Endometrial tumor necrosis factor α (TNF α) is a likely mediator of early luteal phase mifepristone-mediated negative effector action on the preimplantation embryo

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Abstract

Cytokines and growth factors are important mediators of progesterone-regulated endometrial receptivity and embryo development. Early luteal phase administration of a potent antiprogesterin-like mifepristone to the rhesus monkey results in endometrial desynchrony, loss of embryo viability and implantation failure. In the present study, administration of mifepristone (2 mg/kg body weight, s.c.) on day 2 after ovulation resulted in a significant increase ($P < 0.01$) in the level of tumor necrosis factor α (TNF α) in glandular and vascular compartments of endometrium, and in endometrial secretion and luminal fluid on day 6 after ovulation in the rhesus monkey. There was an associated lag in embryonic development, characterized by delayed mitochondrial maturity, poorly developed junctional complexes, a relative absence of intra-cytoplasmic filaments and a high degree of intra-cellular degenerative features. Exposure of TNF α (0, 0.5, 5, 50 ng/ml) to preimplantation stage mouse embryos *in vitro* showed a dose-dependent arrest in growth and development at both morula and blastocyst stages along with ultra-structural features of degeneration similar to those observed in embryos collected from early luteal phase mifepristone-treated monkeys. The *de novo* synthesized and released proteins in terms of trichloroacetic acid precipitable ^{35}S by morulae and blastocysts *in vitro* showed a marked depression following exposure to TNF α compared with control embryos. Based on the above observation and the fact that preimplantation stage embryos express receptors for TNF α , we suggest that increased levels of TNF α in endometrial and luminal compartments around the time of uterine receptivity following early luteal phase administration of mifepristone adversely affect the growth and viability of preimplantation stage embryos.

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Introduction

The viability and growth of preimplantation embryos, as well as implantation of blastocysts, are regulated by a complex network of autocrine and paracrine processes involving a large number of factors (Edwards 1995, Polan *et al.* 1995, Tabibzadeh & Babaknia 1995, Ghosh & Sengupta 1998). Progesterone is essential for endometrial preparation for blastocyst implantation (Ghosh & Sengupta 1998). We have demonstrated previously in the rhesus monkey that a potent antiprogesterin-like mifepristone inhibits blastocyst implantation (Ghosh & Sengupta 1993) because the endometrium becomes desynchronized following early luteal phase mifepristone treatment in this species (Ghosh *et al.* 1996, Sengupta & Ghosh 2000). We have also demonstrated that early luteal phase exposure to mifepristone inhibits the morula-to-blastocyst transition as well as the viability of blastocysts in the rhesus monkey (Ghosh *et al.* 1997, Ghosh *et al.* 2000). We hypothesize that inhibition of progesterone action in the endometrium

alters the profiles of cytokines in endometrium and luminal milieu that in turn affects embryonic growth and viability during the preimplantation stage of gestation. Among the various principal cytokines which may reportedly be involved in these processes, tumor necrosis factor α (TNF α) appears to be a strong candidate (Ghosh & Sengupta 1998). Progesterone inhibits TNF α synthesis and secretion in endometrium (von Wolff *et al.* 1999). TNF α appears to be inhibitory to the viability, growth and differentiation of preimplantation stage blastocysts (Hill *et al.* 1987, Pampfer *et al.* 1994a,b, Wu *et al.* 1999, Ben-yair *et al.* 1997, Kurzawa *et al.* 2001). However, human embryos secrete TNF α *in vitro* until morula stage, but not at blastocyst stage (Lachapelle *et al.* 1993). Thus, it is not known whether TNF α can specifically influence morula-to-blastocyst transition and blastocyst differentiation. In the present study, we have examined this question by investigating: (i) the levels of synthesis and secretion of TNF α by implantation stage endometrium, as well as the morphological characteristics of preimplantation stage

embryos following early luteal phase mifepristone treatment to rhesus monkeys, and (ii) the effect of TNF α on the functional and developmental characteristics of mouse morulae and blastocysts following their *in vitro* exposure to TNF α . Mouse embryos were used in the second part of the study because of the ethical and practical constraints in the use of monkey embryos. Our results lend support to the proposed hypothesis that endometrial and luminal levels of TNF α increase around the time of uterine receptivity following early luteal phase mifepristone action in the rhesus monkey and that TNF α adversely affects the growth and viability of preimplantation stage embryos.

Materials and Methods

Experiments using monkeys

General procedures and treatment groups

Healthy, mature and proven fertile male and female rhesus monkeys (*Macaca mulatta*) that were kept in a semi-natural condition at the Primate Research Facility of the All India Institute of Medical Sciences (New Delhi, India) were used in this study. The details of animal selection, housing, management, monitoring of cycles, mating, laparotomy, endometrial sampling, serum collection and radioimmunoassay for serum estradiol-17 β and progesterone have been described elsewhere (Ghosh & Sengupta 1993, Ghosh *et al.* 1996, Ghosh *et al.* 1997, Ghosh *et al.* 2000, Sengupta & Ghosh 2000). The study design was approved by the Ethics Committee for the Use of Van-human Primates in Biomedical Research of the All India Institute of Medical Sciences.

Female monkeys showing at least two consecutive ovulatory menstrual cycles of normal length (26–32 days) were allocated to two groups. Animals ($n = 25$) 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 of group 1 were injected s.c. with 2 ml vehicle (1:4, benzyl benzoate:olive oil, v/v; $n = 10$) only and animals of group 2 were injected s.c. a single dose of mifepristone (2 mg/kg body weight; $n = 15$) in the same volume of vehicle on day 2 after ovulation in mated cycles. In groups 1 and 2, endometrial samples were collected on day 6 after ovulation and samples only from mated, ovulatory cycles that yielded preimplantation stage embryos were used for further examination in this study. Recovered embryos were assessed for their developmental status under

stereo-zoom microscope as described earlier (Ghosh *et al.* 2000). After retrieval of the embryo, the uterine fluid was kept at -70°C for subsequent estimation of TNF α concentration by enzyme immunoassay as described below. Table 1 shows the details of the embryos recovered from both groups on day 6 after ovulation. The days of ovulation in both groups were assessed from the profiles of estradiol-17 β and progesterone in peripheral serum samples. Steroid radioimmunoassays were performed using antisera and reagents obtained from the World Health Organization Matched Reagent Programme. The methodological details are described elsewhere (Ghosh & Sengupta 1993, Ghosh *et al.* 1996, Ghosh *et al.* 1997, Ghosh *et al.* 2000, Sengupta & Ghosh 2000).

Tissue collection and processing

The procedural details of collection and processing of endometrial samples for Western immunoblotting and immunohistochemistry have been described elsewhere (Ghosh *et al.* 1996, Sengupta & Ghosh 2000). Briefly, endometrial samples were collected on day 6 after ovulation from animals of group 1 ($n = 6$) and group 2 ($n = 7$) by performing laparotomy and fundal hysterotomy following ketamine (12 mg/kg; Vetlar, Parke-Davis, Mumbai, India) anaesthesia. For immunohistochemistry, the tissue samples were quickly washed in ice-cold phosphate buffered saline and then fixed in phosphate buffered neutral paraformaldehyde (4%, w/v) and finally embedded in paraffin wax by a routine procedure. Furthermore, tissue samples were also homogenized in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with 1 mM phenyl methyl sulphonyl fluoride (PMSF). Additionally, tissue samples were also processed for incubation in phenol red free Eagle's minimum essential medium for 16 h at 37°C in a humidified environment of 5% carbon dioxide and 95% air. Tissue homogenates and spent media were used for dot blot and western blot analysis. All chemicals were purchased from Sigma Chemical Co. (St. Louis MO, USA).

Immunoblot, enzyme immunoassay and immunohistochemistry

Profiles of TNF α in tissue spent media were characterized by the SDS-PAGE/western immunoblotting method as described elsewhere (Ghosh *et al.* 1998) using electrophoresis and trans-blot equipment from Bio-Rad (Hercules CA, USA), and the materials and chemicals

Table 1 Preimplantation embryos recovered on day 6 after ovulation^a in monkeys with or without mifepristone treatment on day 2 after ovulation^a.

Group	Number of ovulatory cycles	Number of embryos recovered				
		Degenerated/desynchronized	Morula	Early blastocyst	Blastocyst	Total
1 (vehicle only)	10	1	0	1 ^b	4 ^b	6
2 (mifepristone, 2 mg/kg)	15	2	4 ^b	0	1 ^b	7

Day of ovulation, ^a24 h after preovulatory estradiol rise in peripheral circulation. ^bembryos used in ultrastructural study.

were purchased from Bio-Rad Laboratories and Sigma Chemical Company. Samples of 25 μ g protein content and pre-stained molecular weight markers (Amersham Pharmacia Biotech, Amersham, UK) were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking the nonspecific binding, these membranes were then incubated with neutralizing antibody (R&D Systems, Minneapolis, MN, USA) against TNF α at a working dilution of 1:1500 (stock: 1 mg/ml). Final visualization was achieved by using Vectastain ABC immunoperoxidase kits (Vector Laboratories, Burlingame, CA, USA). The molecular weights and semi-quantitative densitometric analysis of bands were determined using a Gel Scanner GS 670 and Molecular Analyst image analysis software (Bio-Rad). The results are shown as Mr of individual bands and semi-quantitative measures of integral optical density.

Endometrial tissue homogenate (10 μ g of protein) was also taken for dot blot study using nitrocellulose membrane. The blocking of the nonspecific binding, immunoliganding with antibody (R&D Systems) against TNF α and final visualization was achieved using protocol as described above. Respective primary antibody control and secondary antibody control were run to examine the specificity of the procedure. Semi-quantitative estimates of integral optical density were recorded.

The concentrations of TNF α in uterine fluid collected from animals of group 1 ($n = 6$) and group 2 ($n = 7$) yielding embryos were determined by enzyme immunoassay using chemicals from R&D Systems according to the protocol provided by the manufacturer and expressed in terms of total protein estimated by Bio-Rad protein assay reagent (Gogstad & Krutness 1982).

5 μ m paraffin sections were employed for immunohistochemical localization of TNF α using specific antibody (R&D Systems) at a working dilution of 7.5 μ g/ml. Visualization was achieved using Vectastain ABC Peroxidase Elite Kit (Vector Laboratories, Burlingame, USA) and 3,3'-diaminobenzidine hydrochloride (Sigma Chemical Co.) and hydrogen peroxide following a method described previously (Ghosh *et al.* 1997). Respective primary antibody control and secondary antibody control were run simultaneously. Semi quantitative analysis of the area of immunoprecipitation for TNF α on the endometrial sections was done using computer aided image analysis (Quantimet C + , Leica, Germany) as described earlier (Ghosh *et al.* 1998).

The specificity of the antibody binding in western blot and immunohistochemistry was checked by immunoneutralization using recombinant human TNF α (R&D Systems) as described previously (Ho *et al.* 1999).

Detection of TNF-Receptor I (TNF-RI) in monkey blastocysts

Two blastocyst stage embryos were recovered from mated rhesus monkeys on day 6 after ovulation, as described

above, and were fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.2) and washed in cold phosphate buffer followed by dehydration in graded alcohol and embedded in London Resin (LR) white (Electron Microscopy Sciences, Fort Washington, PA, USA). Semi-thin (1 μ m) sections were cut and stained with toluidine blue as detailed previously (Ghosh *et al.* 1996). Prior to immunohistochemical localization of TNF-RI, the endogenous peroxidase in the embryo sections were quenched with 0.3% hydrogen peroxide following the unmasking of antigens with 0.125% (w/v) trypsin for 10 min. After washing the sections in phosphate buffered saline (pH 7.2), the sections were blocked with 2% (v/v) goat serum and exposed to anti-rabbit polyclonal antibody against hTNF-RI (Santa Cruz biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Subsequent visualization steps were performed using Vectastain Elite ABC kit and enhancing solution (Vector Laboratories).

Experiments using mice

Mouse embryo collection and culture

Random-bred sexually mature Swiss albino female mice were subjected to superovulation and allowed to mate with adult males using standardized protocol (Hogan *et al.* 1994). Day 1 of pregnancy was designated from the day of detection of copulatory plug. Uterine horns were flushed with M2 medium (pH 7.4) on the morning of day 4 (Pratt 1987). Morulae and blastocysts with normal appearance under phase contrast microscope were collected and used in different experiments as described below.

Medium M16 (pH 7.6) supplemented with sodium pyruvate (36 μ g w/v), bovine serum albumin (0.4% w/v) along with different concentrations (0, 0.5, 5 and 50 ng/ml) of TNF α (R&D Systems) was used at a ratio of 50 μ l/embryo to culture morulae (group I) and blastocysts (group II) taken in randomly assigned pool of four embryos in humidified 5% carbon dioxide in air at 37°C (Pratt 1987). Cultures were terminated at 24 h because the half-life of TNF α is about 30 h in culture medium (Wincek *et al.* 1991). The study design had been approved by the Institutional Animal Ethics Committee of AIIMS. Tables 2 and 3 show the details of different groups used in this experiment.

Developmental status of cultured mouse embryos

After 24 h culture, embryos were used to examine developmental status under light microscope. Tables 2 and 3 show the number of embryos in group I (morulae) and group II (blastocyst) used and the parameters examined to assess developmental status in this experiment.

Protein synthesis by mouse embryos

In order to examine the protein synthetic ability of morulae (group I) and blastocysts (group II) with or without TNF α (0.5, 5.0, 50.0 ng/ml) *in vitro*, embryos (~100

Table 2 Developmental status of mouse morulae cultured for 24 h in presence of TNF α .

Group	Dose of TNF α (ng/ml)	Total number of experiments	Developmental status (Total number, %)		
			Blastocyst	Arrested	Degenerated
Ia	0.0	50 (6)	41 (82%)	4 (8%)	5 (10%)
Ib	0.5	46 (5)	32 (70%)	2 (4%)	12 (26%) ¹
Ic	5.0	52 (6)	15 (29%) ³	15 (29%) ³	22 (42%) ²
Id	50.0	72 (6)	15 (21%) ³	19 (26%) ³	38 (53%) ³

¹ $P < 0.05$ compared with Ia. ² $P < 0.01$ and ³ $P < 0.001$ compared with Ia and Ib.

Table 3 Developmental status of mouse blastocysts cultured for 24 h in presence of TNF α .

Group	Dose of TNF α (ng/ml)	Total number of experiments	Developmental status (Total number, %)		
			Expanded blastocyst	Hatched blastocyst	Degenerated embryo
Ila	0.0	72 (6)	52 (72%)	16 (22%)	4 (6%)
Ilb	0.5	49 (6)	24 (49%) ¹	12 (25%)	13 (26%) ²
Ilc	5.0	53 (6)	20 (38%) ³	15 (28%)	18 (34%) ³
Ild	50.0	70 (6)	20 (28.5%) ^{3,4}	6 (8.5%) ^{3,4,6}	44 (63%) ^{3,5,7}

¹ $P < 0.05$, ² $P < 0.01$ and ³ $P < 0.001$ compared with group Ila. ⁴ $P < 0.05$ and ⁵ $P < 0.01$ compared with group Ilb. ⁶ $P < 0.01$ and ⁷ $P < 0.05$ compared with group Ilc.

Table 4 Protein synthesis and release by mouse morulae and blastocysts with or without exposure to TNF α *in vitro*.

Embryo stage	Treatment (Number of experiments)	Lysate (cpm $\times 10^{-3}$ /embryo)	Spent medium (cpm $\times 10^{-3}$ /embryo)
Morula	Control (4)	2.0 $\pm$ 0.4	6.7 $\pm$ 0.9
	TNF α , 0.5 ng/ml (5)	1.5 $\pm$ 0.6	3.6 $\pm$ 0.8 ^(**)
	TNF α , 5.0 ng/ml (5)	1.2 $\pm$ 0.5*	3.0 $\pm$ 0.9 ^(**)
	TNF α , 50.0 ng/ml (4)	1.0 $\pm$ 0.4*	1.8 $\pm$ 0.7 ^(**)
Blastocyst	Control (4)	3.1 $\pm$ 0.7*	7.8 $\pm$ 0.4 ^(**)
	TNF α , 0.5 ng/ml (5)	2.4 $\pm$ 0.5 ^(ml0.5)	4.9 $\pm$ 0.9 ^{(b)/(msm0.5)}
	TNF α , 5.0 ng/ml (4)	1.8 $\pm$ 0.4 ^{a/(ml5.0)}	4.2 $\pm$ 0.6 ^{(b)/(msm 5.0)}
	TNF α , 50.0 ng/ml (5)	1.3 $\pm$ 0.3 ^{b/(ml50)}	4.0 $\pm$ 0.4 ^{(b)/(msm50)}

Values are shown as means $\pm$ S.D. * $P < 0.05$ compared with control morula lysate. ^a $P < 0.05$ and ^b $P < 0.01$ compared with control blastocyst lysate. ^(ml0.5), (ml5.0), (ml50) $P < 0.05$ compared with morula lysates after treatment with 0.5 ng/ml, 5.0 ng/ml and 50.0 ng/ml, respectively.

^(*) $P < 0.05$ and ^(**) $P < 0.01$ compared with control morula spent medium. ^(b) $P < 0.01$ compared with control blastocyst spent medium.

^(msm0.5), ^(msm5.0) $P < 0.05$ compared with control morula spent medium after treatment with 0.5 ng/ml, and 5.0 ng/ml, respectively.

^(msm50) $P < 0.01$ compared with control morula spent medium after treatment with 50.0 ng/ml.

embryos per incubation) were cultured for 4 h in M16 medium (as described above) containing 100 μ Ci/ml of ³⁵S-methionine (Neider *et al.* 1987). Embryos were washed and lysed in sample buffer (SDS 0.30% w/v, DTT 3.09% w/v, Tris-HCl 0.44% w/v, Tris base 0.27% w/v) and spent media were also collected. Embryo lysates and media thus collected from 4–5 separate experiments, as shown in Table 4, were further analysed for the measurement of the TCA-precipitable ³⁵S according to the procedure described by Nieder *et al.* (1987) in order to estimate the protein synthesis and release by morulae and blastocysts *in vitro* with or without TNF α .

Ultrastructure of monkey and mouse embryos

Preimplantation embryos appearing normal under light microscope from both groups of monkeys (groups 1 and 2) and both groups of mice (groups I and II) were fixed in 3% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.2), post-fixed in osmium tetroxide followed by dehydration in graded ethanol, and finally embedded in Spurr's resin.

Semithin (0.5 μ m) and thin (70–80 nm) sections were cut and examined as described previously (Ghosh *et al.* 2000).

Statistical analysis

Comparisons between groups for different parameters were performed using the Fisher's exact probability test, Student's *t*-test and analysis of variance followed by multiple comparison test as applicable (Zar 1999). Values are shown as means $\pm$ S.D.

Results

Developmental status of monkey preimplantation stage embryo on day 6 after ovulation following early luteal phase (day 2 after ovulation) administration of mifepristone

As shown in Table 1, the rate of embryo recovery from mifepristone-treated monkeys (47%) was less than that from the control group (60%), however, this difference was not statistically significant. Analysis of developmental

status revealed that 57% (four/seven) of recovered embryos from the mifepristone-treated monkeys (group 2) were morulae and 29% (two/seven) embryos were desynchronized or degenerated. On the other hand, 67% (four/six) of recovered embryos from the control group (group 1) of monkeys were blastocysts, while one out of six (17%) embryos was degenerated. However, these differences were not statistically significant.

Ultrastructural characteristics of monkey preimplantation stage embryos

Among six embryos recovered from group 1 (vehicle only) control treatment animals, one transitional stage embryo and four zonal blastocysts were analyzed for transmission electron microscopic study, while seven preimplantation stage embryos were recovered from monkeys subjected to early luteal phase mifepristone treatment, of which four morulae and one zonal blastocyst were further processed for ultrastructural examination. Figure 1 shows some cardinal ultrastructural features in these embryos obtained from control and mifepristone-treated groups of animals.

As shown in Fig. 1A, the blastomeres in transitional stage embryos from control animals had large nuclei with generally dispersed heterochromatin and round to ovoid shaped mitochondria with dense matrices and without any clear cristae, they were present in clusters along with lipid droplets, while focal areas of cytoplasm lacked any organelles. Distinct inter-blastomere spaces were infrequently present and contained cellular debris. Zonal blastocysts recovered from control animals (group 1) had an enlarged cavity, which was often irregular in shape. Trophoblast cells in embryos collected from control animals exhibited marked polarity with numerous microvilli on an apical surface (Fig. 1B–E). Embryonal trophoblast cells overlying inner cell mass (ICM) cells were flattened and squamous in shape, while underlying cells of ICM showed numerous polyribosomes and coated caveolae and cytoplasmic vesicles were seen on free surfaces of blastomeres (Fig. 1B and C). Mitochondria were either elongated or large ovoid-shaped with discernible lamellar cristae with adjoining short strands of rough endoplasmic reticulum (Fig. 1C–F). Nuclei of ICM blastomeres were large with dispersed heterochromatin, intra-nuclear annulate lamellae, and prominent nuclear pores (Fig. 1B). Trophoblast cells showed long bundles of cytoplasmic filaments, distinctive apical junctions, infoldings of lateral cell membranes and desmosomes, rough endoplasmic reticulum (RER), occasional cisternae of RER filled with amorphous material, numerous plasma membrane-associated coated caveolae, cytoplasmic vesicles, and numerous microvilli on apical cell surfaces (Fig. 1E and F).

Morulae recovered from mifepristone-treated animals during the early luteal phase showed large intercellular spaces; a minimum degree of apposition of lateral cell membranes of blastomeres which, however, had retained their rounded cell shape with few apical microvilli; poorly

developed junctional complexes and relatively small, pleiomorphic mitochondria, which were present in clusters (Fig. 1G). As shown in Figs 1H and I, blastomeres of the single blastocyst retrieved following mifepristone exposure lacked the typical features generally found in such differentiated cells. For example, junctional complexes, though present, were not as well developed or as extensive as observed in blastocysts collected from control animals; mitochondria remained undifferentiated, large, and often showed a high degree of vacuolation, endoplasmic reticulum were poorly developed (Fig. 1I). Furthermore, conspicuous absence of intra-cytoplasmic filaments was a marked feature of trophoblast cells in the single blastocyst obtained from mifepristone-exposed animals. Additionally, few morphological features, generally not seen in blastocysts from control animals, were found in blastomeres of the blastocyst recovered from mifepristone-treated monkeys. These included binucleate cells, large inter-blastomere spaces suggestive of lack of compaction, intra-cytoplasmic vacuoles, autolysosomes, lipofuscin, erythrophagolysosomes, myelinoid and multivesicular bodies. Large intra-cytoplasmic vacuoles containing cellular debris, as well as various cytoplasmic contents, lysosomes, multivesicular bodies, myelinoid bodies and fatty inclusion were also frequently seen in these blastomeres (Fig. 1J and K).

$TNF\alpha$ synthesis and secretion by monkey endometrial samples

There was a significant ($P < 0.01$) increase in the per cent area occupied by immunoprecipitation of $TNF\alpha$ in glandular (group 1, 7.4 ± 1.0 ; group 2, 50.3 ± 6.0) and vascular (group 1, 11.3 ± 2.2 ; group 2, 44.8 ± 4.3) compartments but not in stroma (group 1, 11.5 ± 0.6 ; group 2, 14.6 ± 1.1) on day 6 following mifepristone treatment compared with control group samples in histometric analysis of per cent area of immunoprecipitation (Fig. 2).

Analysis of optical density data from dot blot and western blot experiments also revealed that the level of $TNF\alpha$ in homogenate (0.67 ± 0.07) and spent media (1.62 ± 0.03) was higher ($P < 0.01$) for endometrial samples collected from mifepristone-treated animals compared with that in homogenate (0.28 ± 0.04) and spent medium (0.46 ± 0.03) obtained from control animals. As shown in Fig. 3, western blot analysis revealed two bands (17 and 35 kD) in spent media from both groups. Since both bands are absent in antibody-preincubated negative control samples, we presume that the two bands in endometrial secretion arose from monomeric and dimeric $TNF\alpha$ presentation. Although we did not further investigate this issue, similar observation has been reported earlier (Ameloot *et al.* 2001).

The uterine fluid concentration of $TNF\alpha$ was significantly ($P < 0.01$) higher in mifepristone-treated animals (4.4 ± 0.5 ng/100 μ g protein) compared with control animals (1.2 ± 0.3 ng/100 μ g protein).

Figure 1 Ultrastructural characteristics of blastomeres of embryos from control (group 1) monkeys (A–F) and from mifepristone-treated (group 2) monkeys (G–K). *Arrow*, junctional complex; *Brackets*, myelinoid bodies; *c*, caveolae; *cd*, cellular debris; *Et*, embryonic trophoblast; *f*, filaments; *ICM*, inner cell mass; *I*, lipid droplet; *ly*, lysosome; *m*, mitochondria; *mb*, multivesicular body; *mv*, microvilli; *N*, nucleus; *rer*, rough endoplasmic reticulum; *ZP*, zona pellucida. Bars: 2 µm (C, D); 3 µm (F, H–K); 5 µm (E); 6 µm (G); 10 µm (A, B).

TNF-RI in the preimplantation stage monkey embryo

As shown in Fig. 4, TNF-RI was localized in both inner cell mass and polar trophoblast cells in monkey blastocysts.

Developmental status of mouse preimplantation stage embryos following TNF α exposure in vitro

Tables 2 and 3 show the details of developmental characteristics of morulae and blastocysts collected on day 4 of

gestation and cultured for 24 h *in vitro* in the presence of different concentrations of TNF α .

As shown in Table 2, at morula stage TNF α , at a concentration of 0.5 ng/ml (Group 1b), resulted in a marginally higher ($P < 0.05$) incidence of degeneration, while there was no significant change in the per cent morulae developed into blastocysts compared with controls (Group 1a). The number of embryos showing degenerated and arrested features, however, increased following exposure to higher concentrations (5 and 50 ng/ml) of TNF α , as compared

Figure 2 Immunohistochemical localization of TNF α antigen in endometrial tissue collected on day 6 after ovulation from control (group 1) monkeys (A) and from mifepristone-treated (Group 2) monkeys (B–D). Compared with the control sample, mifepristone treatment resulted in a high degree of immunostaining for TNF α in the apical zone of glandular epithelial cells (B) and in the vascular compartment (C). No immunostaining was found when the primary antibody was immunoabsorbed with TNF α antigen (D) as described in the Materials and Methods section. Bars: 10 μ m (C); 20 μ m (A, B, D).

with group Ia (control) and group Ib (TNF- α , 0.5 ng/ml). As a result, the per cent of morulae undergoing blastocyst development in group Ic (TNF- α , 5 ng/ml) and group Id (TNF- α , 50 ng/ml) was significantly ($P < 0.001$) less than that found in group Ia (control) and group Ib (TNF- α , 0.5 ng/ml).

As shown in Table 3, when blastocysts were incubated with different doses of TNF α , a dose dependent increase ($P < 0.01$ for group IIb, and $P < 0.001$ for groups IIc and IId) in the per cent of embryos showing obvious degeneration, and a dose dependent decrease ($P < 0.05$ for group IIb, and $P < 0.001$ for groups IIc and IId) in the development of embryos to form expanded blastocysts as compared with those in control group embryos (group IIa) were recorded. No statistically significant change was observed in the per cent of blastocysts degenerated or expanded between group IIb (TNF α , 0.5 ng/ml) and group IIc (TNF α , 5 ng/ml). Also, no change was observed in the per cent blastocysts expanded between group IIc (TNF α , 5 ng/ml) and group IId (TNF α , 50 ng/ml). However, the per cent of blastocysts degenerated was higher in group IId as compared with group IIb ($P < 0.01$) and group IIc ($P < 0.05$). On the other hand, there was no change in the per cent number of blastocysts hatched following exposure to 0, 0.5, and 5.0 ng/ml TNF α in culture medium; however it decreased significantly ($P < 0.01$) with exposure to very high concentrations of TNF α (50 ng/ml) as compared with all other treatment groups.

Ultrastructural characteristics of mouse preimplantation stage embryos following TNF α exposure *in vitro*

Morulae and blastocysts collected on day 4 from mated female mice, without apparent abnormality as seen under stereozoom microscope were subjected to *in vitro* culture

in medium containing different doses of TNF α (0, 0.5, 5 and 50 ng/ml) for 24 h following which apparently normal looking blastocysts were fixed for transmission electron microscope (TEM) examination. Figure 5 highlights the ultrastructural features of blastocysts developed from morulae and blastocysts, respectively, after 24 h culture with or without TNF α . TEM examination revealed that morulae that did develop into blastocysts *in vitro*, however, showed a higher degree of degenerative features in blastomeres following exposure to TNF α , especially at higher concentrations (5 ng/ml and 50 ng/ml) studied (Fig. 5C and D) compared with control embryos (Fig. 5A and B).

Figure 3 Western immunoblot for TNF α in spent medium of endometrial tissue collected on day 6 after ovulation from control (lane 1) and mifepristone-treated (lane 2) monkeys. Lane 3 shows spent medium of sample used in lane 2 that had been preincubated with neutralizing antibody as described in the Materials and Methods section. Optical density of both bands (Mr 35 and 17 kD) was considered for densitometric analysis.

Figure 4 Rhesus monkey blastocyst (1 µm section) showing immunolocalization for TNF-R1 (A). A contiguous 1 µm embryo section showed no staining for TNF-R1 following incubation in medium lacking the primary antibody (B). Bar: 25 µm.

All blastocysts in this group (control and TNF α -exposed) were in either substage one or two of their development with clear blastocoelic cavities according to the classification described earlier (Nadijcka & Hillman 1974). Ultra-structural examination of blastocysts from the control group revealed close clustering of mitochondria which were beginning to show cristae, but matrix remained mostly electron-dense; mitochondria were found closely associated with short strands of RER and small to large cisternae of RER were often engorged with amorphous inclusions; lipid droplets were occasionally lined with discrete glycogen particles. A large nucleus bearing 'honeycomb' nucleoli and evenly dispersed chromatin was characteristically observed. Distinctive apical and basolateral junctional complexes were discernible in such control group blastocysts (Fig. 5A and B). Following exposure to TNF α , morulae developed into blastocysts but several degenerative features were clearly distinguishable: embryonic trophoblast showed loss of polarized features with apical as well as basolaterally distributed microvilli in cells; these cells were largely cuboidal and irregular in shape and showed a high degree of fatty inclusions, reactive lysosomes, lipofuscin, degenerative vacuoles, intra-cytoplasmic villi, blastomeres showing large intercellular spaces, woolly transformation of mitochondria and marked distortion in nuclear membrane contour more predominantly in blastomeres of the inner cell mass (Fig. 5C and D). These features were commonly not seen in the control group blastocysts.

Blastocysts incubated for 24 h in control medium underwent development to substage three type differentiation with ICM showing epiblast and proximal endoderm layer (Fig. 5E and F). Mitochondrial differentiation was found to be distinctive at this stage of development; polarization of cytoplasmic organelles characterized by distribution of mitochondria and Golgi zones in peri-nuclear location was seen (Fig. 5E and F). Blastocysts exposed to TNF α at varying doses showed various degrees of cellular degenerative features (Fig. 5G–J) which included large

intra-cytoplasmic vacuoles enclosing cellular debris, lipid droplets, lipofuscin containing large residual bodies, large conglomerations of glycogen, primitive types of myelinoid structures reminiscent of such bodies found in monkey blastocysts recovered from mifepristone-exposed animals (Fig. 1J and K), loss of mitochondrial differentiation and numerous primitive junctional complexes unlike blastocysts from the control group. Although stereological analysis was not done in the present study, the relative abundance of such degenerative features on subjective analysis was considered fewer in blastomeres of blastocysts exposed to TNF α compared with that found in morulae exposed to TNF α .

Protein synthesis in mouse preimplantation stage embryos following TNF α exposure *in vitro*

Table 4 shows the data of ^{35}S -methionine labeled proteins in spent media as well as in lysates of morulae (group I) and blastocysts (group II) with and without TNF α (0.5, 5.0, and 50.0 ng/ml) treatment *in vitro*. It is evident from the data that embryonic protein synthesis as well as protein secretion were higher ($P < 0.05$) at blastocyst stage compared with morula stage between respective sets of different treatments (for example between control-treated morulae and blastocysts, 0.5 ng TNF α /ml treated morula and blastocysts, 5.0 ng TNF α /ml treated morula and blastocysts, and 50 ng TNF α /ml-treated morula and blastocysts) and that exposure to 5.0 and 50.0 ng TNF α per ml medium resulted in statistically significant ($P < 0.05$) inhibition of embryonic protein synthesis both at morula and blastocyst stages, while protein secretion by morula stage and blastocyst stage embryos was significantly ($P < 0.01$) affected even at 0.5 ng TNF α per ml medium.

Discussion

The results of the present study confirm that subcutaneous administration of mifepristone at a dose of 2 mg per kg

body weight adversely affects the growth of preimplantation stage embryos, particularly from morula stage onwards (Ghosh *et al.* 1997, 2000) and that mouse preimplantation stage embryos exposed to TNF α *in vitro* exhibited failure of morula-to-blastocyst transition in a dose-dependent manner. Furthermore, ultrastructural examination revealed that mouse embryos exposed *in vitro* to TNF α showed many similarities in their ultrastructural features to blastomeres of embryos exposed *in vivo* to mifepristone with loss of trophoblast cell polarity, lack of mitochondrial maturity and a higher abundance of degenerative features. The degenerative features like the high degree of fatty inclusion, lipofuscin, myelin bodies and inter-blastomere spaces as observed in the blastomeres of embryos retrieved from mifepristone-exposed monkeys and in the blastomeres of mouse embryos exposed *in vitro* to TNF α are reminiscent of the reported ultrastructural characteristics of blastomeres of t^{12}/t^{12} and tw^{32}/tw^{32} mouse embryos (Hillman *et al.* 1970, Hillman & Hillman 1975). Interestingly, both t^{12}/t^{12} and tw^{32}/tw^{32} embryos show delay in the process of morula-to-blastocyst transition (Hillman *et al.* 1970, Hillman & Hillman 1975), and lose their viability at very early stages of gestation (Nadijcka & Hillman 1975). We have earlier reported that monkey embryos exposed *in vivo* to mifepristone fail to yield evolvable implantation (Ghosh *et al.* 1997). Psychoyos & Prapas (1987) observed that administration of mifepristone to rats on days 1 and 2 of pregnancy resulted in a blockade of the morula-to-blastocyst transition.

The possibility that TNF α could be an effector candidate in mediating the embryotoxic effect of mifepristone is further substantiated by the fact that: (i) endometrial synthesis and secretion of TNF α is increased resulting in its higher concentration in luminal milieu following early luteal phase mifepristone treatment, and (ii) preimplantation blastocyst stage monkey embryos exhibit the receptor for TNF α (TNF-R1).

Although it is possible that mifepristone may directly act on preimplantation stage embryos, indirect evidence that: (i) mifepristone does not affect the growth of preimplantation stage embryos of the rhesus monkey (Wolf *et al.* 1990), and (ii) there was no detectable mifepristone in the

Figure 5 Ultrastructural characteristics of mouse blastocysts developed *in vitro* following 24 h culture of morulae in control medium (A, B) and in medium containing 0.5 ng/ml (C) and 50 ng/ml (D) TNF α . Ultrastructural features of mouse blastocysts grown for 24 h in control medium (E, F) and in medium containing 0.5 ng/ml (G), 5.0 ng/ml (H, I) and 50 ng/ml (J) TNF α . Arrow, junctional complex; asterisk, intracytoplasmic vacuole; brackets, myelinoid bodies; cd, cellular debris; cv, intracytoplasmic vacuole; Ep, central epiblast; Et, embryonal trophoblast; g, glycogen; gl, perinuclear Golgi zone; l, lipid droplet; lp, lipofuscin; Lt, elongated lateral trophoblast; ly, lysosome; m, mitochondria; mb, multivesicular body; mo, vacuolated mitochondria; mw, woolly mitochondria; mv, microvilli; N, nucleus; Pent, proximal endoderm; rer, rough endoplasmic reticulum; ZP, zona pellucida. Note that the inset area in (E) is shown in enlarged form in (F). Bars: 2 μ m (F); 3 μ m (B, I, J); 4 μ m (C); 6 μ m (G, H); 10 μ m (A, E); 15 μ m (D).

luminal fluid following luteal phase administration of mifepristone (2 mg/kg body weight) to rhesus monkeys (unpublished data), casts serious doubt on this possibility. We have demonstrated earlier that low dose mifepristone applied during the early luteal phase does not affect the serum concentrations of estradiol-17 β and progesterone and thereby ovarian functions in the rhesus monkey (Ghosh & Sengupta 1993). It now appears likely that early luteal phase antiprogesterin action affects endometrial function in a way that the luminal environment becomes hostile and cannot support preimplantation embryo growth (Antonakis *et al.* 1994, Beir-Hellwig *et al.* 1994, Ding *et al.* 1994, Psychoyos *et al.* 1995, Qui *et al.* 2001). This notion is indeed substantiated by the observation that passive immunization against progesterone in the mother during early pregnancy resulted in arrested embryonic growth in mice, rats and ferrets (Wang *et al.* 1984, Rider & Heap 1986, Phillips *et al.* 1988).

Mifepristone is a potent anti-glucocorticoid and anti-progesterone (Philibert, 1984). Generally, mifepristone-mediated inhibition of glucocorticoid activity in mammalian tissues, as well as inhibition of progesterone action specifically in endometrial cells, tend to increase TNF α (Gaillard *et al.* 1985, Busso *et al.* 1987, Lazar *et al.* 1992, Tabibzadeh *et al.* 1995). In an interesting study, Laird *et al.* (1996) have demonstrated that there was a down regulation of TNF α production by secretory phase endometrium in the presence of progesterone *in vitro*. Therefore, we anticipated that early luteal phase application of mifepristone blocks progesterone and glucocorticoid action in the implantation stage endometrium and upregulates endometrial synthesis and secretion of TNF α . The results of the present study have now indeed demonstrated that mifepristone administration on day 2 after ovulation results in increased endometrial synthesis and secretion of TNF α on day 6 after ovulation.

In the next phase, we tested the hypothesis that a high level of luminal TNF α affects embryo growth. Because of practical and ethical constraints this hypothesis could not be tested using monkey embryos, and therefore it was tested using mouse embryos. TNF α affects morula-to-blastocyst transition and blastocyst growth *in vitro* in a dose-dependent manner. Morphological changes, based on ultrastructural examination, and functional changes, based on the profile of proteins synthesized and released by apparently normal-looking embryos exposed to TNF α *in vitro*, were significantly different compared with those found in embryos in control treatment. The possibility that TNF α present in culture medium can adversely affect the growth of the preimplantation stage embryo is also substantiated by several reports based on studies using different models. TNF α has been shown *in vivo* and *in vitro* to reduce the viability of mouse blastocysts, and rat blastocysts along with high levels of apoptotic cell death (Pampfer *et al.* 1994a,b, Wu *et al.* 1999, Kurzawa *et al.* 2001). The observation in the present

study that preimplantation stage embryos express receptors for TNF α corroborates well with earlier studies on mouse preimplantation stage embryos (Pampfer *et al.* 1994b, 1995).

Morula-to-blastocyst transition is a critical step in the process of embryogenesis characterized by ultrastructural polarization (Enders *et al.* 1990, Ducibella *et al.* 1995, Ohsugi *et al.* 1999) and specific profiles of proteins synthesized at this developmental stage (Zheng *et al.* 1993, Shi *et al.* 1994, Sasaki *et al.* 1999). Mouse blastocysts allowed to develop *in vivo* are classified into four sub-stages based on their ultrastructural features: substage one, when blastocoelic cavity is well formed but not expanded, substage two, when the cavity is expanded but ICM cells do not show any clear-cut differentiation, substage three, when ICM shows differentiation into epiblast and proximal endoderm, and substage four, when visceral endoderm develops and forms a continuous layer around the blastocyst cavity (Nadijcka & Hillman 1974). Embryos in the control group showed development until substage three, which was not achieved by embryos exposed to TNF α . It is interesting to note that in the present study, though a population of morulae underwent differentiation into blastocysts, their development was significantly compromised after TNF α exposure *in vitro*, characterized by a loss of cell polarity in trophoblast cells, lack of mitochondrial maturity, and a higher occurrence of inter-blastomere space, intra-cytoplasmic vacuoles, lysosomes and lipofuscin and multivesicular bodies (Ghadially 1988). It appears that input from the immediate microenvironment can influence these processes (Neider *et al.* 1987, Enders *et al.* 1989). A subgroup of embryos both in mice and monkeys had escaped the adverse effects putatively mediated by TNF α . In the present study, one out of seven embryos could attain blastocyst stage, and in our previous reports one out of twelve embryos, and five out of sixteen embryos could attain blastocyst stage despite application of mifepristone to rhesus monkeys (Ghosh *et al.* 1997, 2000). In mice, more than 20% of morulae could develop into blastocysts and a similar percentage of blastocysts could expand and hatch even in the presence of TNF α , despite a dose-dependent increase in the number of degenerated embryos. It has been shown in mouse that the morphological event of cavitation at the morula stage can occur even when either DNA replication was inhibited (Dean & Rossant 1984) or cytokinesis was blocked (Pratt *et al.* 1981). It is also possible that these embryos do not express the functional receptors for TNF α or the proteins required for their down-stream signal transduction (Takahashi *et al.* 1995, Sridhar *et al.* 2001). The significance of this observation is only speculative at this point in time. It may be conjectured that the group of embryos, which are relatively resistant to TNF α , may escape environmental hostility and thereby give an advantage of providing effort toward establishing pregnancy despite the possibility that such environmental hostility may cause some defect in the organism. Alternatively, based on

experiments using TNF α positive and TNF α knock-out mice, Toder *et al.* (2003) concluded that TNF α boosts death signaling to kill the embryo if initial damages triggered by detrimental stimuli culminate in structural anomalies and stimulate protective mechanisms if the repair of these damages may prevent maldevelopment.

It is to be noted that morulae which could develop in mifepristone-treated female monkeys, and in the presence of TNF α in mouse embryo cultures, exhibited a higher abundance of ultrastructural defects in their blastomeres along with a deficit in protein synthesis. Despite the fact that some events in embryogenesis may proceed in a fixed action pattern even in a hostile environment, environmental deficit or hostility can leave an unfavourable imprint, which in turn may adversely affect the required sufficiency and time synchrony of interactions between the embryo and endometrium resulting in failure of blastocyst implantation. This notion is supported by the observation that apparently normal-looking preimplantation stage embryos retrieved from luteal phase mifepristone-treated monkeys could not support implantation and evolutive pregnancy (Ghosh *et al.* 1997), and that apparently normal-looking mouse blastocysts exposed to TNF α *in vitro* failed to implant (Wuu *et al.* 1999).

Thus, we conclude that delay in embryo development along with the onset of degenerative changes observed in monkey preimplantation stage embryos following mifepristone exposure may be contributed by increased TNF α concentrations in uterine luminal fluid. The possibility that early luteal phase mifepristone administration can affect the equilibrium of a larger network of cytokines in the endometrium, involving transforming growth factor β (TGF β), interferon γ (IFN γ) and interleukins (ILs), besides TNF α (Antanakis *et al.* 1994, Rotello *et al.* 1991, Shaarawy & Nagui 1997) resulting in irreversible damage to the preimplantation embryo needs to be examined. Additionally, the relative depletion of specific growth factors like epidermal growth factor (EGF) and insulin like growth factors (IGFs) in the endometrium may also potentiate detrimental effect of TNF α (Wu *et al.* 1996, Ho *et al.* 1999) on embryo growth following luteal phase administration of mifepristone.

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