.* 1996). We have reported previously that administration of mifepristone on day 2 after ovulation results in differential profiles of secretory proteins (Ghosh *et al.* 1997a). Thus, progesterone antagonism with mifepristone treatment during the early luteal phase induces changes which render the endometrium non-receptive and the luminal milieu inhospitable for blastocyst implantation (Ghosh *et al.* 1997b). Besides progesterone, a significant degree of association exists between endometrial maturation and the levels of several cytokines in endometrial cells in relation to blastocyst implantation (see Tabibzadeh & Babaknia 1995).

Leukaemia inhibitory factor (LIF) is a glycoprotein with a remarkable range of biological actions in various tissue systems (Kurzrock *et al.* 1991, Hilton 1992). LIF is expressed in mouse uterine endometrial glands, especially on the fourth day of pregnancy when implantation occurs; such well-timed expression of LIF in the endometrium in this species is crucial for successful blastocyst implantation (Stewart *et al.* 1992). LIF expression is also high around the time of implantation in the human (Charnock-Jones *et al.* 1994, Arici *et al.* 1995).

The β type transforming growth factors (TGF β) are a family of structurally homologous dimeric proteins and they play important roles in the regulation of cell surface molecules, cell proliferation, tissue remodelling, extracellular matrix formation and immunomodulation (Ellingsworth 1990, Clemens 1991). Each of the five forms of TGF β is initially synthesized as part of a larger precursor molecule with the mature form of TGF β at the C-terminus. Following proteolytic cleavage, the two portions of the precursor remain together and are secreted as a biologically inactive, noncovalently-bound complex (latent TGF β) consisting of dimers of both the precursor remainder and mature TGF β . The precursor remainder is designated the latency associated peptide (LAP). Through a complex and largely unclear mechanism activation of latent TGF β occurs (Miyazono *et al.* 1993). Expression of TGF β has been shown to be high in the preimplantation stage endometrium of mouse (Das *et al.* 1992) and it has been implicated in embryo development (Paria *et al.* 1992). TGF β isoforms display a differential expression in ovine endometrium during the peri-implantation period (Dore *et al.* 1996). TGF β 1 expression by human endometrial stromal cells is increased by oestradiol and progesterone (Arici *et al.* 1996) and this cytokine has been shown to be present in maternal endometrium in first-trimester samples (Kauma *et al.* 1990).

Vascular endothelial growth factor (VEGF) is a dimeric growth factor which is a specific mitogen for endothelial cells and thereby influences angiogenesis (Ferrara *et al.* 1992). Variant 189 of VEGF also influences vascular permeability (Connolly *et al.* 1989, Keck *et al.* 1989). This growth factor has been shown to be expressed in epithelial and stromal cells of human endometrium (Charnock-Jones *et al.* 1993). Furthermore, it has been suggested that VEGF participates in the increased permeability and angiogenesis occurring in the uterine vascular bed during implantation in the rodent (Chakraborty *et al.* 1995).

In the present study, our aim was to examine the effect of early luteal phase administration of mifepristone, at a dose which inhibits blastocyst implantation, on endometrial levels and secretion of LIF, TGF β and VEGF during implantation using the rhesus monkey as a primate model. To this effect, implantation stage endometrial samples were collected on day 6 after ovulation from proven fecund cycles with or without mifepristone treatment on day 2 after ovulation and were semi-

quantitatively analysed for the above cytokines using immunohistochemistry and Western blot methods.

Materials and Methods

Animals and treatment

Healthy, mature and proven fertile male and female rhesus monkeys (*Macaca mulatta*) kept in a semi-natural condition at the Primate Research Facility of the All India Institute of Medical Sciences (AIIMS, New Delhi, India) were used in this study. The details of animal selection, housing and management have been described elsewhere (Ghosh & Sengupta 1992, Ghosh *et al.* 1996). The present study was approved by the Ethics Committee for Use of Non-Human Primates in Biomedical Research, AIIMS.

A vaginal swab from each female monkey was assessed daily. Females ($n=19$) showing ovulatory menstrual cycles of normal length (26–32 days) were allowed to cohabit with males during days 8–16 of their menstrual cycles. Vaginal smears were checked daily for the presence of spermatozoa. Female monkeys were randomly assigned to two treatment groups: control (group 1, $n=8$) and mifepristone treatment (group 2, $n=11$). Female animals from group 1 were injected s.c. with 2 ml vehicle (1:4, benzyl benzoate:olive oil, v/v) on day 2 after their presumed day of ovulation in successfully mated cycles. Animals assigned to group 2 were injected s.c. with a single dose of RU486 (2 mg/kg body weight) in the same volume of vehicle on day 2 after ovulation in mated cycles. We have shown previously that this treatment schedule is one hundred per cent efficacious in preventing implantation in monkeys (Ghosh *et al.* 1993). The days of ovulation in mated cycles were assessed from the daily profiles of oestradiol-17 β (E $_2$) and progesterone (P $_4$) in peripheral serum samples. Steroid radioimmunoassays were performed according to the method of Sufi *et al.* (1988) using antisera and chemicals obtained from the WHO Matched Reagents Programme. The methodological details are described elsewhere (Ghosh & Sengupta 1989a,b).

Antibodies

The source, specification and dilution of different antisera used for Western blotting (WB) and immunohistochemistry (IHC) are given in Table 1. All antibodies (IgG) were polyclonal except that for TGF β 1, and were purchased from R&D System (Minneapolis, MN, USA). Sensitivities of antibodies were assessed from direct ELISA as suggested by the supplier. Dilutions of stocks of antibodies for WB and IHC as shown in Table 1, were optimized based on 3–5 points titrations. According to the supplier, antibodies for LIF and VEGF have no cross reactivity with other cytokines, that for TGF β 1 has less than 1% cross reactivity with TGF β 2–5, TGF β pan (TGF β 1–3, 5) has cross

Table 1 Characteristics of primary antibodies used

	Specification of antigen	Specification of antisera	Sensitivity* (ng/well)	Dilution**	
				WB	IHC
Cytokine					
LIF	rhLIF	Goat IgG	2	5×10^2	5×10
TGF β 1	rhTGF- β 1	Mouse IgG ^a	10	5×10^2	5×10
TGF β 1 LAP	rhLAP	Goat IgG	2	10^3	10^2
TGF β pan	rhTGF- β s	Rabbit IgG	—	10^3	10^2
VEGF	rhVEGF	Goat IgG	1	10^3	10^2

*Sensitivity was assessed by direct ELISA using WB dilution. **Dilutions of stock (1 mg antibody/ml) were precalibrated based on 3–5 titration points and the information provided by the manufacturer.

^aMonoclonal; rh, recombinant human.

reactivity with TGF β 1, 2, 3 and 5, while TGF β 1 LAP cross reacts only with TGF β 1 LAP and latent TGF β 1, but not with mature TGF β 1. Specificity of antibody liganding and visualization in WB and IHC were assessed by (i) omitting primary antibodies, (ii) replacing primary antibodies with unrelated immunoglobulins from the same species and from other species, (iii) omitting secondary antibodies, (iv) replacing labelled secondary antibodies with unrelated labelled immunoglobulins from the same species and from other species, and (v) preincubating the antibodies with recombinant human antigens (R&D System). Labelled and unlabelled immunoglobulins, non-immune sera and visualization kits (horseradish peroxidase) were purchased from Vector Laboratories (Burlingame, CA, USA).

Tissue collection and processing

The procedural details of embryo retrieval, collection of endometrial samples and their dating have been described elsewhere (Sengupta *et al.* 1988, 1989, Ghosh *et al.* 1993, 1996). Briefly, endometrial dating and embryo age were determined from the presumed day of ovulation (D₀) based on serum profiles of E₂ and P₄ (Sengupta *et al.* 1989, Ghosh & Sengupta 1992, Ghosh *et al.* 1997a). Accordingly, endometrial samples were collected on day 6 after ovulation by performing laparotomy and fundal hysterotomy following ketamin (12 mg/kg; Vetlar; Parke-Davis, Mumbai, India) anaesthesia. The presence of a fresh corpus luteum was also checked. Tissue samples were collected only from those animals which yielded a pre-implantation stage embryo on flushing of the entire reproductive tract with sterile Earle's balanced salt solution (EBSS; Gibco-BRL, New York, USA), pH 7.4. Using this criterion, tissue samples were obtained from 6 control animals (group 1) and 7 mifepristone-treated animals (group 2).

Tissue samples were processed for chemical fixation in phosphate-buffered neutral formaldehyde followed by paraffin embedding as described elsewhere (Ghosh *et al.* 1993, 1996). Paraffin-embedded tissue samples were used for

IHC. Furthermore, tissue samples were processed for incubation in phenol red-free Eagle's minimum essential medium for 16 h at 37 °C in a humidified environment of 5% oxygen and 95% air (Basha *et al.* 1980). Spent media were used for WB analysis.

Western immunoblotting

Profiles of experimental cytokines in the incubation medium were characterized by the SDS-PAGE/Western immunoblotting method as described by Ausubel *et al.* (1994) using electrophoresis and trans-blot equipment from Bio-Rad Laboratories (Richmond, CA, USA). The materials and chemicals were purchased from Bio-Rad Laboratories and Sigma Chemical Co. (St Louis, MO, USA). Prestained molecular markers (Amersham International, Amersham, Bucks, UK) were run with each gel as standard. Samples of the same protein content (10 μ g) were separated on 15% SDS-PAGE gels, the proteins were transferred to nitrocellulose membranes which were incubated with primary antibodies. Final visualization was achieved by using Vectastain ABC immunoperoxidase kits (Vector Laboratories) and diaminobenzidine hydrochloride (Sigma Chemical Co.). Positive control, negative control and other controls, as given above, were run to examine the specificity of the procedure. The molecular weights of individual bands were determined based on profile analysis with a calibrated molecular weight standard curve, and semi-quantitative densitometric analysis based on profile analysis, and peak integrations of individual and pooled immunopositive bands were performed using Molecular Analyst image analysis software and a Gel Scanner GS 670 (Bio-Rad Laboratories). The results are shown as M_r of individual bands and semi-quantitative measures (optical density (OD).mm) of pooled profiles of bands, because the basis of protein cleavage in electrophoretic resolution was not examined in the present study.

Immunohistochemistry

Immunohistochemical staining for experimental cytokines was performed with formaldehyde-fixed, deparaffinized

Figure 1 Western immunoblot profiles for (A) LIF, (B) TGF β 1, (C) TGF β 1 LAP, (D) TGF β pan and (E) VEGF in media incubated with endometrium collected on day 6 after ovulation from control (group 1, C) and mifepristone-treated (group 2, R) animals.

tissue sections using the method described previously (Ghosh *et al.* 1993, 1996). The source, specification and dilution of different antisera used are given in Table 1. Final visualization was achieved by using Vectastain ABC peroxidase kits (Vector Laboratories), freshly made diaminobenzidine hydrochloride (Sigma Chemical Co.) and hydrogen peroxide. Positive controls (human osteoblasts for TGF β , human endometrium for VEGF and LIF) and other controls, as stated above, were run to assess the specificity of immunostaining. The immunohistochemically stained sections were analysed microscopically to estimate morphometrically the areas of immunoprecipitation in different endometrial compartments, namely glandular, stromal and vascular compartments in the functionalis zone using a Leica microscope and a precalibrated computer-assisted video image analysis system (QWIN-Quantimet 500 C+, Leica, Cambridge, UK). In endometrial functionalis, glands of multiple dimensions and different types of blood vessels are seen. Only glands and vessels which were in true cross section were measured using model systems described by Schipper *et al.* (1989) and Lowe (1984), and collectively termed glandular compartment and vascular compartment respectively. The area of lamina propria minus these two segments was taken as stroma. The details of histometric measurements are given elsewhere (Ghosh *et al.* 1993, 1996) and the methodological details are given by Baak & Oort (1983), Russ (1990), and Hamilton & Allen (1995). Briefly, glandular (at $\times 25$) and vascular (at $\times 40$) compartments were detected using an interactive planimeter analyser only in cases where discernibility of these structures was distinct, and the immunopositive areas were measured in a particular compartment (segment) by detecting positive profiles in digitized images based on an optimized grey level threshold after shading correction and pixel calibration against the standard provided by the manufacturer.

Statistical analysis

Statistical analyses of densitometric and morphometric data were performed using χ^2 -test and a modified *t*-test, as applicable (Samuels 1991). Data are shown as means $\pm$ S.E.M.

Results

Seventy-five per cent of control animals and about sixty-four per cent of mifepristone-treated animals yielded preimplantation embryos. The observed difference in the yield of preimplantation embryos between groups was not statistically significant.

As shown in Fig. 1, all experimental cytokines were detected in 2–3 immunopositive bands, and there was no significant change in the band characteristics between the two treatment groups. Furthermore, the total densitometric scores of pooled band profiles from Western blot analysis failed to detect any change in the *in vitro* secretory levels of LIF, TGF β 1 and latent TGF β 1 (LAP) between the two treatment groups (Table 2). However, there was a significant ($P < 0.01$) increase in TGF β pan secretion from endometrial samples from mifepristone-treated animals (group 2) compared with control (group 1) endometrial samples (Table 2). In contrast, there was a significant ($P < 0.01$) decrease in the *in vitro* VEGF secretion from endometrial samples obtained from group 2 (mifepristone-treated) compared with those from group 1 (Table 2).

Table 3 shows the morphometric analysis of the area of immunopositive precipitate for different experimental cytokines in three endometrial compartments – gland, stroma and blood vessels – in the functionalis zone. Figures 2–4 show the representative microphotographs for LIF, TGF β 1, TGF β pan and VEGF from both treatment groups. No significant changes were noted for LIF and

Table 2 Densitometric analysis of immunopositive bands of cytokines in Western blots

Cytokine	Total immunopositive score (OD.mm)		
	Control (n=6)	Mifepristone (n=7)	Significance of difference (P)
LIF	1.3 $\pm$ 0.3	1.6 $\pm$ 0.3	NS
TGF β 1	2.2 $\pm$ 0.6	2.5 $\pm$ 0.4	NS
TGF β 1 LAP	0.5 $\pm$ 0.1	0.5 $\pm$ 0.05	NS
TGF β pan	1.2 $\pm$ 0.1	1.5 $\pm$ 0.1	<0.01
VEGF	2.1 $\pm$ 0.5	0.6 $\pm$ 0.1	<0.01

For procedural details, see Materials and Methods. NS, non-significant.

Table 3 Morphometric analysis of immunohistochemical staining in three endometrial compartments – gland, stroma and blood vessels. Group 1 (control) n=6, group 2 (RU486-treated) n=7

Cytokine	Area of immunopositive precipitation (per cent)								
	Gland			Stroma			Blood vessel		
	Group 1	Group 2	P value	Group 1	Group 2	P value	Group 1	Group 2	P value
LIF	35.2 $\pm$ 7.0	39.7 $\pm$ 6.8	NS	10.1 $\pm$ 2.8	12.0 $\pm$ 3.2	NS	68.5 $\pm$ 4.2	84.1 $\pm$ 4.9	<0.05
TGF β 1	10.2 $\pm$ 3.5	49.0 $\pm$ 7.9	<0.01	6.5 $\pm$ 1.4	9.9 $\pm$ 3.0	NS	19.1 $\pm$ 3.6	45.2 $\pm$ 7.6	<0.01
TGF β 1 (LAP)	72.7 $\pm$ 8.2	69.3 $\pm$ 6.0	NS	24.1 $\pm$ 2.0	31.6 $\pm$ 2.8	NS	82.0 $\pm$ 7.3	88.8 $\pm$ 4.3	NS
TGF β (pan)	10.9 $\pm$ 3.8	29.5 $\pm$ 4.9	<0.02	3.4 $\pm$ 1.5	4.9 $\pm$ 1.5	NS	19.9 $\pm$ 3.5	45.5 $\pm$ 10.0	<0.05
VEGF	61.0 $\pm$ 4.0	47.5 $\pm$ 3.1	<0.05	5.9 $\pm$ 1.4	8.6 $\pm$ 1.6	NS	84.4 $\pm$ 5.0	60.2 $\pm$ 4.0	<0.05

NS, non-significant.

latent TGF β 1 (LAP) in glandular epithelial compartments between the two treatment groups (Table 3). As shown in Fig. 2, LIF was observed mainly at the luminal side of the glandular epithelium. Significant increases in the scores of TGF β 1 (P <0.01) and TGF β pan (P <0.02) were noted in the endometrial glandular compartment of mifepristone-treated animals (group 2) compared with control (group 1) animals (Fig. 3). A high degree of nuclear staining for TGF β 1 was frequently observed in mifepristone-treated endometrium (Fig. 3b). The VEGF score was decreased (P <0.05) in RU486-treated endometrial glandular epithelium compared with control endometrial gland (Table 3, Fig. 4). Generally, relatively low to moderate amounts of precipitate for TGF β pan, TGF β 1, VEGF, and LIF were observed in the stromal compartment (Table 3, Figs 2–4). There was, however, no significant change in the score of any cytokine tested in the stromal compartment in the two treatment groups. In the vascular compartment of mifepristone-treated (group 2) endometrial samples, there was an increase in the immunopositive score of LIF (P <0.05; Fig. 2b,d) and in that of TGF β 1 (P <0.01) and TGF β pan (P <0.05) compared with those from group 1 control samples (Table 3). There was no change in the area of TGF β 1 LAP in the vascular compartment between the two groups. In contrast, the immunopositive area of VEGF in endometrial blood vessels following RU486 treatment

decreased (P <0.05) compared with the control group (Table 3, Fig. 4).

Discussion

We have demonstrated previously that administration of a potent anti-progestin, such as RU486, on day 2 after ovulation in a mated cycle inhibits blastocyst implantation in the rhesus monkey (Ghosh & Sengupta 1993) and this was associated with marked retardation in endometrial secretory maturation (Ghosh *et al.* 1996) and significant decline in the viability of preimplantation embryos (Ghosh *et al.* 1997a). However, there was no change in serum concentrations of ovarian steroid hormones (Ghosh & Sengupta 1993, Ghosh *et al.* 1996). It was hypothesized in the present study that an alteration in the levels of cytokines such as LIF, TGF β and VEGF at the endometrial level may mediate the anti-nidatory action of mifepristone.

There is substantial indirect evidence to suggest an involvement of LIF in the process of implantation. Endometrial LIF expression is essential for blastocyst implantation and embryo growth in a variety of species including primates (Fry *et al.* 1992, Shen & Leder 1992, Stewart *et al.* 1992, Robertson *et al.* 1993, Kauma & Matt

Figure 2 Immunohistochemical localization of LIF in endometrial samples from control (a,b) and mifepristone-treated (c,d) animals. Haematoxylin counterstained. $\times 500$ (a,c) and $\times 790$ (b,d).

1995, Lopata 1996). Available studies in the human also indicate that endometrial LIF synthesis and secretion are high during the mid-luteal phase, that is, around the time of blastocyst implantation (Charnock-Jones *et al.* 1994, Kojima *et al.* 1994, Arici *et al.* 1995, Chen *et al.* 1995, Cullinan *et al.* 1996, Vogiagis *et al.* 1996). Several cytokines which are influenced by progesterone have been found to enhance endometrial LIF expression (Arici *et al.* 1995). Ace & Okulicz (1995) also observed that expression of LIF was up-regulated in progesterone-dominated monkey endometrium.

In the present study, mid-luteal phase endometrial secretion of LIF *in vitro* resolved into two bands at 56 and 27 kDa. LIF molecules are highly glycosylated, single chain molecules varying in molecular masses from approximately 38–67 kDa resulting from differential

glycosylation of a protein of approximately 26 kDa in the human (Hilton *et al.* 1988, Hilton 1992). In the present study, LIF secretion by endometrial samples collected on day 6 after ovulation of proven fecund cycles was not affected by early luteal phase mifepristone treatment in monkeys. Immunohistological examination also failed to detect any change in the level of LIF in the glandular epithelial compartment. Our observation on glandular LIF level following RU486 treatment in monkeys does not agree with the one reported by Gemzell-Danielsson *et al.* (1997), in which a significant decrease in LIF was noted in mid-luteal phase human endometrium following early luteal phase mifepristone treatment. However, unlike the human study, we have used endometrial samples only from proven fecund cycles. It is notable that the embryo may stimulate endometrial LIF production (Plachot *et al.*

Figure 3 Immunohistochemical localization of TGF β 1 (a,b) and TGF β pan (c,d) in endometrial samples from control (a,c) and mifepristone-treated (b,d) animals. A high degree of nuclear staining for TGF β 1 in the mifepristone-treated endometrial sample (b) is evident. Haematoxylin counterstained. $\times 500$ (a,b) and $\times 790$ (c,d).

Figure 4 Immunohistochemical localization of VEGF in endometrial samples from control (a) and mifepristone-treated (b) animals. Haematoxylin counterstained. $\times 790$.

1996). We have shown previously in the rhesus monkey that the endometrium on day 6 of a fecund cycle has distinct morphological and biochemical characteristics which distinguish it from the endometrium on day 6 of non-mated ovulatory cycles (Ghosh & Sengupta 1988, 1989b, Sengupta *et al.* 1988, Ghosh *et al.* 1993). The results of the present study support an earlier observation that LIF production in human endometrium *in vitro* was not affected by a low dose of RU486 (Chaouat *et al.* 1995). Similar to earlier reports in the human (Charnock-Jones *et al.* 1994, Arici *et al.* 1995, Chen *et al.* 1995, Vogiagis *et al.* 1996), we also observed only low to moderate levels of LIF in the stromal compartment of mid-luteal phase endometrium in the rhesus monkey. There was, however, a marginal increase in the level of LIF in the vascular compartment. Thus, it remains to be investigated further whether LIF plays the same critical role in the process of implantation in primates as has been demonstrated in mice (Stewart *et al.* 1992).

TGF β may also play a significant role in preimplantation stage endometrium and its level may be affected around the time of blastocyst implantation as a result of early luteal phase application of mifepristone. Expression of TGF β has been shown to be high in mouse and ovine preimplantation stage endometrium (Das *et al.* 1992, Dore *et al.* 1996), which has been implicated in embryo development (Paria *et al.* 1992). Furthermore, TGF β 1 expression by human endometrial stromal cells is regulated by ovarian steroid hormones (Casslen & Ohlsson 1981, Gong *et al.* 1991, Arici *et al.* 1996) and this cytokine has been shown to be present in first-trimester maternal endometrial samples (Kauma *et al.* 1990). In an elegant study, Casey and MacDonald (1996) have suggested that TGF β may interact in a very complex manner in progesterone-dominated endometrium in defining its functional destiny.

In the present study, TGF β 1, TGF β 1 LAP and TGF β pan were immunodetected in three bands in the range of 50–45, 30–25 and 20–15 kDa respectively. Similar immunodetectable bands have been observed in other species (Lyons *et al.* 1988, Miyazono *et al.* 1988, R&D Systems 1994). The fact that a lower score of TGF β 1 LAP compared with TGF β 1 was detected in Western blots possibly supports the notion that TGF β s are secreted in mature forms to act upon adjacent cells (Lyons *et al.* 1988). On the other hand, a higher score of TGF β 1 LAP compared with TGF β 1 was observed in immunohistology presumably due to higher sensitivity of antibody against LAP compared with that against TGF β 1 (see Table 1). The immunodetectable levels of TGF β 1 and TGF β pan were dramatically enhanced in endometrium together with higher secretion of TGF β pan following RU486 treatment, with no change in TGF β 1 LAP. It appears that endometrial receptivity towards blastocyst implantation is significantly associated with the level of TGF β 1 in the endometrium; it tends to be low at that time in the rhesus

monkey. Following early luteal phase mifepristone administration, there was a significant increase in endometrial TGF β during the peri-implantation period, resulting in desynchronisation of epithelial cell maturation and consequent failure of implantation. TGF β is inhibitory to epithelial cell mitosis, and it also induces apoptosis (Rotello *et al.* 1991, Murphy & Ballejo 1994). Apoptosis is significantly high in mifepristone-treated endometrium (Ghosh *et al.* 1992, Rotello *et al.* 1992, Ghosh *et al.* 1996). TGF β in endometrium is also very high during menstruation (Casey & MacDonald 1996). In fact, it was suggested by Gold *et al.* (1994) that TGF β may play a significant paracrine role between different endometrial compartments, and simultaneous overexpression or accumulation of TGF β , especially in glandular epithelium, is indicative of dysregulated growth control. Interestingly, Gold *et al.* (1994) also observed a high degree of nuclear TGF β 1 staining in abnormal endometrial glands and stroma. Such a higher degree of nuclear TGF β 1 staining may be associated with its inhibitory activity on DNA synthesis and inductive effect on apoptosis (Ignatz & Massague 1987, Rotello *et al.* 1991).

Expression of endometrial VEGF is high during implantation and it presumably participates in increased vascular permeability and angiogenesis occurring in maternal endometrium during blastocyst implantation (Sharkey *et al.* 1993, Chakraborty *et al.* 1995, Smith 1996). Thus, we hypothesized that following early luteal phase mifepristone treatment, endometrial VEGF could be affected at the time of implantation resulting in implantation failure. In the present study, endometrial VEGF was detected in two bands at 46 and 28 kDa. It has been noted by others that VEGF is a dimeric protein (~ 46 kDa) consisting of two identical (~ 23 kDa) subunits (see Ferrara *et al.* 1992). Similar to the observation in women (Charnock-Jones *et al.* 1993), immunodetectable VEGF was primarily observed in the epithelium of implantation stage endometrium in the present study. Although band characteristics in Western blot did not change with mifepristone treatment, there was a significant decrease in the levels of VEGF in endometrium and in its secretion *in vitro* following mifepristone treatment. Although we have no clear idea about the regulation of VEGF in endometrium by oestradiol and progesterone, a cell line (RL-95) has been shown to express significantly higher levels of VEGF in response to progesterone (Smith 1996).

Although it is true that *in vitro* secretion of a cytokine and its histochemical distribution do not provide direct information about the secretory status of the test cytokine *in situ*, such information generally provides an effective operative knowledge (Ballock & Roberts 1993). Thus, the observation that the experimental cytokines were affected not only in the glandular compartment, but also in the vascular compartment of preimplantation stage endometrium following early luteal phase mifepristone treatment appears important because changes in the

vascular bed are integral to the process of implantation (Finn & Porter 1975, Ghosh *et al.* 1993, Abberton & Rogers 1995). It is generally known that VEGF and fibroblast growth factor promote angiogenesis, LIF inhibits angiogenesis and TGF β can both inhibit and promote angiogenesis depending on its concentration and overall endocrinology and paracrinology of the tissue (Klagsbrun & D'Amore 1991, Pepper *et al.* 1992, 1993, 1995, Casey & MacDonald 1996). LIF, TGF β and VEGF are also known to influence leucocytic margination and emigration, vascular integrity and remodelling, as well as organisation of extravascular matrix (Ignatz & Massague 1987, Schainberg *et al.* 1988, Hilton 1992, Murphy & Ballejo 1994, Smith 1996). Therefore, the observed changes in the cytokines characterized by high LIF and TGF β together with decreased VEGF in the vascular compartment are suggestive of a major functional change in this compartment. Previous morphological studies also revealed that small blood vessels were affected together with microhaematoma and enhanced emigration of leucocytic cells into the extracellular matrix in mid-luteal phase endometrium after mifepristone administration in human volunteers (Johannisson *et al.* 1989) and in monkeys (Ghosh *et al.* 1996).

To our knowledge, this is the first report documenting a pooled status of LIF, TGF β and VEGF in glandular and vascular compartments of implantation stage endometrium with or without early luteal phase mifepristone treatment in a primate species. The observed changes in these cytokines in endometrium are indicative of dysregulated glandular maturation and vascular incompetence resulting in endometrial inadequacy and loss of preimplantation embryo viability following early luteal phase mifepristone treatment in the rhesus monkey (Ghosh *et al.* 1996, 1997a).

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