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EFFECT OF VAGINALLY ADMINISTERED FUMAGILLIN ON IMMUNOHISTOCHEMICAL DISTRIBUTION OF LEUKEMIA INHIBITORY FACTOR, INTERLEUKIN 6, TRANSFORMING GROWTH FACTOR β AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN IMPLANTATION STAGE ENDOMETRIUM OF THE RHESUS MONKEY

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Abstract : Intravaginal administration of an anti-angiogenic agent, fumagillin, during blastocyst implantation inhibits pregnancy establishment in a dose-related manner in the rhesus monkey. In the present study, mated female rhesus monkeys were vaginally inserted with tampons containing vehicle (group 1; n=5) and test agent (fumagillin, 4 mg/animal; group 2; n=6) on cycle day 20, and endometrial tissue samples were collected on cycle day 24 from all monkeys and processed for histological examination and imminohistochemical localization for LIF, IL-6, TGF-β and VEGF. Concentrations of estradiol-17β, progesterone and chorionic gonadotrophin in peripheral circulation were determined. From the serum profiles of the hormones, 2 monkeys in group 1, and 1 monkey in group 2 appeared pregnant. However, endomertial morphology revealed histological evidence of pregnancy in 3 out of 6 fumagillin-treated animals. Histometric analysis of immunohistochemical staining in epithelial, stromal and vascular compartments revealed that per cent areas occupied by immunoprecipitate for the cytokines studies did not change in epithelial and stromal compartments, except that for TGF-β which was higher ($P<0.05$) in epithelial compartment in group 2. No change was observed in immunoprecitpitation areas for IL-6 in epithelial, stromal and vascular compartments. On the other hand, changes ($P<0.05$) for LIF, TGF-β and VEGF were evident in the vascular compartment. It is possible that disparate responses observed in glandular, stromal and vascular compartments in implantation stage endometrium following fumagillin treatment actually caused from associated decline in progesterone concentration in peripheral circulation. It is also possible that fumagillin, an angiostatic agent, affects the synthesis and secretion of cytokines primarily in the vascular compartment of implantation stage endometrium, and thereby manifests differential responses in epitheial, stromal and vascular compartments.

Key words : endometrium, fumagillin, IL-6, immunohistochemistry, implantation, LIF, rhesus monkey, TGF-β, VEGF

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INTRODUCTION

Blastocyst implantation is a critical and complex process towards the establishment of pregnancy in eutherian mammals. During implantation and placentation, increased vascular permeability, reduced vascular impedence, increased angiogenesis, as well as cellular proliferation in different cell types are seen in maternal endometrium, while in the conceptus trophoblast cells undergo rapid proliferation and vascularization (1). In women and in other menstruating non-human primates, the vascular supply in the fetal membranes is established before the time point at which the first menstrual period is missed (2). Thus, it appears possible to inhibit the process of blastocyst implantation by local application of angiostatic and anti-mitotic agents (3). We have previously reported that intravaginal administration of an anti-angiogenic agent like fumagillin during blastocyst implantation inhibits the establishment of pregnancy in a dose-related manner in rhesus monkeys (4). We consider that the local angiostatic action of the agent results in its contraceptive action. The dose (4 mg/animal) at which establishment of pregnancy was inhibited was, however, found to cause a decline in luteal phase progesterone level (4). Since progesterone is essential for nidation in monkeys and women (5), there is a likelihood that fumagillin mediated decline in serum progesterone level during the implantation window results in its contraceptive action. Furthermore, we have reported that intra-vaginal administration of anti-nidatory dose of fumagillin affected glandular and vascular functions in implantation stage endometrium in the rhesus monkey (6). Based on our earlier observation that the immunohistochemical distribution of cytokines like leukemia inhibitory factor (LIF), transforming growth β (TGF-β) and vascular endothelial growth factor (VEGF) in different cellular compartments in endometrial sample on day 6 after ovulation are affected with the induction of endometrial refractoriness to blastocyst implantation following a single dose application of a high affinity antiprogestin - mifepristone (RU486) - on day 2 after ovulation (7), it appears that cytokines like LIF, TGF-β and VEGF in implantation stage endometrium play significant role in the process of induction of endometrial receptivity to blastocyst implantation. In the present study, we report our observation on the effect of vaginally administered anti-nidatory dose of fumagillin on immunohistochemical distribution of LIF, interleukin-6 (IL-6), TGF-β and VEGF in implantation stage endometrium using the rhesus monkey as a non-human primate model.

METHODS

Animals

Sexually mature, proven fertile male and female rhesus monkeys (*Macaca mulatta*) were individually housed in cages having access to natural light and fed with standard pellet diet supplemented with fresh fruits and soaked gram, peanut and water *ad libitum*. Females showing two consecutive cycles of normal length (26 to 32 days) were assigned for treatment in the third cycle, when they were allowed to cohabitate with male monkeys between cycle days 8 to 16 (treatment cycle). Successful

insemination was assessed from microscopic examination of vaginal swabs during the mating period. The details of the protocol are given elsewhere (4, 6). The study was approved by the Ethics Committee for the Biomedical Use of Non-Human Primates and performed in the Primate Research Facility of the All India Institute of Medical Sciences.

Experimental procedures

Female monkeys (n = 12) were randomly assigned to two groups. Animals belonging to group 1 (control; n = 6) and group 2 (fumagillin, 4 mg/animal; n = 6) were vaginally inserted with tampons (Tampex, USA) bearing dialysis tubing containing vehicle and test agents on cycle day 20 of mated cycles as described previously (4, 6). Blood samples were collected daily by venipuncture from cycle day 8 until day of tissue collection and were used to determine the peripheral levels of circulating estradiol-17β (E_2), progesterone (P) and chorionic gonadotrophin (mCG) as described previously (4, 6, 9, 10). Serum samples were collected and stored at -20°C until hormone assays were performed. Monkeys with hormonal profiles showing ovulation and establishment of luteal function were selected for the present study.

Endometrial tissue samples were collected on cycle day 24 from all monkeys by performing laparotomy and fundal hysterotomy following ketamine (12 mg/kg; Ketlar, Parke-Davis, Mumbai, India) anaesthesia. The presence of corpus luteum was checked before tissue collection. Accordingly, one animal in the control group (group 1) was not included, because ovulation was not detected in this animal. The procedural details of tissue collection and processing have been described elsewhere (6). Briefly, tissue samples (group 1, n = 5; group 2, n = 6) were fixed in phosphate-buffered neutral paraformaldhyde (4%, w/v) and embedded in paraffin wax using a routine procedure.

Immunohistochemistry and histometry

Tissue sections (5 μm) were employed for immunohistochemistry using a method described earlier (7, 11). Briefly, tissue sections were deparaffinized and hydrated through graded alcohol to phosphate buffered saline (PBS). Endogenous peroxidase activity was then quenched with 0.3% hydrogen peroxide in methanol. Sections were incubated overnight at 4°C with the primary antibody. The details of the antibodies employed in the study are given in Table I. Sensitivity of antibody liganding for all antibodies was pre-calibrated by diluting the stock (1 mg/ml) and performing 4-5 points titration and based on the information provided by the manufacturer. Final visualization was achieved using the ABC kit (Vector Laboratories, Burlinghame, CA, USA) and freshly made diaminobenzidine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) with hydrogen peroxide as described previously (7, 11). Specificities of liganding for different antibodies used, as well as, specificity for visualization protocol were provided by the manufacturers and were further assessed by omitting primary antibodies, replacing primary antibodies with unrelated immunoglobulins from same species, omitting secondary antibodies and replacing

labelled secondary antibody with unrelated immunoglobulins from same species and other species. The reagents were purchased from Vector Laboratories (Burlinghame, CA, USA). All immunostaining procedures were performed in a single run. Duplicate sections were lightly counterstained with haemotoxylin to facilitate the identification of cellular elements. The immunohistochemically stained sections were analysed microscopically to histometrically estimate the areas of immunoprecipitation in glandular, stromal and vascular compartments in functionalis zones using a Leica microscope and a pre-calibrated computer assisted video image analysis system (QWin-S, version 2.3; Leica, Cambridge, UK). The details of histometric measurements are given elsewhere (7, 10, 11). Briefly, glandular, stromal and vascular components were visualized using an interactive planimeter analyzer only in cases where discernibility of these structures was distinct and immunopositive areas were measured in a particular compartment (segment) by detecting positive profiles in digitized images based on an optimized gray level threshold after shading correction and pixel calibration against the standard provided by the manufacturer.

Statistics

Statistical analysis of quantitative measurements were performed using modified *t*-test (12). The probability level of $P = 0.05$ was taken as the limit of significance. The data are shown as means±SEM.

RESULTS

A total of 12 animals were assigned to the present study, out of which 1 in control group (group 1) failed to ovulate. Thus, 11 animals (5 in group 1, and 6 in group 2) were utilized in the experiment. Table II gives the summary of reproduction profiles of animals in groups 1 and 2 following respective treatments.

Fig. 1 shows the serum concentrations of E_2 and P during the luteal phase of

TABLE I: Characteristics of primary antibodies used.

Cytokines	*Specification of antigen*	*Specification of antisera*	*Sensitivity* (ng/well)*	*Dilution***
LIF	rhLIF	Goat IgG	2	5×10
IL-6	rhIL-6	Goat IgG	1	10^2
TGF-β1	rhTGF-β1	Mouse IgG[a]	2	5×10
TGF-β pan	rhTGF-βs	Rabbit IgG	n	10^2
VEGF	rhVEGF	Goat IgG	1	10^2

*Sensitivity was assessed by direct ELISA at 1 µg/ml. **Dilutions of stock (1 mg/ml) were precalibrated based on 4–5 titration points and the information provided by the manufacturer. [a]Monoclonal; rh, recombinant human; n, not determined.

TABLE II: Summary of observed reproductive profiles in animals subjected to control and fumagillin treatment.

Group	*Monkey number*	*Day of ovulation [cycle day]*	*Duration of treatment [days after ovulation]*	*mCG detection [days after ovulation]*	*Comments*
1, Control (vehicle only)	C1	14	6–10	ND	NP
	C2	10	10–14	ND	NP
	C3	13	7–11	11	P
	C4	13	7–11	11	P
	C5	X	X	X	X
	C6	14	6–10	ND	NP
2, Fumagillin (4 mg/animal)	F1	9	11–15	ND	NP
	F2	12	8–12	ND	NP
	F3	10	10–14	ND	P[a]
	F4	9	11–15	15	P
	F5	13	7–11	ND	NP
	F6	9	11–15	ND	P[a]

X, no ovulation. ND, not detected. NP, non-pregnant. P, pregnant.
[a]based on histological analysis.

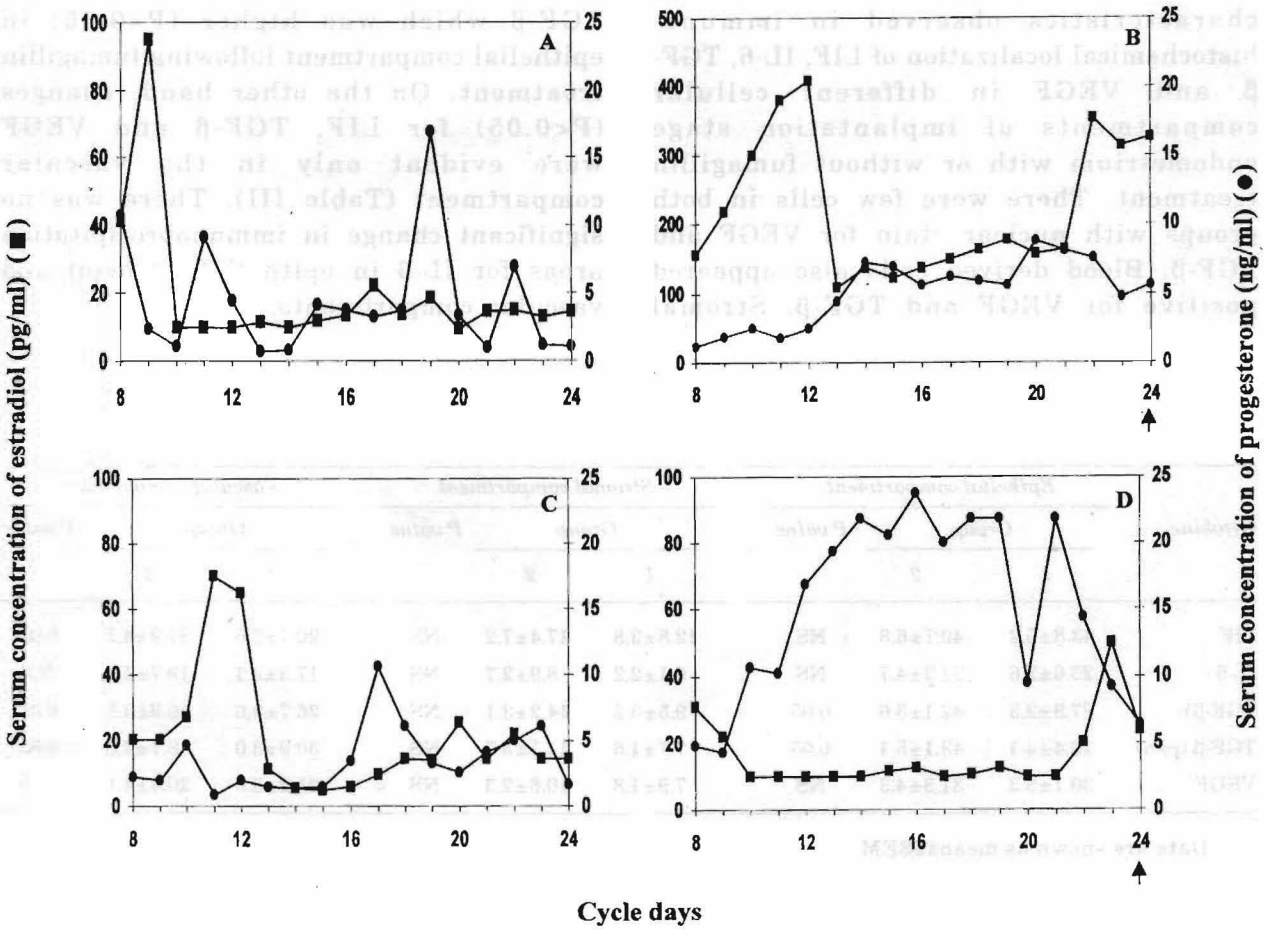

Fig. 1: Serum concentrations of estradiol-17β (■) and progestrone (●) in control (A, C2; B, C3) and fumagillin (C, F2; D, F4) treated monkeys. *Arrows* indicate the first day of detectable mCG in serum samples.

animals representative of control and treatment groups. From the serum profiles of these hormones, 2 monkeys (C3 and C4) in group 1, and 1 (F4) in group 2 appeared pregnant (Table II). On pooled analysis, however, the area under curve for serum progesterone during days 20 to 24 (that is the period of intravaginal treatment was found to be significantly ($P<0.05$) less in group 2 (9 ± 0.8) compared with that in group 1 (16 ± 1.2).

Fig. 2 highlights some of the characteristics observed in immunohistochemical localization of LIF, IL-6, TGF-β and VEGF in different cellular compartments of implantation stage endometrium with or without fumagillin treatment. There were few cells in both groups with nuclear stain for VEGF and TGF-β. Blood derived cells also appeared positive for VEGF and TGF-β. Stromal extracellular matrix was found to be positive in a variable way for VEGF and TGF-β. Overall, IL-6 staining was relatively less in all compartments except in few decidual cells.

Table III shows the data from histometric analysis of immunohistochemical staining in epithelial, stromal and vascular compartments. It revealed that per cent areas occupied by immunoprecipitate for the cytokines studied did not change in epithelial and stromal compartments, except that for TGF-β which was higher ($P<0.05$) in epithelial compartment following fumagillin treatment. On the other hand, changes ($P<0.05$) for LIF, TGF-β and VEGF were evident only in the vascular compartment (Table III). There was no significant change in immunoprecipitation areas for IL-6 in epithelial, stromal and vascular compartments.

TABLE III : Morphometric analysis of immunohistochemical staining in epithelial, stromal and vascular compartments.

Cytokine	*Epthelial compartment*			*Stromal compartment*			*Vascular compartment*		
	Group		*P value*	*Group*		*P value*	*Group*		*P value*
	1	*2*		*1*	*2*		*1*	*2*	
LIF	43.8±5.2	40.7±6.8	NS	12.8±3.8	17.4±7.2	NS	20.7±2.6	37.9±4.3	0.05
IL-6	25.6±2.6	22.3±4.7	NS	6.1±2.2	8.9±2.7	NS	17.3±3.1	19.7±4.6	NS
TGF-β1	27.9±3.3	42.1±3.6	0.05	9.5±3.2	14.2±3.1	NS	26.7±3.6	46.9±3.5	0.05
TGF-β (pan)	19.4±4.1	43.1±5.1	0.05	7.7±1.6	11.1±3.7	NS	30.9±3.0	48.7±3.5	0.05
VEGF	30.1±3.3	31.3±4.3	NS	7.9±1.8	10.6±2.3	NS	31.2±3.0	20.4±4.1	0.05

Data are shown as means±SEM.

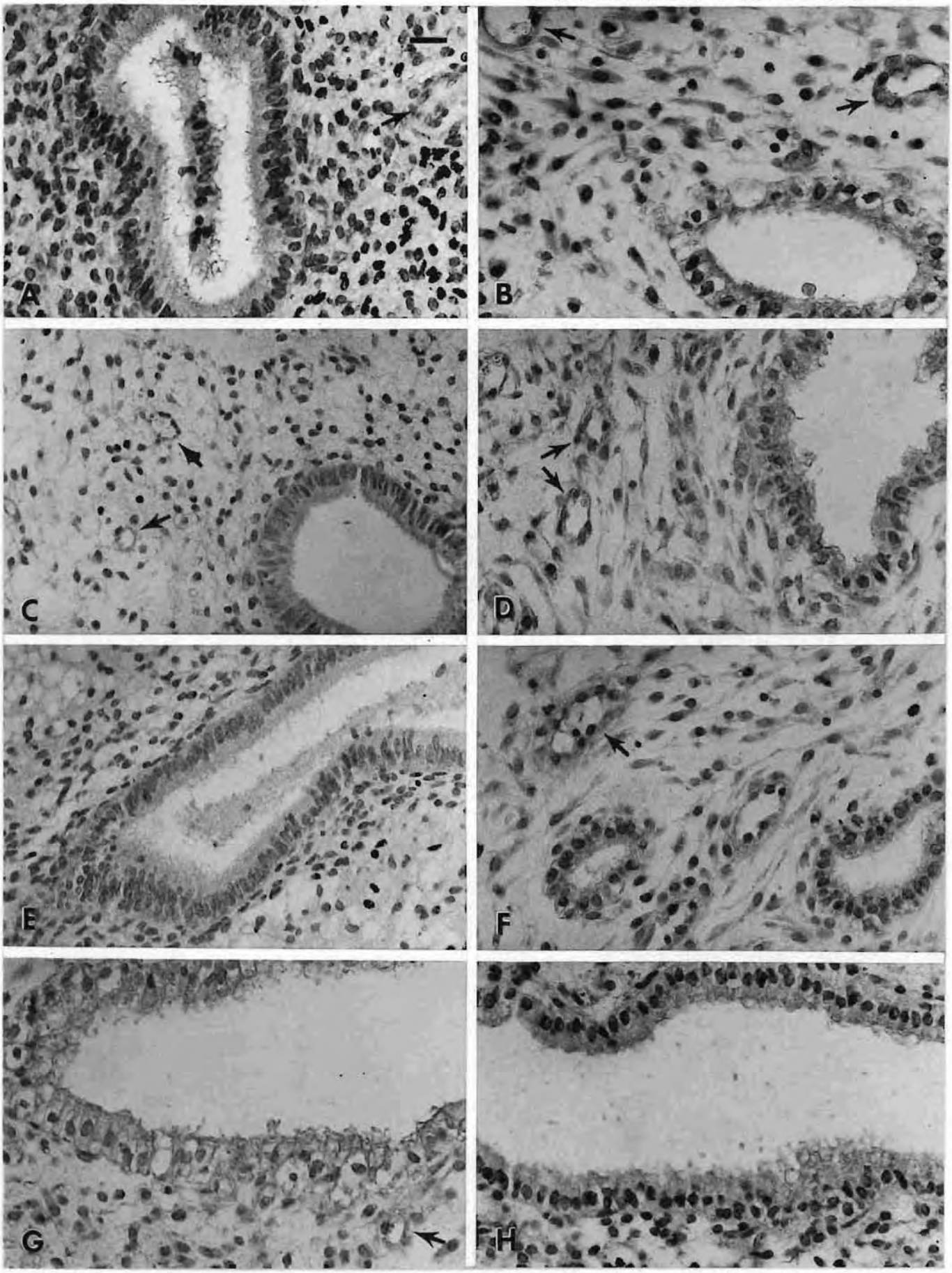

Fig. 2 : Immunohistochemical localization of LIF (A,B), IL-6 (C,D), TGF-β (E,F), and VEGF (G,H) in endometrial samples of control (A,C,E,G) and fumagillin treated (B,D,F,H) animals. *Arrows* indicate blood vessels. Haematoxylin counterstained. Bar=200 μm.

DISCUSSION

We have previously reported that intravaginal administration of 1 mg fumagillin during days 20 to 26 of mated ovulatory cycles did not result in inhibition of embryo implantation in monkeys, while administration of 2 mg fumagillin resulted in inhibition of establishment of pregnancy in 2 out of 4 monkeys, and administration of 4 mg inhibited establishment of pregnancy in all 7 treated animals (4). From our previous reports, it appears that fumagillin-mediated antifertility effect could be mediated through its local action on vascularity at endometrial level and, thus, it may render endometrium hostile for implanting blastocyst (6).

Out of three animals in group 2, which exhibited epithelial plaque cell response as the morphological evidence of initiation of implantation (1, 6), only one showed the endocrinological profile in terms of serum E_2, P and mCG typical of pregnancy cycle. On pooled analysis, it was however revealed that P concentration was lower during days 20 to 24 in fumagillin-treated animals compared with the control animals. In a previous study, we also noted that intravaginal administration of 4 mg fumagillin during day 20 to 26 of mated ovulatory cycles resulted in a clear decline of luteal phase P, while treatment with 1 mg and 2 mg fumagillin did not cause any significant change in the luteal phase P concentrations (4). Collectively, it appears that intravaginal administration of 4 mg fumagillin inhibited the function of corpus luteum in monkeys, which led to the decreased production of P. Inadequate luteal phage P thus affected endometrial function in an adverse manner resulting in failure of pregnancy establishment (8, 13).

In the present study, we observed that anti-nidatory effect of fumagillin was associated with altered levels of cytokines in glandular epithelium and vascular compartment. In the glandular compartment, the level of TGF-β was found to be increased in fumagillin exposed endometrium, while immunopositive areas for LIF, TGF-β and VEGF were altered in vascular compartment of fumagillin treated endometrial samples. It appears feasible that an increase in LIF and a decrease in VEGF concentrations in the vascular compartment were associated with the angiostatic action of fumagillin (14, 15). Interestingly, the immunopositive areas of LIF and VEGF were not affected in the epithelial compartment. Also, there was no significant change in the levels of IL-6 in any compartment. While all of these cytokines have been implicated in the process of endometrial receptivity and blastocyst implantation (8), IL-6 does not appear to be obligatory in the process of normal fertility (16, 17).

The fact that TGF-β increases in epithelial and vascular compartments following application of anti-nidatory dose of fumagillin appears intriguing, especially because TGF-β has been suggested to play significant role in the process of endometrial preparation and blastocyst implantation and development (18–20). However, it appears possible that TGF-β may mediate biphasic role on endometrium depending on its local concentration: at an optimal concentration it tends to enhance the

functional orchestration between epithelial, stromal and vascular compartments, while at a higher concentration it tends to dysregulate their functions, to inhibit DNA synthesis and to induce apoptosis (7, 8, 21, 22).

Since these cytokines are regulated by ovarian steroid hormones (23), it is possible that the inhibitory action of fumagillin on ovarian P production could indirectly compromise endomemtrial differentiation and cytokine production. However, it is noteable that cytokine expression in the vascular compartment was primarily affected, while levels of LIF and VEGF were not affected in the glandular compartment of fumagillin exposed endometrial samples. Thus, there are two possibilities. Firstly, there may exist disparate response loop in glandular, stromal and vascular compartments to the tissue P concentration (24). In fact, it has been demonstrated that partial inhibition of P action by the administration of a high affinity antiprogestin like mifepristone affects primarily the vascular compartment in luteal phase endometrium (10, 25). Secondly, it is also possible that fumagillin which a potent angiostatic agent affects the synthesis and secretion of cytokines primarily in the vascular compartment in implantation stage endometrium (26), and thereby manifests differential responses in epithelial, stromal and vascular cells. Using the same experimental design, we have earlier, demonstrated that fumagillin treatment did not result in a remarkable change in any of the functionally critical morphological parameters of epithelial compartment in endometrium, while its vascular competence and function were affected, characterized by increased extravasation and extravascular micro-hematoma (6).

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