Effect of tumour necrosis factor α on rat blastocyst growth and glucose metabolism

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Tumour necrosis factor α (TNF- α) synthesis has recently been described in the uterus during the preimplantation phase of pregnancy. The present study was undertaken to determine whether preimplantation embryos are a potential target for TNF- α in rats. First, the expression of TNF- α receptors by blastocysts was demonstrated by ligand binding assay with human ^{125}I -labelled TNF- α and reverse transcription–amplification for the p60 receptor form. The functionality of these receptors was then assessed by incubating blastocysts with 3 nmol mouse TNF- α l^{-1} *in vitro* and determining their morphology and the number of cells after 24 h. At that concentration, cell proliferation in blastocysts was inhibited by TNF- α but this was not accompanied by any change in the morphology of the embryos. Similar results were obtained when lower doses of TNF- α (30 and 300 pmol l^{-1}) were tested. The rate of glucose consumption of rat blastocysts exposed to 3 nmol TNF- α l^{-1} was not altered when they were incubated with the cytokine for 4 h, but the rate of glucose incorporation decreased over the same period. Our data indicate that rat blastocysts are responsive to physiological concentrations of TNF- α and that this cytokine has the potential to influence the preimplantation development of rats.

Introduction

It is almost 20 years since tumour necrosis factor α (TNF- α) was first identified in the serum of immunologically challenged mice (Carswell *et al.*, 1975). Since that time researchers have been exploring the many pleiotropic activities that it assumes in a wide array of biological systems. The best-known actions of TNF- α include the rejection of transplanted tumours, reactions to inflammatory and immunological agents, and the development of the cachectic condition that accompanies chronic illness (Fiers, 1991; see Vassalli, 1992 for review). Although macrophages have been identified as the primary cellular source of TNF- α (Männel *et al.*, 1980), many different tissues and cell types can synthesize this cytokine upon adequate stimulation (Giroir *et al.*, 1992). Even more impressive are both the ubiquitous distribution of TNF- α -specific receptors in virtually all organs and tissues (Goodwin *et al.*, 1991; Lewis *et al.*, 1991) and the spectrum of possible biological reactions to TNF- α at the cellular level. *In vitro*, this cytokine can stimulate the growth of fibroblasts (Vilcek *et al.*, 1986), repress the differentiation of myoblasts (Miller *et al.*, 1988), elicit the secretion of ACTH by pituitary cells (Bernardini *et al.*, 1990), inhibit the lipoprotein lipase activity in adipocytes (Price *et al.*, 1986) and induce the degeneration of oligodendrocytes (Selmaj and Raine, 1988).

Considering the above observations, the recently reported indications that TNF- α might also play an important role in the regulation of the reproductive system are not surprising. In the female rat, local TNF- α synthesis has been described in the ovary (Roby and Terranova, 1989; Sancho-Tello *et al.*, 1992) and the uterus (Yelavarthi *et al.*, 1991). During the peri-implantation phase of pregnancy in this species, TNF- α is predominantly synthesized by the epithelial cells lining the uterine lumen and the decidualized cells adjacent to the implanted embryo (Yelavarthi *et al.*, 1991). Although rat ovarian thecal cells (Zachow *et al.*, 1992; Brännström *et al.*, 1993) and rat placental trophoblasts (Hunt *et al.*, 1989, 1990) have been identified as responsive to TNF- α , it is still unclear whether the development of embryos could also be directly influenced by TNF- α before or at the time of implantation. Previous attempts to address this issue using mouse embryos have failed to detect any significant effect of TNF- α *in vitro* during the preimplantation and peri-implantation developmental phases unless very high concentrations were used (Hill *et al.*, 1987; Haimovici *et al.*, 1991; Wincek *et al.*, 1991; Lachapelle *et al.*, 1993). In the present study, we report that rat blastocysts express TNF- α -specific receptors before their implantation, an absolute prerequisite for any biological effect of TNF- α , and that when this cytokine is added at physiological concentrations to the culture medium *in vitro* it influences the rates of cell proliferation and glucose incorporation by the blastocysts.

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Materials and Methods

Reagents

Human recombinant ^{125}I -labelled TNF- α and D-[5- ^3H]glucose were purchased from Amersham (Bucks). Mouse recombinant TNF- α was purchased from Boehringer (Mannheim), and rabbit anti-mouse TNF- α polyclonal antibody from Calbiochem (San Diego, CA). Taq DNA polymerase used in PCR was obtained from Cetus Perkin Elmer (Emeryville). Earle's balanced saline solution (EBSS), Dulbecco's PBS and glucose-free Ham's F-10 medium were purchased from Gibco (Paisley), α -D(+)-glucose and polyvinylpyrrolidone were from Sigma (St Louis, MO). Moloney murine leukemia virus (MMLV) reverse transcriptase was purchased from United States Biochemicals (Cleveland, OH).

Embryos

Sexually mature male and female Wistar rats from our breeding centre were mated overnight and checked the next morning (day 1 of pregnancy) for the presence of a vaginal plug. On day 5, embryos at the blastocyst stage were recovered from the uterus by inserting a 26-gauge needle into the utero-tubal junction and gently flushing 0.4 ml prewarmed Ham's F-10 medium through each uterine horn. Embryos from different litters were pooled before being randomly distributed among culture groups in batches of 8–10 embryos.

Ligand binding

The presence of TNF- α receptors at the cell surface of rat blastocysts was tested by radioligand binding assay (Paria and Dey, 1990) with ^{125}I -labelled TNF- α . Embryos were released from their zona pellucida with 0.4 g pronase l^{-1} and allowed to recover from the enzymatic treatment for 2 h in EBSS supplemented with 1 mmol glutamine l^{-1} , 1 mmol pyruvate l^{-1} , 3 g BSA l^{-1} , 100 iu penicillin ml^{-1} and 100 μg streptomycin ml^{-1} . The blastocysts were then incubated for 30 min at 37°C with 70 pmol ^{125}I -labelled TNF- α l^{-1} (final specific activity was 600 Ci mmol^{-1}) in PBS containing 1 g BSA l^{-1} , washed repeatedly in ice-cold PBS with the same concentration of BSA, and fixed in 40 g paraformaldehyde l^{-1} in PBS for 15 min before their transfer onto polylysine-coated slides. The preparations were cytospun at 45 g for 5 s, dehydrated through increasing grades of alcohol and applied directly against an autoradiographic film (Davis *et al.*, 1989). Control experiments were carried out in parallel with a 500-fold molar excess (35 nmol l^{-1}) of unlabelled TNF- α added to displace the radioactive probe.

mRNA analysis

Total RNA was extracted from pools of 30 embryos in the presence of 20 μg *E. coli* ribosomal RNA using a micro-adaptation of the guanidium isothiocyanate-phenol chloroform isolation method (Chomczynski and Sacchi, 1987), and reverse-transcribed for 90 min at 37°C in a volume of 50 μl with 80 μmol random cDNA primers l^{-1} and 100 iu MMLV

reverse transcriptase per reaction (Pampfer *et al.*, 1992). After enzymatic heat inactivation, 5 μl of the reverse-transcribed sample was added to 95 μl of PCR mixture containing 1 μmol PCR primers l^{-1} and 5 iu Taq polymerase (Pampfer *et al.*, 1992). Amplification consisted of 40 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min. PCR primers complementary to nucleotides 287–307 and 710–730 of the cDNA sequence that encodes the p60 form of the rat TNF- α receptor (TNF- α Rp60) (Himmeler *et al.*, 1990) were designed to generate an amplicon of 444 basepairs (bp). On the basis of the great similarity (78%) between rat TNF- α Rp60 and human TNF- α Rp55 cDNAs in the targeted region, and the possibility that their intron-exon structure may be conserved, it was assumed that the amplification of contaminating genomic DNA would produce a distinct amplicon. Preliminary amplification of DNA purified from rat uterine cells with the same TNF- α Rp60 primers produced an amplicon of about 1100 bp (data not shown). PCR primers complementary to nucleotides 184–203 and 405–424 of the rat β -actin cDNA sequence (Nudel *et al.*, 1983) were used in control reactions. The β -actin-specific primers generated an amplicon of 241 bp from cDNA and 327 bp from genomic DNA. Total RNA from about 5×10^5 uterine cells (obtained by flushing the reproductive tract) was analysed in parallel for β -actin and TNF- α Rp60 mRNA expression. PCR reactions were subjected to electrophoresis on 2% agarose gels in the presence of 1 μmol ethidium bromide l^{-1} .

Culture

The culture medium was Ham's F-10 supplemented with 6 mmol glucose l^{-1} , 1 mmol glutamine l^{-1} , 1 g of either BSA or polyvinylpyrrolidone l^{-1} and antibiotics as above (De Hertogh *et al.*, 1991) with or without TNF- α . Incubations were carried out at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Control medium contained the same final concentration of TNF- α vehicle solution as did the TNF- α medium. During incubation, the embryo:volume ratio was maintained at one blastocyst per 25 μl of medium. In some control experiments, the culture medium containing TNF- α was incubated with a 500-fold molar excess of neutralizing anti-TNF- α antibody for 1 h at 37°C before use. The morphology of the blastocysts and the number of cells were determined at both the start and the end of the incubation time. The developmental stages were scored according to the following criteria: blastocyst with a nascent blastocoele, 7; blastocyst with clearly demarcated trophoctoderm and inner cell mass, 8; expanded blastocyst with enlarging blastocoele, 9; hatching blastocyst, 10; and fully hatched blastocyst, 11 (Gates, 1965). The number of cells was estimated using a differential fluorochrome staining technique that allows for the separate counting of inner cell mass and trophoctoderm cells in individual blastocysts (Pampfer *et al.*, 1990). Embryos were denuded with 0.4 g pronase l^{-1} , incubated for 30 min at 37°C in 10% rabbit anti-rat antiserum in EBSS and treated for 30 min at 37°C with 5% guinea-pig serum in EBSS to immunolyse the trophoctoderm cells. This treatment resulted in the permeabilization of these cells to two DNA fluorescent dyes, bisbenzimidazole (20 μg ml^{-1}) and propidium iodide (10 μg ml^{-1}), whereas the

Fig. 1. Presence of tumour necrosis factor α (TNF- α) receptors at the cell surface of rat blastocysts. (a) Two blastocysts were incubated with ^{125}I -labelled TNF- α and transferred onto glass slides before autoradiography. A positive signal was obtained for each embryo. The size of the spots was larger than that of the embryos owing to the diffusion of γ particles into the film emulsion. (b) Two blastocysts were treated as above in the presence of a molar excess of unlabelled TNF- α . No autoradiographic signal was obtained. Scale bar: 3 mm.

inner cell mass was permissive to bisbenzimidazole only. Cells containing nuclear fragments were counted as dead cells and the dead cell index of each embryo was calculated in both inner cell mass and trophoblast cell lineages as described by Pampfer *et al.* (1990).

Glucose metabolism

Groups of three blastocysts were placed in metabolic chambers in 5 μl Ham's F-10 supplemented with 280 μmol glucose l^{-1} , 1 g BSA l^{-1} , 25 mmol Hepes l^{-1} , antibiotics and D-[5- ^3H]glucose (final specific activity was 1.1 Ci mmol^{-1}) (Dufrasnes *et al.*, 1993) with or without TNF- α . Glucose consumption was measured after 4 h of incubation at 37°C. Previous experiments have shown that about 82% of the $^3\text{H}_2\text{O}$ released by rat blastocysts in 4 h were recovered in the NaOH chemical trap. At the end of the incubation, the embryos were recovered, washed and the amount of radiolabelled glucose that had been incorporated determined (Dufrasnes *et al.*, 1993).

Statistical analysis

Statistical differences between control and TNF- α culture groups were analysed by unpaired Student's *t* test or one-way analysis of variance, as appropriate. Comparisons between distributions of number of cells were carried out using the χ^2 test.

Results

TNF- α receptors

The presence of TNF- α receptors was visualized at the cell surface of rat blastocysts by means of a radiolabelled ligand-binding technique (Fig. 1). Autoradiographic signals for ^{125}I -labelled TNF- α were repeatedly detected on these embryos and

Fig. 2. Presence of mRNA transcripts for the p60 form of the tumour necrosis factor α (TNF- α) receptor in rat blastocysts. (a) Total RNA from three different batches of about 50 embryos (BL) was reverse transcribed and amplified for rat β -actin. The expected 241 bp amplicon (arrowhead) was visualized after electrophoresis. Total RNA from uterine cells present in the flushing medium (FLM) generated the same amplicon as well as a product probably derived from the amplification of genomic DNA. (b) The same blastocyst preparations were amplified for the p60 form of the TNF- α receptor and produced the expected 444 bp amplicon (arrowhead). No RNA specific amplicon was obtained from uterine cells but a product with the predicted size (about 1100 bp) of a genomic TNF- α Rp60 amplicon was detected.

the binding of ^{125}I -labelled TNF- α was completely displaced by the addition of a 500-fold molar excess of unlabelled TNF- α .

Blastocysts were then tested by reverse transcription and PCR for the presence of mRNA encoding the p60 form of the TNF- α receptor (Fig. 2). Total cDNA was prepared from three batches of embryos and first analysed using β -actin PCR primers. An amplicon of the predicted size for β -actin (241 bp) was generated using cDNA from all three embryo batches. cDNA prepared from uterine debris produced the same fragment and a larger product that was identified by its size as resulting from the amplification of contaminating genomic DNA. When TNF- α Rp60 primers were used in similar PCR reactions, they generated a 444 bp amplicon that was confirmed as deriving from the cDNA for TNF- α Rp60 by restriction enzyme mapping (data not shown). No amplicon of this size was produced when total cDNA from uterine debris was amplified with the same set of TNF- α Rp60 primers, indicating that the positive reactions in the embryo cDNA samples were not due to the presence of contaminating uterine cells. A weak PCR band of about 1100 bp was visible in the uterine cell cDNA sample analysed with the TNF- α Rp60 primers, and probably resulted from the amplification of genomic DNA present in that preparation.

Embryo growth

After 24 h of incubation, no significant difference was found in the morphology of blastocysts that were grown in the presence or absence of 3 nmol TNF- α l^{-1} (Table 1). In each culture group, about 65% of the blastocysts were expanded, 25% were in the process of hatching out of their zona pellucida and 10% were fully hatched. The morphological development of these embryos in the presence or absence of TNF- α was not modified when the medium was supplemented with polyvinylpyrrolidone instead of BSA.

Table 1. Effect of 3 nmol tumour necrosis factor α (TNF- α) l^{-1} on the morphology of rat blastocysts incubated for 24 h

Culture medium	Morphological score					
	At start of incubation		At end of incubation (24 h)			
	Control	Number of embryos	Control	Number of embryos	TNF- α	Number of embryos
Ham's F10 + BSA	7.83 $\pm$ 0.07	29	9.40 $\pm$ 0.13	30	9.21 $\pm$ 0.08	29
Ham's F10 + PVP	8.06 $\pm$ 0.05	49	9.28 $\pm$ 0.06	54	9.21 $\pm$ 0.06	52

Results are given as means $\pm$ SEM.
PVP: polyvinylpyrrolidone.

Table 2. Effect of 3 nmol tumour necrosis factor α (TNF- α) l^{-1} on cell proliferation by rat blastocysts incubated for 24 h

Culture medium	Cell lineage	Cell number					
		At start of incubation		At end of incubation (24 h)			
		Control	Number of embryos	Control	Number of cells	TNF- α	Number of embryos
Ham's F10 + BSA	ICM	11.54 $\pm$ 0.63		18.96 $\pm$ 0.75		16.04 $\pm$ 0.76 ^b	
	TE	22.46 $\pm$ 0.77		47.82 $\pm$ 1.74		44.12 $\pm$ 1.62	
	Total	34.34 $\pm$ 1.23	24	66.73 $\pm$ 2.24	22	60.15 $\pm$ 2.25 ^a	26
Ham's F10 + PVP	ICM	12.93 $\pm$ 0.57		17.42 $\pm$ 0.56		15.76 $\pm$ 0.53 ^a	
	TE	24.46 $\pm$ 1.07		50.50 $\pm$ 1.22		45.47 $\pm$ 1.13 ^b	
	Total	37.68 $\pm$ 1.53	45	68.06 $\pm$ 1.53	50	61.22 $\pm$ 1.35 ^b	45

Results are given as means $\pm$ SEM.

The superscripts a and b indicate significant differences between control and TNF- α -treated groups at $P < 0.05$ and $P < 0.01$, respectively.

ICM: inner cell mass; PVP: polyvinylpyrrolidone; TE: trophectoderm cells.

To analyse these embryos further, the number of cells was counted at the start of the incubation period and after 24 h in the presence or absence of 3 nmol TNF- α l^{-1} (Table 2). In control medium supplemented with BSA, the total number of cells doubled during the culture to reach an average of 67 cells per blastocyst. There were 10% fewer cells in blastocysts that had been grown in the presence of TNF- α ($P < 0.05$). This decrease amounted to 15.4% in the inner cell mass and 7.8% in the trophectoderm cells when calculated separately. When embryos were cultured in a medium supplemented with polyvinylpyrrolidone instead of BSA, the overall decrease induced by 3 nmol TNF- α l^{-1} in the total number of cells was of the same order of magnitude ($P < 0.01$), but the cellular deficiency was evenly distributed between the inner cell mass and the trophectoderm cells. The negative effect of TNF- α was fully neutralized by an anti-TNF- α antibody, since its addition in 500-fold molar excess corrected the cell distribution shift induced by TNF- α (Fig. 3). Anti-TNF- α antiserum or normal serum IgG had no effect on blastocyst proliferation

when used alone at the same excess concentration (data not shown).

To ascertain whether the inhibitory effect of TNF- α could be reproduced at more physiological concentrations, the cell proliferation experiments were repeated using a culture medium supplemented with polyvinylpyrrolidone and 30 or 300 pmol TNF- α l^{-1} in addition to the previous dose of 3 nmol l^{-1} . In the presence of 30 pmol TNF- α l^{-1} , the total number of cells was decreased by 9%, with 12% of the difference occurring in the inner cell mass and 8% in the trophectoderm cells; the same results were obtained with 300 pmol l^{-1} ($P < 0.05$) (Fig. 4). The morphology of the blastocysts was not modified by TNF- α at any of the three doses. The data therefore indicated that the maximal inhibition of cell proliferation by TNF- α was probably about 10% in rat blastocysts and that this effect could be achieved *in vitro* with 30 pmol TNF- α l^{-1} .

To determine whether the cellular deficiency associated with TNF- α was mediated by the cytostatic or cytotoxic properties

Fig. 3. Neutralization of the decrease in number of cells induced by tumour necrosis factor α (TNF- α) in rat blastocysts. Embryos were incubated in control medium ($\square$), with 3 nmol TNF- α l⁻¹ ($\square$) or with 3 nmol TNF- α l⁻¹ neutralized with an anti-TNF- α antibody ($\blacksquare$), and the total number of cells was counted after 24 h. The distribution pattern, shifted to the left following exposure to TNF- α ($P < 0.05$), was corrected by neutralizing the cytokine. More than 50 blastocysts were tested in each group.

of the cytokine, preparations of blastocysts exposed to 3 nmol TNF- α l⁻¹ for 24 h were carefully analysed for the presence of dead cells in the inner cell mass and trophectoderm cell lineages (Table 3). Dead cells were identified by their scattered arrays of irregular nuclear fragments. Although there was a clear increase in both inner cell mass and trophectoderm dead cell indices following culture for 24 h compared with initial values, addition of 3 nmol TNF- α l⁻¹ did not modify these proportions in a significant manner. Comparable results were obtained irrespective of whether BSA or polyvinylpyrrolidone was added to the culture medium.

Glucose metabolism

Blastocysts were cultured for 4 h in the presence or absence of 3 nmol TNF- α l⁻¹ and assessed for glucose utilization (Fig. 5). Addition of TNF- α at that concentration failed to modify the rate of glucose consumption through the glycolytic pathway, but decreased the rate of incorporation of glucose-derived compounds into blastocysts ($P < 0.05$).

Discussion

The study reported here was designed to determine whether rat blastocysts are a potential target for TNF- α when they are preparing for their implantation in the uterus. Previous reports have demonstrated that uterine epithelial and decidual cells are primary sites of TNF- α synthesis during early pregnancy in rats (Yelavarthi *et al.*, 1991), and similar results have been obtained when both mouse and human reproductive tissues were investigated during the same gestational

Fig. 4. Influence of different doses of tumour necrosis factor α (TNF- α) on cell proliferation by rat blastocysts. Embryos were incubated in control medium or with increasing concentrations of TNF- α , and the number of inner cell mass cells (ICM) ($\square$) and the number of trophectoderm cells (TE) ($\square$) were counted after 24 h. More than 40 embryos were tested in each group and the results are given as means $\pm$ SEM. *Differences between control embryos and TNF- α -treated embryos were statistically significant ($P < 0.05$) for both number of ICM cells and number of TE cells at the three doses tested.

period (Chen *et al.*, 1991; Hunt *et al.*, 1993). Transient increases in both mRNA and protein content have been detected for TNF- α on day 1 of pregnancy and during the peri-implantation period in the mouse uterus (Sanford *et al.*, 1992; De *et al.*, 1993).

The aim of the first part of our investigation was to determine whether rat blastocysts express TNF- α receptors. ¹²⁵I-labelled TNF- α binding was observed on the cell surface of blastocysts and this ligand binding was eliminated in the presence of competing unlabelled TNF- α . Displacement of ¹²⁵I-labelled TNF- α by lymphotoxin α (TNF- β), which also binds to TNF- α receptors (Schoenfeld *et al.*, 1991), was not investigated because there has been no published evidence of lymphotoxin α synthesis in the pregnant uterus. The expression of TNF- α receptors by rat blastocysts was further supported by the detection of corresponding mRNAs using the reverse transcription-PCR technique. Although two distinct TNF- α receptors have been identified in mice (Goodwin *et al.*, 1991; Lewis *et al.*, 1991) and in humans (Fiers, 1991; Vassalli, 1992), only one of these receptors (TNF- α Rp60) has been molecularly cloned in rats (Himmeler *et al.*, 1990). Reverse transcription-PCR analysis of different batches of embryos indicated that at least the TNF- α Rp60 form is expressed in rat embryos at the blastocyst stage. The two TNF- α receptors that have been characterized in other species are known to initiate distinct signal transduction pathways in responsive cells, and recent comparative studies have demonstrated the predominant role of the TNF- α p60 receptor over the Rp80 form in many cellular systems (Tartaglia *et al.*, 1991). It is possible that rat embryos co-express both TNF- α receptor forms during the

Table 3. Effect of 3 nmol tumour necrosis factor α (TNF- α) l^{-1} on dead cell indices in rat blastocysts incubated for 24 h

Culture medium	Cell lineage	Dead cell index (%)					
		At start of incubation		At end of incubation (24 h)			
		Control	Number of embryos	Control	Number of embryos	TNF- α	Number of embryos
Ham's F10 + BSA	ICM	≤ 0.50		5.07 ± 1.84		7.27 ± 1.43	
	TE	≤ 0.50		1.17 ± 0.61		0.66 ± 0.35	
	Whole embryo	≤ 0.50	24	2.17 ± 0.57	22	2.36 ± 0.50	26
Ham's F10 + PVP	ICM	≤ 0.50		6.84 ± 1.49		4.35 ± 0.92	
	TE	≤ 0.50		≤ 0.50		≤ 0.50	
	Whole embryo	≤ 0.50	45	1.67 ± 0.34	50	1.35 ± 0.27	45

Results are given as means $\pm$ SEM.

ICM: inner cell mass; PVP: polyvinylpyrrolidone; TE: trophectoderm cells.

Fig. 5. Influence of tumour necrosis factor α (TNF- α) on the rates of glucose consumption and incorporation by rat blastocysts. (a) Embryos were incubated in control medium (control) or with 3 nmol TNF- α l^{-1} (TNF- α) in the presence of D-[5- 3 H]glucose and tested for their glucose consumption after 4 h, as measured by the quantitative recovery of 3 H $_2$ O in a NaOH chemical trap. (b) At the end of the incubation period, the embryos were washed and assessed for the incorporation of glucose. Control and TNF- α results were significantly different from each other (* $P < 0.05$). More than 50 batches of three embryos were tested in each group and the results are given as means $\pm$ SEM.

preimplantation period but, if earlier observations in the mouse are of any relevance, this seems unlikely. Previous studies have indeed shown that only the p60 form of the TNF- α receptor, and not the p80 form, is expressed in early post-implantation embryos (Goodwin *et al.*, 1991) and this comparison has recently been extended to the preimplantation period (R. De Hertogh, Y.-D. Wun and S. Pampfer, unpublished).

Once the presence of TNF- α receptors on rat embryos was established, their function was studied by examining different developmental parameters in blastocysts following their incubation for 24 h with TNF- α . Concentrations of about 25 pmol l^{-1} have been detected in human follicular fluids (Wang *et al.*, 1992) and in peritoneal fluids associated with endometriosis (Taketani *et al.*, 1992). Circulating concentrations of TNF- α in obese rats can reach 10 pmol l^{-1} (Hotamisligil

et al., 1993) and previous experiments demonstrating the action of TNF- α on rat placental and ovarian cells were carried out with doses from 0.01 nmol l^{-1} to 50 nmol l^{-1} (Hunt *et al.*, 1989, 1990; Andreani *et al.*, 1991; Zachow *et al.*, 1992; Brännström *et al.*, 1993). In the study reported here, doses of 30 and 300 pmol l^{-1} , and 3 nmol TNF- α l^{-1} were tested on rat blastocysts. Incubations were performed in a culture medium supplemented with either BSA or a synthetic macromolecule (polyvinylpyrrolidone) (Cholewa and Whitten, 1970) because commercially available BSA preparations sometimes contain co-purified contaminants that might interfere with the experiments (Gardner and Kaye, 1991).

The three different TNF- α doses significantly inhibited the rate of cell proliferation in rat blastocysts, without affecting their morphological appearance. The extent of this inhibition was approximately 10% of the total number of cells and equally distributed between the two embryonic cell lineages studied (the inner cell mass and trophectoderm cells). These two cellular populations are known to contribute to distinct embryonic components following implantation, with the inner cell mass cells giving rise to all of the fetal germ layers and the trophectoderm cells participating in the formation of the embryo-maternal interface (Hebel and Stromberg, 1986). Although the extent of the cellular decrease induced by TNF- α is small, it is important to consider that any cell loss caused by arrested cell multiplication or cell death in the inner cell mass lineage before implantation will result in a much larger deficiency a few cell divisions later, when this type of cell is expected to form the primitive egg cylinder. Previous experimental studies have clearly demonstrated that failure to provide enough inner cell mass cells prior to this critical developmental phase was incompatible with a successful post-implantation embryogenesis, even when placental derivatives appeared normal (Tam, 1988).

Our data suggest that an early exposure to a physiological dose of TNF- α may diminish the ability of rat blastocysts to generate viable fetuses. It should also be remembered that TNF- α often needs to synergize with other cytokines to

achieve full efficiency (Fiers, 1991; Vassalli, 1992) and that many of these co-factors are secreted by uterine cells during the preimplantation period (Pampfer *et al.*, 1991). Surprisingly, the inhibitory effect of TNF- α on cell proliferation was apparently not mediated by a marked increase in the occurrence of cell death, suggesting that TNF- α is embryostatic rather than embryotoxic on early rat embryos when tested in the absence of other factors.

As a preliminary step towards understanding how TNF- α could mediate its action on preimplantation embryonic growth, the possibility that TNF- α may alter glucose metabolism was investigated in blastocysts. Rat embryos at this stage have recently been shown to take up and use glucose actively, mostly through the glycolytic metabolic pathway, during the blastocyst phase (Brison and Leese, 1991; Dufrasnes *et al.*, 1993). Insulin, which can modulate cell proliferation in rat blastocysts *in vitro* (De Hertogh *et al.*, 1991), was found to do so without affecting glucose metabolism (Dufrasnes *et al.*, 1993). At concentrations within the 1–10 nmol l⁻¹ range, TNF- α stimulates glucose uptake in cell types such as muscle cells (Lee *et al.*, 1987), fibroblasts (Cornelius *et al.*, 1990) and vascular endothelial cells (Pekala *et al.*, 1990). In these cells, the effect of TNF- α was mediated through the upregulation of type-1 glucose transporters, a form of the Na⁺-independent glucose transporter family that is also present in mouse preimplantation embryos (Hogan *et al.*, 1991; Aghayan *et al.*, 1992). To test whether TNF- α could interfere with embryonic glucose metabolism, blastocysts were cultured in the presence of 3 nmol TNF- α l⁻¹ and D-[5-³H]glucose during a period that was too short to allow the cytokine to affect embryonic cell proliferation. TNF- α did not modify the rate of glycolysis in the treated embryos but decreased the incorporation of radio-labelled glucose into macromolecules. Because TNF- α can also enhance the rate of glycogen breakdown in muscle cells (Lee *et al.*, 1987), these results suggest a possible association between TNF- α and the pattern of glycogen accumulation in rat blastocysts.

TNF- α is presumably one of the most pleiotropic cytokines to have been characterized so far, and its implication in several biological mechanisms involved in the host defence against infection and cancer means that its synthesis must be vital in many instances. Clearly, such a critical status has severe drawbacks when it becomes unbalanced, and it is now well established that TNF- α can also induce extremely deleterious effects when released in an inappropriate dose with respect to time (Fiers, 1991; Vassalli, 1992). Previous demonstrations that TNF- α is locally synthesized in the pregnant uterus along with the data presented here raise interesting questions as to the role of this cytokine in the regulation of early pregnancy.

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