Endocrine (1995) 3, 557-562

Expression and localization of messenger RNA for tumor necrosis factor receptor (TNF-R) I and TNF-RII in pregnant mouse uterus and placenta

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The temporal and cell-specific localization of tumor necrosis factor (TNF) receptor mRNAs in the uterus and placenta during pregnancy in the mouse was investigated. Messenger RNA for TNF and the TNF receptors (TNF-RI, p55/p60 and TNF-RII, p75/p80) was assessed by northern blot and *in situ* hybridization. TNF, TNF-RI and TNF-RII specific transcripts were present on days 7 through 18 of pregnancy. Relative concentrations of TNF mRNA decreased from days 7 to 18 with levels being higher in the uterus than the placenta. In contrast TNF-RI mRNA levels were constant throughout gestation and no differences were seen between steady state levels in the uterus and placenta. Two transcripts for TNF-RII (3.6 and 4.5 kb) were identified in all tissues. Steady state levels of TNF-RII mRNA increased throughout gestation and levels were higher in the placenta than in the uterus. On day 9 of gestation, TNF-RI and TNF-RII mRNAs were localized to undecidualized endometrium, mesometrial decidual cells, and the developing placenta. In addition, muscle cells contained TNF-RI but not TNF-RII mRNA. By day 15 of gestation, TNF-RI and TNF-RII transcripts were primarily localized to the uterine epithelium and trophoblast giant cells and spongiotrophoblast cells in the placenta. The results of these studies reveal the uterine and placental cell-specific expression of TNF receptor mRNAs during pregnancy in the mouse and provide insight into the cellular targets of TNF action.

Keywords: messenger RNA; mouse; placenta; tumor necrosis factor; tumor necrosis factor receptors; uterus

Introduction

The expression of TNF mRNA and protein in the uterus and placenta has been described (Yelavarthi *et al.*, 1991; Hunt *et al.*, 1992; Philippeaux & Piguet, 1993; Roby & Hunt, 1994; Roby & Hunt, 1995) and has provided the rationale for several hypotheses as to the function of uterine/placental TNF (Hunt, 1993; Hunt *et al.*, 1994; Pollard & Mitchell, 1993; Tabibzadeh *et al.*, 1993). In order for TNF to exert biological function it must first bind to specific membrane receptors, TNF-RI (p55/60) and/or TNF-RII (p75/80). Human placental membrane preparations exhibit high affinity and low affinity TNF receptor binding sites (Eades *et al.*, 1988; Aiyer & Aggarwal, 1990; Hayakawa *et al.*, 1991). However, TNF receptors have not been localized to specific types of cells. The presence of TNF-R in the mouse uterus and placenta has not been established.

The uterus and placenta are made up of several different cell types expressing varied functional phenotypes. In order to understand the potential roles of uteroplacental TNF during pregnancy, it is necessary to define the cell-specific expression of TNF receptors. This report describes the uterine and placental cell-specific expression of TNF-RI and TNF-RII mRNA during pregnancy in the mouse and, thus, identifies the uterine and placental cells which are the probable sites of direct TNF binding and action. These data provide insight into the cell-specific sites of TNF action and support the postulate that TNF has important pregnancy-associated functions.

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Received 2 March 1995; accepted 2 May 1995

Results

Characterization of TNF receptor probes

Preliminary experiments were performed to characterize the TNF-RI and TNF-RII probes prepared in our laboratory using cell lines previously shown to express messenger RNA for TNF and the specific TNF-R forms. The RAW mouse macrophage cell line stimulated with LPS expresses TNF mRNA (Yelavarthi *et al.*, 1991); the EL4 mouse lymphoma cell line expresses only TNF-RI mRNA (Goodwin *et al.*, 1991); and the 70Z/3 mouse pre-B lymphocyte cell line expresses only TNF-RII mRNA (Goodwin *et al.*, 1991).

As shown in Figure 1, the TNF probe recognized a single transcript of approximately 1.9 kb in the RAW cell line. In addition, TNF transcripts were detected in RNA from 70Z/3 cells and more weakly from the EL4 cell RNA. The TNF-RI probe hybridized with a single transcript of approximately 2.6 kb, the reported size for the mouse TNF-RI mRNA, in the EL4 cell but not the 70Z/3. The TNF-RII probe hybridized with two transcripts of approximately 4.5 and 3.6 kb, in agreement with the reported sizes for the TNF-RII mRNAs. TNF-RII mRNA was present in 70Z/3 and RAW RNA but not EL4 RNA.

Detection of TNF, TNF-RI and TNF-RII mRNAs in pregnant uterus and placenta by northern blot hybridization analysis

TNF The mouse uterus and placenta expressed TNF mRNA on days 7 through 18 of pregnancy (Figure 2). Densitometric analysis revealed that steady state levels of TNF mRNA in the intact uterus did not change greatly from day 7 to day 9 (Figure 3). On days 12, 15 and 18 the placenta and uterus were analysed separately. Steady state levels of TNF mRNA were somewhat higher in the uterus than in the placenta. TNF mRNA levels in both the uterus and placenta decreased between days 12 and 18 of pregnancy (Figure 3).

TNF receptors The mouse uterus and placenta contained transcripts from both TNF receptor genes. TNF-RI expression was relatively constant in both the uterus and placenta from day 7 through day 18 of gestation (Figures 2 and 3). In contrast, steady state levels of TNF-RII mRNA increased from day 7 to day 9 in the intact uterus. During the second half of pregnancy, the placenta expressed markedly higher levels of TNF-RII mRNA than the uterus. The steady state

levels of TNF-RII mRNA in the uterus remained relatively constant from days 12 to 18 while the steady state levels in the placenta peaked at day 15 and decreased slightly by day 18. The two TNF-RII transcripts of approximately 4.5 and 3.6 kb were expressed at all time points (Figure 2). Expression of the 3.6 kb transcript was always lower than the 4.5 kb transcript, although the ratio of 4.5 to 3.6 did not change as determined by densitometry (data not shown). Densitometric analysis of the 4.5 kb transcript is illustrated in Figure 3. Data presented in Figures 2 and 3 are representative of three independent experiments.

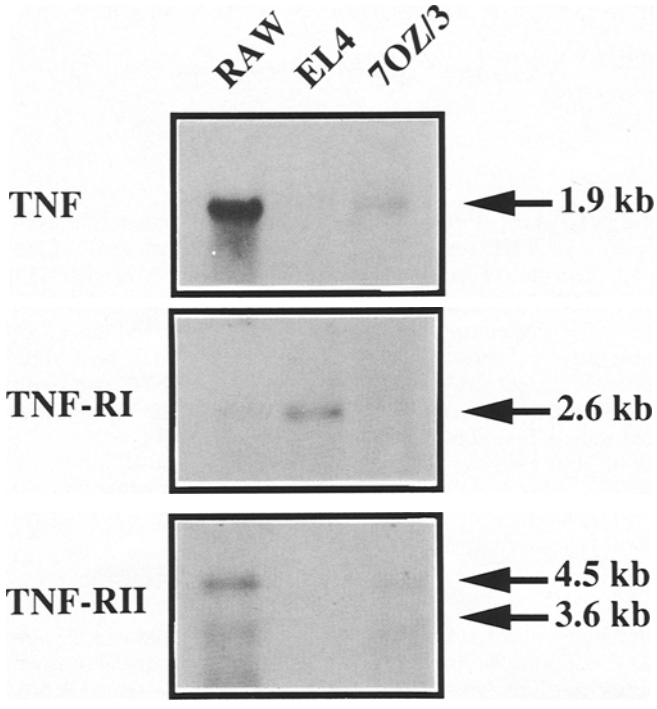

Figure 1 Characterization of the TNF, TNF-RI and TNF-RII probes. Poly (A)$^+$ RNA (2 μg/lane) isolated from cells was fractionated by formaldehyde gel electrophoresis and transferred to nitrocellulose. RAW.264, a mouse macrophage cell line, expresses TNF mRNA when stimulated with LPS.EL4, a mouse lymphoma cell line, expresses TNF-RI mRNA and 70Z/3, a mouse pre-B lymphocyte, expresses TNF-RII mRNA. The sizes of the mRNAs were estimated by comparison with the migration of RNA standards in the same gel and are indicated on the right

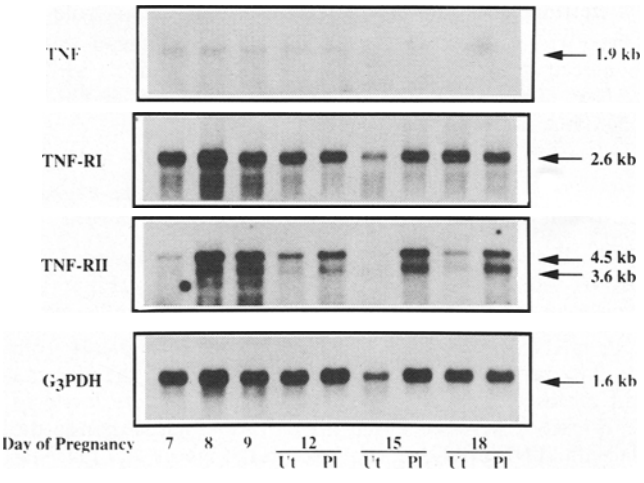

Figure 2 Northern analysis for TNF, TNF-RI and TNF-RII mRNAs during pregnancy. Poly(A)$^+$ RNA (2.5 μg/lane) was isolated from intact uteri on days 7, 8 and 9 of pregnancy and from uteri and placentas separately on days 12, 15 and 18 of pregnancy. RNAs were fractionated by formaldehyde gel electrophoresis and transferred to nitrocellulose. Northern blots were hybridized with ^{32}P-labeled cRNA (TNF and TNF-RI) or cDNA (TNF-RII and G3PDH) probes. The sizes of the mRNAs were estimated by comparison with the migration of RNA standards in the same gel and are indicated on the right. Data are representative of three independent experiments

Localization of TNF, TNF-RI and TNF-RII mRNAs in pregnant uterus and placenta by in situ *hybridization analysis*

In situ hybridization was performed for TNF, TNF-RI and TNF-RII on intact uteri collected on the same days of pregnancy as has been assessed by northern blot hybridization. The cell specific localization of TNF mRNA during gestation in the mouse has been previously reported. In the present studies TNF mRNA was localized to uterine epithelium, decidua, and trophoblast cells as already reported and therefore these data not shown. The cellular localization of TNF-RI and TNF-RII on day 9 and day 15 of pregnancy will be described and are representative of the localization observed throughout pregnancy. *In situ* hybridization results for the boxed areas in Figure 4 are shown in Figures 5 and 6.

TNF receptors On day 9 of pregnancy TNF-RI mRNA was primarily localized to the undecidualized endometrium and myometrium (Figure 5A). Hybridization signals were also observed in the developing placenta, although signal intensity was lower than that observed in the myometrium. Cells in the decidua capsularis (or anti-mesometrial decidua) did not contain detectable TNF-RI mRNA. However, a weak hyb-

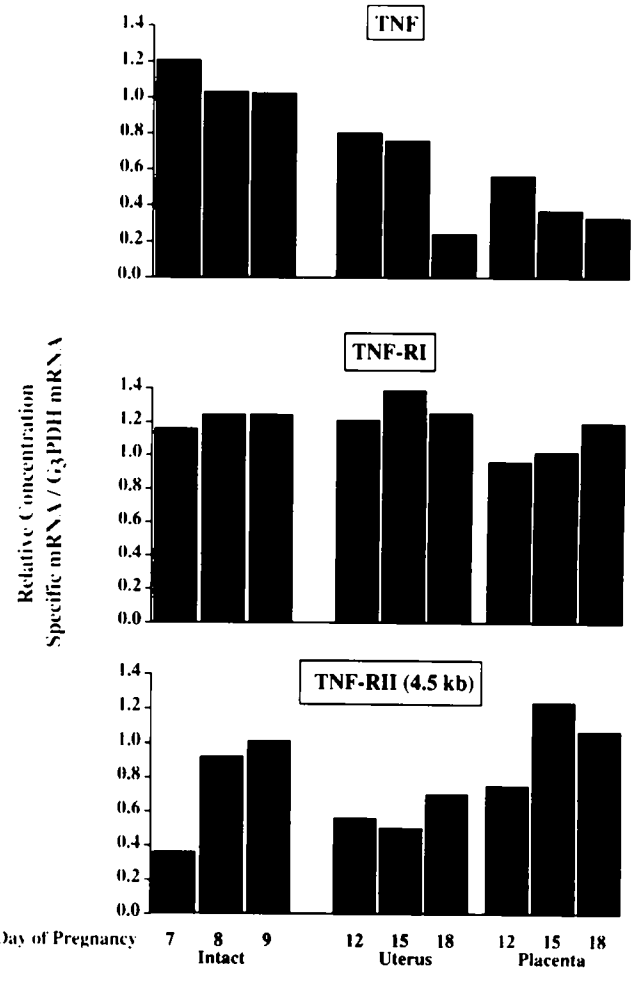

Figure 3 Densitometric analysis of TNF, TNF-RI and TNF-RII mRNA expression during gestation. Densitometric analysis of the northern blots shown in Figure 2 is expressed as the relative concentration of the density of specific hybridization to the density of hybridization with G3PDH probe. Data are representative of three independent experiments

ridization signal was identified in some mesometrial decidual cells.

TNF-RII was also primarily localized to the undecidualized endometrium on day 9 of pregnancy (Figure 5C). In contrast to TNF-RI, TNF-RII mRNA was not detected in the myometrium. However, weak hybridization signals were present in the developing placenta. Similarly to TNF-RI, TNF-RII mRNA was localized to mesometrial decidual cells and individual cells distributed throughout the decidua (not shown).

Later in gestation, on day 15, the distribution of TNF-RI and TNF-RII transcripts was very similar. Receptor mRNAs were localized to trophoblast giant cells, spongiotrophoblast cells and glycogen cells in the junctional zone of the placenta. In addition, intense hybridization signals were observed in the uterine epithelium (Figure 6).

The controls for all *in situ* hybridization experiments included the use of labeled sense orientation probes or non-specific DNA in place of the antisense probe. These controls which were done for each tissue in every experiment were invariably negative.

Discussion

This report describes the expression of TNF and TNF-R mRNA from 2 days after implantation to just prior to parturition in the mouse. This is the first report describing the cellular localization of TNF-RI and TNF-RII mRNA in the mouse uterus and placenta. The presence of TNF mRNA and protein in the uterus and placenta has been established (Chen *et al.*, 1991; Yelavarthi *et al.*, 1991; Hunt *et al.*, 1992, 1993; Roby & Hunt, 1994, 1995). In addition, the cellular sources, including uterine epithelium and placental trophoblasts, have been demonstrated. Currently, the function of TNF in pregnancy is not known, however, the cellular localization of TNF receptors provides insights into the possible roles for TNF during pregnancy.

There are two TNF receptors, TNF-RI and TNF-RII, which bind TNF with different affinities, affect different intracellular signalling pathways, and hence presumably mediate different cellular responses (Tartaglia & Goeddel, 1992). The role of each receptor is currently under intense study (Gehr *et al.*, 1992; Kruppa *et al.*, 1992; Vandenabeele *et al.*, 1992; Wong *et al.*, 1992; Tartaglia *et al.*, 1993). Although the responses mediated via each receptor do not appear to be exclusive, in general, TNF-RI appears to mediate cytotoxicity (Tartaglia *et al.*, 1991; Wong *et al.*, 1992) while TNF-RII is associated with proliferation (Tartaglia *et al.*, 1991; Gehr *et al.*, 1992).

Northern hybridization analysis in the present study indicated that the mRNA for TNF-RI is present at a relatively constant level throughout pregnancy, however, a shift in the cell-types expressing TNF-RI mRNA from early to late pregnancy was revealed by *in situ* hybridization analysis. In contrast to the constant levels of TNF-RI mRNA, TNF-RII mRNA levels increased as pregnancy progressed. During the second half of pregnancy TNF-RII mRNA was more abundant in the placenta as compared to the uterus. The results of this study are similar to the expression patterns of TNF-RI and TNF-RII mRNA reported for the human placenta (Yelavarthi & Hunt, 1993). TNF-RI mRNA was expressed at a constant level in tissues assessed from first trimester and term placentas while mRNA for TNF-RII fluctuated (Yelavarthi & Hunt, 1993). The same study indicated that specific TNF-R protein was present in the same cells expressing specific TNF-R mRNA. One exception to this was observed in first trimester tissues where stromal cells contained TNF-RI mRNA but not protein (Yelavarthi & Hunt, 1993). Correlation of the TNF-R mRNA described in the present study with receptor protein levels awaits the generation of specific antibodies. TNF-R mRNA and protein patterns may be either similar or different. Taken together, these data indicated that the overall level of TNF-R mRNA increases as pregnancy progresses and that there is a shift in the cells expressing receptor mRNA from endometrium and myometrium early in pregnancy to placenta and epithelium late in pregnancy.

TNF has been shown to be associated with preterm labor (Romero *et al.*, 1989) and has been hypothesized to be involved in premature myometrial contraction. However, the contractile activity of murine myometrium isolated from day 18 of pregnancy was not affected by *in vitro* treatment with TNF (Oshiro *et al.*, 1993). From the data presented in the present study, it would appear that TNF receptors are not abundant in the mouse myometrium late in gestation which might explain the results from the contractility study (Oshiro *et al.*, 1993). Other studies have demonstrated that vascular smooth muscle contraction is diminished after TNF treatment *in vitro* (McKenna *et al.*, 1988; Busse & Mulsch, 1990). It would be interesting to determine the effect of TNF earlier in gestation on induced myometrial activity when TNF might serve a protective role in reducing myometrial contraction at an inappropriate time. The abundance of TNF-R in the myometrium early in pregnancy might also indicate a role for TNF in the increase in vascular permeability that occurs

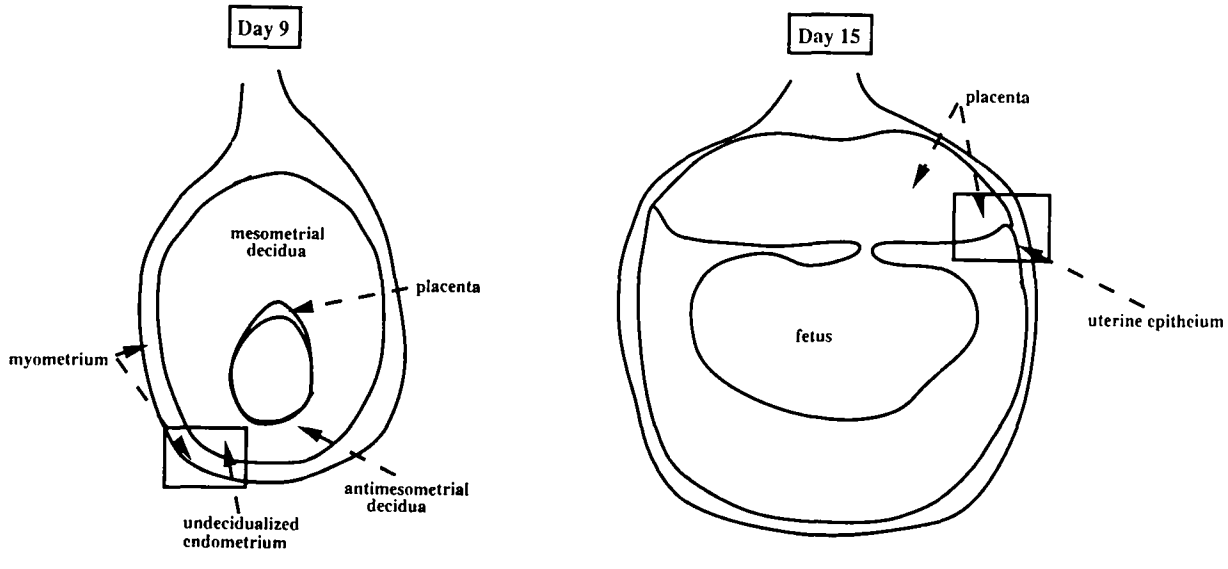

Figure 4 Line drawings of the mouse uterus and placenta on day 9 and day 15 of pregnancy. The boxed areas represent the regions shown in Figures 5 and 6

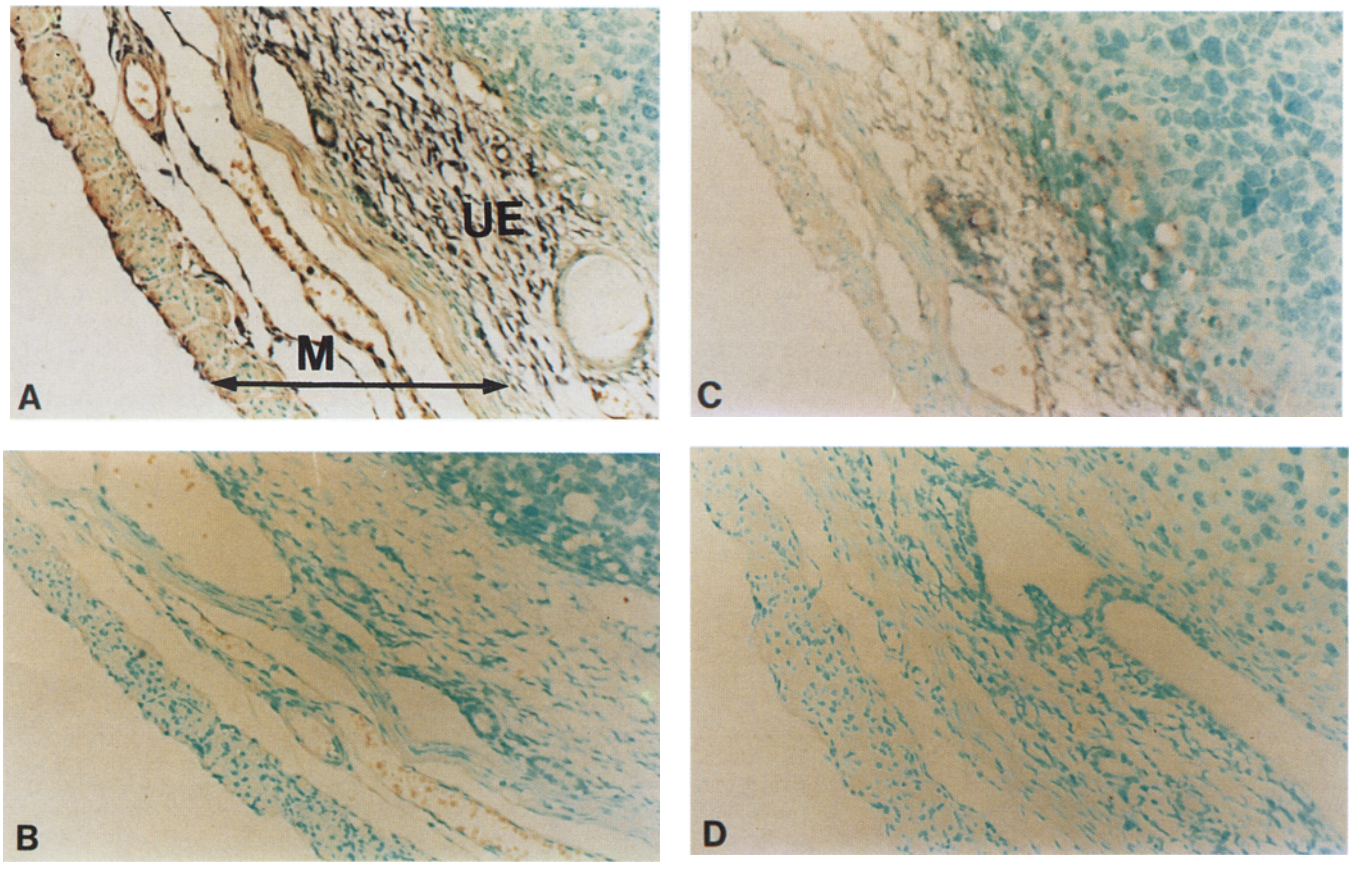

Figure 5 *In situ* hybridization analysis for TNF-RI and TNF-RII mRNA on day 9 of pregnancy. *In situ* hybridization experiments were conducted using a 498 bp antisense cRNA specific for TNF-RI (**A**) and a 930 bp cDNA specific for TNF-RII (**C**). TNF-RI mRNA (**A**) is localized to the undecidualized endometrium and muscle. TNF-RII mRNA (**C**) is localized to the undecidualized endometrium. The sense version of the TNF-RI cRNA and nonspecific DNA served as controls for the TNF-RI and TNF-RII probes (**B** and **D**, respectively) and exhibited no hybridization. UE: undecidualized endometrium; M: myometrium. Original magnification, 200 X

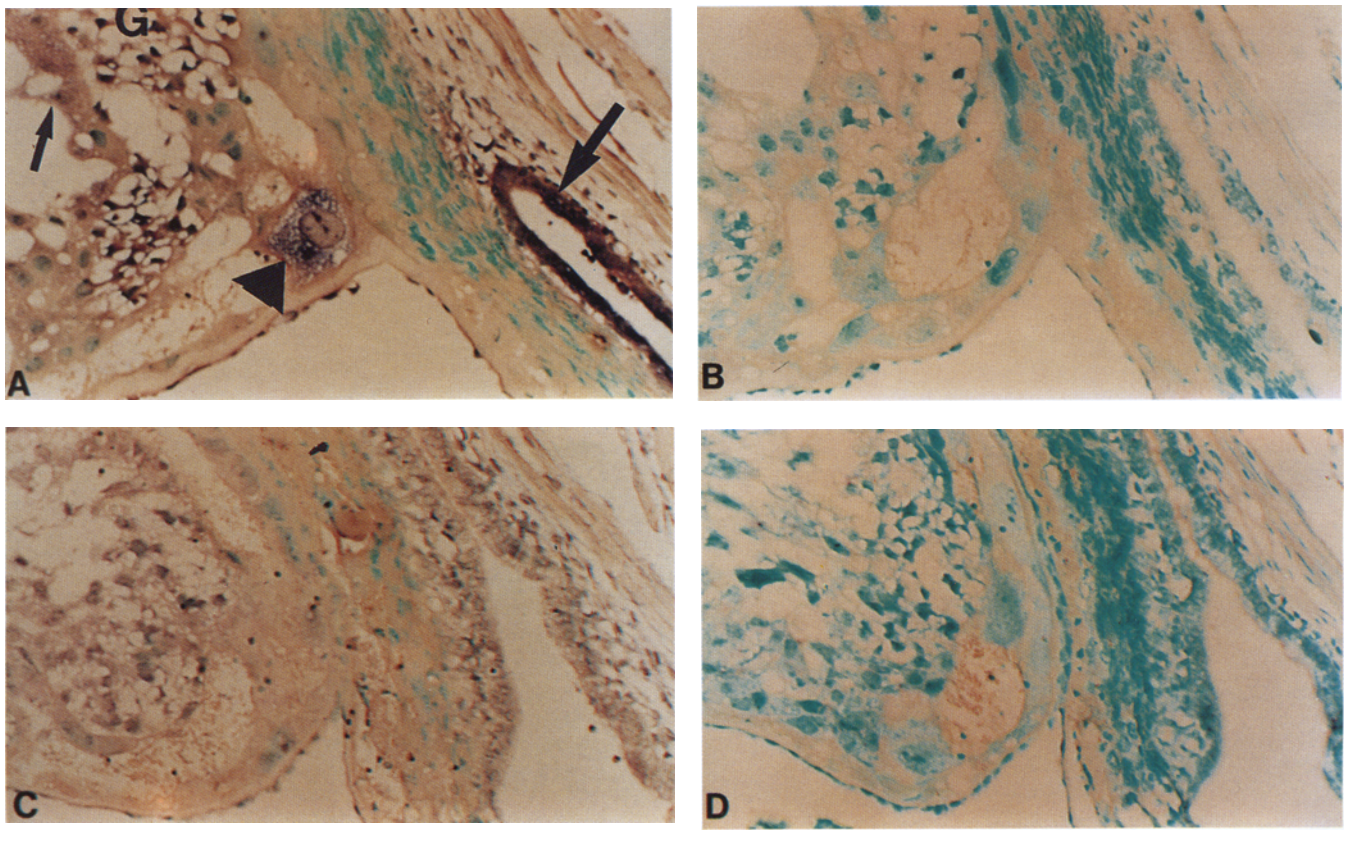

Figure 6 *In situ* hybridization analysis for TNF-RI and TNF-RII mRNA on day 15 of pregnancy. TNF-RI mRNA (**A**) is localized to trophoblast giant cells, spongiotrophoblast and glycogen cells. A strong hybridization signal is present in the uterine epithelium. TNF-RII mRNA (**C**) is localized to trophoblast giant cells, spongiotrophoblast and glycogen cells in the placenta and the uterine epithelium. The sense version of the probe and nonspecific DNA (**B** and **D**, respectively), exhibit no hybridization signal. Tissue sections were counterstained with methyl green. Arrow head: trophoblast giant cell; small arrow: spongiotrophoblast

early in gestation. It is well established that TNF causes many vascular changes, including increasing vascular permeability (Clauss *et al.*, 1992). Results from the present study indicate that the biological action of TNF on the myometrium would be mediated via TNF-RI.

The significance of an abundance of TNF-R mRNA localized to the uterine epithelium is not known. However, the ligand binding domain of the TNF-R can be shed from the cell surface and serve as a binding protein, and it has been hypothesized that a shed form of the TNF-R might serve a protective role against the destructive effects of excessive TNF (Fernandez-Botran, 1991). Infection during pregnancy has been shown to result in elevated levels of TNF (Romero *et al.*, 1989). In addition, the concentration of TNF binding protein in amniotic fluid and urine increases during pregnancy in women (Austgulen *et al.*, 1992). The increased binding protein was hypothesized to function in 'mopping up' excess TNF that might have deleterious effects on the pregnancy.

The observation that TNF and TNF-R mRNA are present in trophoblast cells throughout pregnancy implies that TNF has a direct, and possibly autocrine effect on trophoblast cell function. Previous studies have investigated the effects of TNF on trophoblast cell proliferation and hormone production. In regard to proliferation, TNF has been shown to function as an autocrine growth factor for human choriocarcinoma cells *in vitro* through TNF-RI (Yang *et al.*, 1993), to inhibit the proliferation of rat trophoblast cell lines *in vitro* (Hunt *et al.*, 1990) and to have neither proliferative nor antiproliferative effects on murine trophoblast primary cultures (Drake & Head, 1990). Although these results do not appear to be consistent, the model systems used in each case are different. The effect of TNF on trophoblast proliferation is probably related to the state of differentiation of the cell.

Hormone production is an important function of the trophoblast, and TNF alters hCG production by human trophoblast cells (Li *et al.*, 1992). Although the murine placenta does not produce a chorionic gonadotropin, other hormones, such as the placental lactogens (Colosi *et al.*, 1987) might be regulated, at least in part, by TNF. The data from the present study indicates that TNF might function directly on the trophoblast cells through either or both TNF-RI and TNF-RII.

This study defines the cell-specific localization of TNF-R mRNA in the mouse uterus and placenta and therefore identifies the possible cellular sites of direct TNF action.

Materials and methods

Animals, hormone treatments, tissue collection and cell culture

Female Swiss mice (age, 2–3 months) were purchased from Harlan (Indianapolis, IN). The mice were maintained on a 14:10 h light:dark schedule with food and water provided *ad libitum*. The protocols for all animal experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Three females were caged with a single male and pregnancy was identified by the presence of a vaginal plug (day 1 of pregnancy). Mice were euthanized by inhalation of metophane. Uteri collected on each day of pregnancy were fixed overnight in freshly prepared, cold 4% paraformaldehyde for later embedding in paraffin for *in situ* hybridization (Yelavarthi *et al.*, 1991), or were quickly frozen in liquid N_2 for later extraction of RNA. Two to six implantation sites from at least four different animals were assessed by *in situ* hybridization for each day of pregnancy. *In situ* hybridizations were repeated on each tissue at least four times. For RNA isolations, intact uteri and separated uteri and placentas were pooled for each day of pregnancy. Northern analysis was performed on three different pools in three different experiments. The RAW.246 mouse macrophage cell line was maintained in culture and was stimulated with 10 $\mu g\ ml^{-1}$ of lipopolysaccharide (LPS) as described previously (Hunt *et al.*, 1993). The EL4 mouse lymphoma cell line (TIB29) and the 70Z/3 mouse pre-B lymphocyte cell line (TB158) were obtained from the American Type Culture Collection, Gaithersburg, MD, and were maintained in accordance with their directions. Cell pellets were prepared from subconfluent cultures. Adherent cells were trypsinized, and all cells were pelleted by centifugation and stored at −80°C until RNA extraction.

Probes

A 298 bp PvuII–PvuII fragment of mouse TNFα cDNA (Fransen *et al.*, 1985; Murray & Martens, 1989) cloned into pGEM3 (Promega, Madison, WI) was generously provided by Dr C Marten (Affimax Research Institute, Palo Alto, CA). cDNAs for TNF-RI and TNF-RI were provided by Genetech Inc, South San Francisco, CA. A 498 bp fragment of TNF-RI cDNA and a 930 bp fragment of TNF-RII cDNA were subcloned into pSP72. A 1.2 kb fragment of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (R.W. Allen, American Red Cross Blood Services, St Louis, MO) was subcloned into pGEM-3Z. For *in situ* hybridization experiments, the cDNAs served as templates for polymerase directed synthesis of digoxigenin-labeled antisense and sense cRNA (TNF and TNF-RI) or cDNA (TNF-RII) (Genius Labeling Kit, Boehringer Mannheim, Indianapolis, IN) as described previously (Chen *et al.*, 1993). For Northern analysis the cDNAs served as templates for polymerase directed synthesis of ^{32}P-labeled antisense cRNA (TNF and TNF-RI) or cDNA (TNF-RII and G3PDH) (Melton *et al.*, 1984; De *et al.*, 1989). Reagents for the synthesis of probes were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Restriction enzymes and polymerases were purchased from Promega (Madison, WI). Unless otherwise noted all other chemicals and reagents were purchased from Sigma Chemical Co., St Louis, MO.

In situ *hybridization*

In situ hybridization conditions were as previously reported (Chen *et al.*, 1993) with the following exceptions. Prior to hybridization, the tissue sections were treated with 1 μg/ml of proteinase K for 30 min at 37°C. Following overnight hybridization at 47°C in a humidified chamber, the tissue sections were washed extensively, concluding with 0.1 × SSC at 47°C for 30 min. Hybridization was detected by first incubating the tissue sections with a biotinylated monoclonal anti-digoxigenin antibody, and then with streptavidin conjugated to alkaline phosphatase. Incubation in a BCIP-containing substrate yielded a brown-to-purple precipitate at the site of hybridization. The tissue sections were counterstained with methyl green

Northern blot hybridization

TNF and TNF receptor mRNA was detected by northern blot analysis in samples of poly(A)$^+$ RNA that had been isolated from cells, uteri, or placentas using the Mini RiboSep Ultra kit (Collaborative Biomedical Products, Bedford, MA), and separated by electrophoresis on 1% agarose gels. Prehybridization, hybridization (2×10^6 c.p.m. of labeled probe/ml), and posthybridization steps were performed as previously described (Roby *et al.*, 1993; Yang *et al.*, 1993).

Acknowledgements

This work was supported by a grant from the National Institutes of Health (HD29156) to J.S.H., and a core grant to the Kansas Mental Retardation Research Center (HD02528). K.F.R. was supported by a National Research Service Award.

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