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Tenascin is induced at implantation sites in the mouse uterus and interferes with epithelial cell adhesion

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SUMMARY

Expression of tenascin, an extracellular matrix protein associated with morphogenetic events and altered states of cellular adhesion, was examined in mouse uterus during the peri-implantation period. A uniform low level expression of tenascin was detected in stromal extracellular matrix during the estrous cycle and days 1 through 4 of early pregnancy. During the period of blastocyst attachment (day 4.5), an intense deposition of tenascin fibrils was located in the extracellular matrix of stroma immediately subjacent to the uterine epithelium surrounding the attaching blastocyst. This localized intensity of tenascin expression was both spatially and temporally restricted. By day 5.5, differentiation of stroma in the immediate area around the embryo to form the primary decidual zone was accompanied by a reduced amount of tenascin expression in the form of fragmented fibrils. Tenascin also could be induced by an artificial stimulus in uterine stroma of mice that had been hormonally prepared for implantation. The ability of artificial stimuli to induce tenascin expression suggested that the tenascin-inducing signals were derived from uterine cells, presumably lumenal epithelium, rather than embryonic cells. Consistent with this, conditioned medium from primary cultures of uterine epithelium was found to induce tenascin expression (2- to 4-fold) in isolated uterine stroma. Artificial stimuli generated a temporal pattern of tenascin expression similar to that observed during early pregnancy; however, in the artificially induced model, tenascin was induced in stroma immediately subjacent to lumenal epithelium along the entire length of the uterus. Purified tenascin and a recombinant tenascin fragment consisting of alternatively spliced fibronectin type III repeats, interfered with maintenance of uterine epithelial cell adhesion to Matrigel. In contrast, other recombinant tenascin fragments or fibronectin had no effect in this regard. Tenascin had no effect on adhesion of uterine stroma. Collectively, these results suggest that stimulation of TN expression in stromal extracellular matrix in vivo occurs via hormonally regulated, epithelial-mesenchymal interactions and serves as an early marker for uterine receptivity and the attachment phase of implantation. Furthermore, tenascin may facilitate embryo penetration by disrupting uterine epithelial cell adhesion to underlying basal lamina.

Abbreviations: ECM, extracellular matrix; hCG, human chorionic gonadotropin; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TN, tenascin; UE, uterine epithelia

Key words: tenascin, implantation, uterus, mouse, epithelium, cell adhesion

INTRODUCTION

Embryo implantation involves a series of cell-cell and cell-matrix interactions. During the peri-implantation period, rodent uterine epithelial cells (UE) undergo a hormonally regulated transition to a receptive state characterized by an apical surface that is permissive to blastocyst attachment and an apparent reduction in basolateral adhesion (El-Shershaby and Hinchliffe, 1974; Smith and Wilson, 1974; Schlafke and Enders, 1975). After attachment to the apical surface of UE, trophoblast must either move between cells or remove UE in order to arrive at the basal lamina (Schlafke et al., 1985). By the time the trophoblast penetrate the basal lamina, the adjacent stroma have differentiated into the decidual cells of the primary decidual zone (Parr et al., 1986), a process involving extensive extracellular matrix (ECM) remodeling and cellular realignment (Wewer et al., 1986; Glasser et al., 1987; Senior et al., 1988; Farrar and Carson, 1992).

Identification of molecules that modulate these interactions is key to our understanding of the implantation process. Tenascin (TN) is one of a group of ECM proteins that have been proposed to regulate adhesive interactions by promoting cellular detachment from substrata (Sage and Bornstein, 1991). For example, in adult intestine, TN is found at junctions

between epithelia and stroma in a gradient increasing from the base of the crypts to the tips of villi and underlying epithelia where cell shedding occurs (Probstmeier et al., 1990; Aufderheide and Ekblom, 1988). In contrast, during embryonic morphogenesis or oncogenesis, TN is generally localized to stromal ECM adjacent to rapidly proliferating epithelia in areas where cell migration or epithelial branching occur (Erickson and Lightner, 1988; Chiquet-Ehrismann et al., 1986). Although the precise functions of TN have yet to be confirmed, it has been suggested that TN facilitates penetration of mesenchymal tissue by 'loosening' stromal contacts with ECM components such as fibronectin (Chiquet-Ehrismann, 1991; Aufderheide and Ekblom, 1988). Interaction of TN with chondroitin sulfate proteoglycans may further compromise the ability of cells to interact with ECM components via neighboring integrins (Chiquet-Ehrismann, 1991; Murphy-Ulrich et al., 1991). Chondroitin sulfate proteoglycans appear to be major ligands of TN (Hoffman and Edelman, 1987) and themselves exert anti-adhesive activities in various systems (Perris and Johansson, 1987; Yamagata et al., 1989) including mouse embryo outgrowth assays (Carson et al., 1992). Chondroitin sulfate proteoglycans also are the predominant proteoglycans produced by mouse uterine stroma (Jacobs and Carson, 1991). Therefore, it is of interest to determine if TN is expressed in uterine tissue in a fashion that might contribute to the morphogenetic events of implantation.

In the present study, TN expression was examined in mouse uteri during the estrous cycle and peri-implantation period. The results indicate that TN can be induced in stroma of receptive, but not non-receptive, uteri by decidualizing stimuli, including blastocysts. TN expression was temporally and spatially restricted to the stroma subjacent to UE surrounding an implanting blastocyst, appearing only after the blastocyst had hatched and presumably initiated attachment. Expression of TN may contribute to UE penetration by reducing basolateral adhesion of the UE during the attachment phase of implantation and facilitate tissue remodeling occurring during formation of the primary decidual zone.

MATERIALS AND METHODS

Materials

CF-1 mice were purchased from SASCO (Omaha, NE). Pregnant mare serum gonadotropin was from Calbiochem (San Diego, CA). Antibodies and sources were as follows: fluorescein-conjugated, species-specific donkey antibody to rabbit IgG and species-specific sheep antibody to rat IgG, Amersham Corp. (Arlington Heights, IL); rabbit antibodies to chicken and human TN (Chiquet-Ehrismann et al., 1986; Lightner et al., 1989); rat monoclonal antibody recognizing mouse TN (Tn 12; Aufderheide and Ekblom, 1988) generously provided by Dr Peter Ekblom (Uppsala University, Sweden); rabbit antibody to laminin and rabbit antibody to collagen type IV, Collaborative Research, Inc. (Bedford, MA); rat monoclonal antibody to mouse laminin B2 chain, Chemicon (Temecula, CA); species-specific, rhodamine-conjugated donkey anti-rat IgG, Jackson Immunoresearch Labs (West Grove, PA); rabbit antibody to desmin, Sigma Chemical Co. (St Louis, MO); rabbit antibody to mouse ZP2/ZP3, the generous gift of Dr Paul Wassarman (Roche Institute, NJ); rabbit antisera to the basement membrane proteoglycan, perlecan, generously provided by Dr John Hassell (Eye and Ear Institute of Pittsburgh, PA). Purified human TN and recombinant TN fragments were prepared and characterized as described (Aukhil et al., 1993). Matrigel was purchased from Collaborative Research, Inc. (Bedford, MA). Human chorionic gonadotropin and p-nitrophenyl-β-D-N-acetylglucosamine were purchased from Sigma Chemical Co. (St Louis, MO). O.C.T. imbedding medium was obtained from Miles, Inc. (Elkhart, IN). ^{125}I-Protein A (30 μCi/μg) was purchased from ICN (Irvine, CA). Trypsin/EDTA solution was purchased from Irvine Scientific (Santa Ana, CA). Anocell culture dish inserts were from Whatman, Inc. (Clifton, NJ).

Natural and artificial implantation model

Virgin female mice, strain CF1 (6 weeks old), were superovulated by intraperitoneal injection of 5 U pregnant mare serum gonadotropin followed 48 hours later by injection of 5 U human chorionic gonadotropin (hCG). Females were immediately caged individually with CF1 males of proven fertility. Times of pregnancy are given as 12 hour intervals from the time of hCG injection. Hatching of blastocysts from zonae began at 96 hours. Unhatched blastocysts at 108 hours constituted 20% of the total embryos recovered. Many hatched blastocysts at 108 hours displayed signs of initial adhesion, i.e., adhering UE, but could still be flushed from the uterus. For experiments in which an artificial stimulus substituted for the blastocyst, females were superovulated and mated according to the regimen described above. Uteri were unilaterally (right side) ligated at the utero-tubal junction on day 2. On day 4.5, the ligated horn was injected intralumenally with 50 μl of oil. Uteri were collected at 6 hour intervals from the time of oil injection.

Primary cultures of uterine stroma and epithelia

Primary cultures of mouse uterine stroma were prepared from random cycling or day 4.5 pregnant superovulated mouse uteri from which blastocysts had been flushed. Stroma were isolated and purity was assessed as previously described (Jacobs and Carson, 1991); however, trypsin/EDTA solution was used in the isolation procedure instead of collagenase/dispase. Stromal cells were plated on glass coverslips or wells in culture medium consisting of Dulbecco's modified Eagle's medium: Ham's F12, 1:1 (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin for an attachment period of 1-2 hours after which treatments were initiated. Medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum as indicated. In some experiments, uterine stroma were cultured in serum-free medium conditioned by polarized UE prepared as described previously (Jacobs et al., 1990) and cultured on Anocell culture dish inserts.

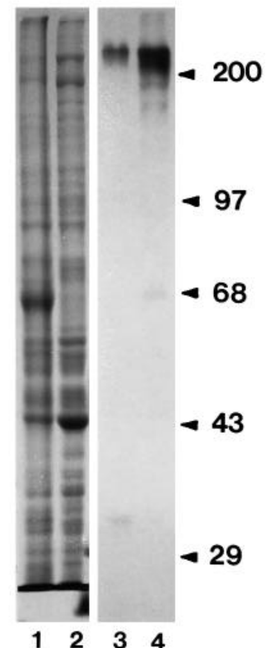

Fig. 1. Specificity of anti-tenascin. Total cell-associated protein from endometrial scrapings 24 hours after oil induction in day 4.5 pregnant uteri (100 μg per lane; lanes 1 and 3) and stroma cultured 3 days in medium plus 10% (v/v) fetal calf serum (100 μg per lane, lanes 2 and 4) were separated by SDS-PAGE (Coomassie stained gel, lanes 1 and 2) and transferred to nitrocellulose as described in Materials and Methods. Probing with rabbit anti-chick TN (autoradiograph, lanes 3 and 4) detected a major band at M_r 265×10^3 in both samples, with an additional major band at M_r 240×10^3 and minor bands at M_r 205×10^3 and 185×10^3 in the extract of cultured stroma (lane 4). The position of pre-stained molecular weight standards are indicated (×10^{-3}) to the right. The M_rs in the figure are those of the unstained proteins.

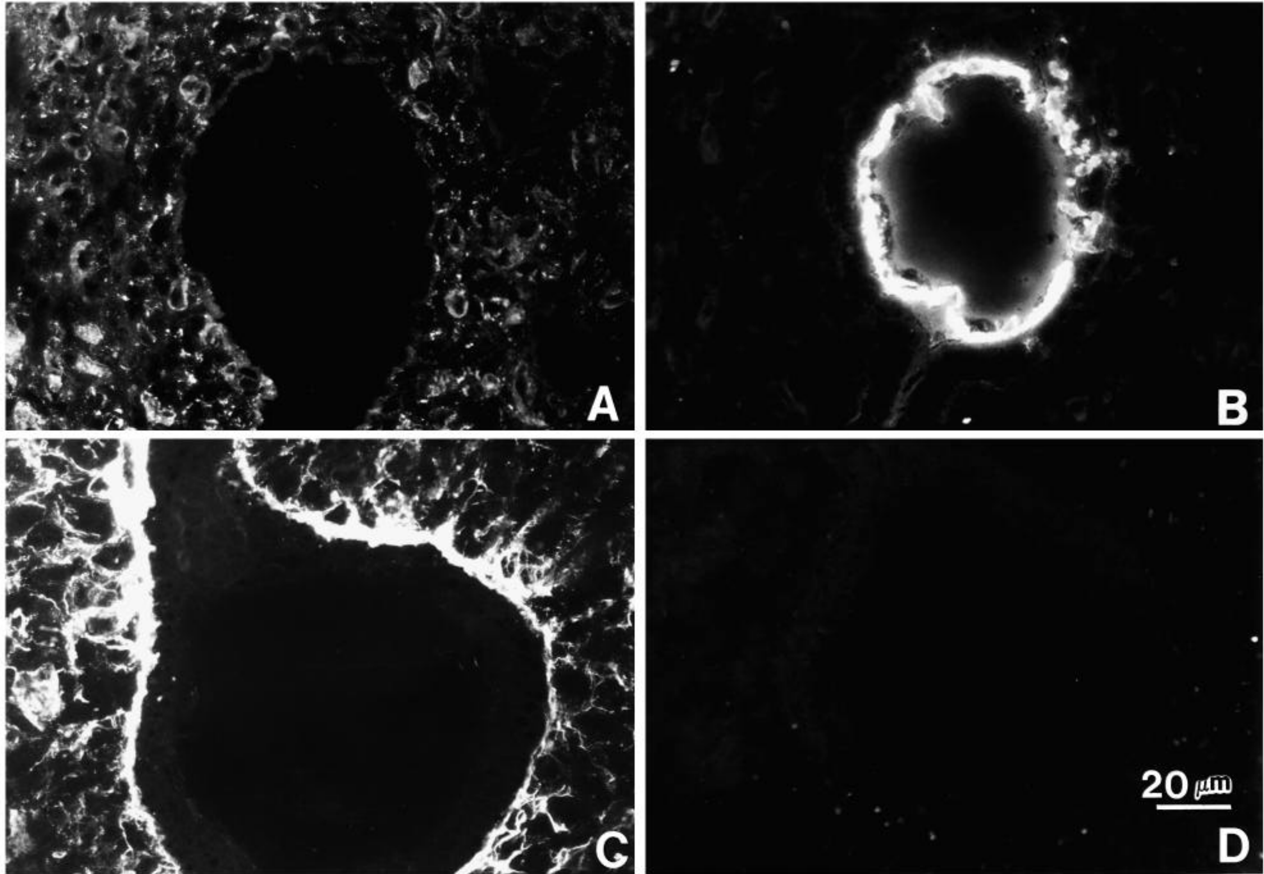

Fig. 2. Induction of tenascin during the attachment phase of implantation. Sections of pregnant mouse uterus collected between 96 and 108 hours (A,B) or at 108 hours (C,D) post hCG injection were probed with antibodies to TN (A,C) and zona pellucida proteins 2 and 3 (B,D). TN expression in stroma around the antimesometrial end of the lumen containing an unhatched embryo positive for zona pellucida proteins 2 and 3 was equivalent to that present in uterine stroma of cycling mice. Surrounding an expanded hatched blastocyst negative for zona proteins 2 and 3, intense TN expression was present in a fibrillar array which radiated into the stromal compartment from the area of basal lamina underlying the lumenal epithelium. The antimesometrial side is toward the top of each panel. Magnification is indicated in D.

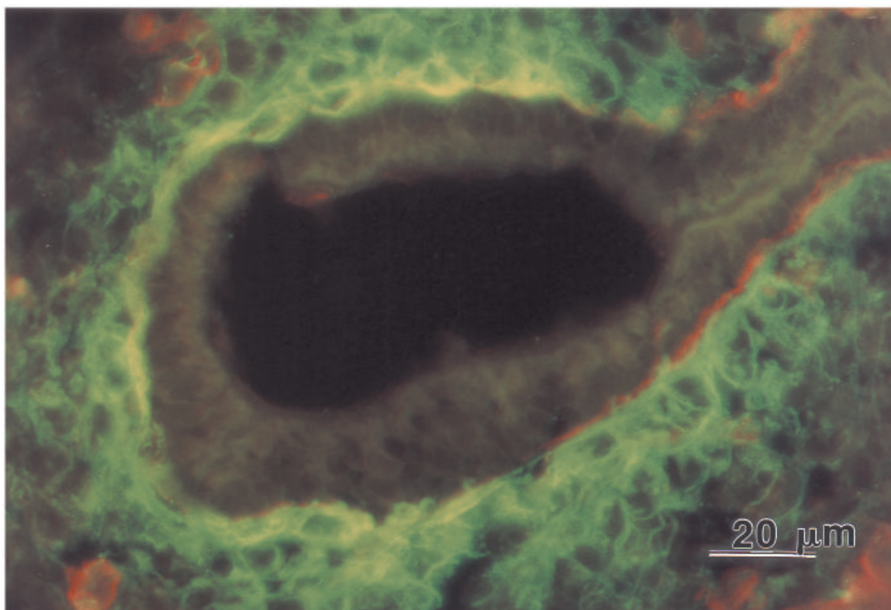

Fig. 3. Tenascin fibrils do not extend beyond the basal lamina. An implantation site containing a hatched blastocyst was doubly stained with rabbit antisera to TN (fluorescein) and a rat monoclonal antibody to mouse laminin B2 (rhodamine). The photograph reveals that TN staining juxtaposes and radiates from the stromal side of the basal lamina underlying the site. Staining is not observed on the epithelial side of the basal lamina. Magnification is indicated on the figure.

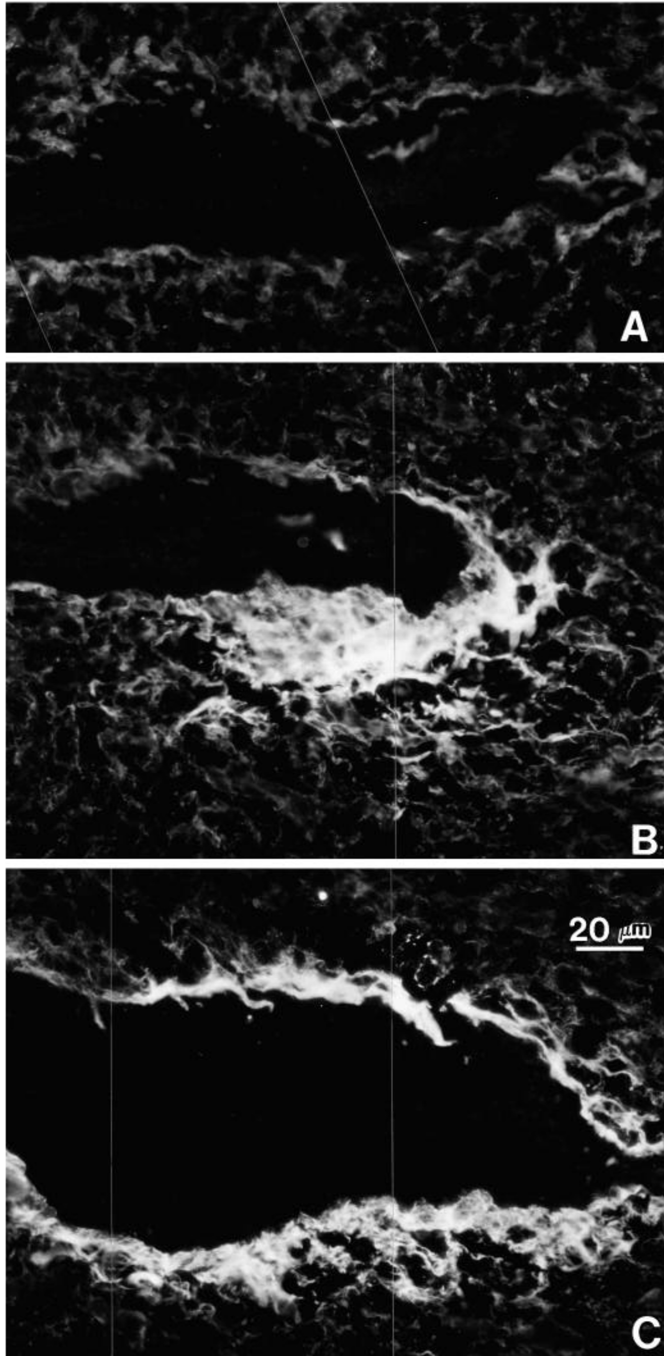

Fig. 4. Spatial restriction of tenascin induction during implantation. Serial sections of an attachment phase implantation site (108-110 hours post hCG) probed with antibody to TN demonstrates the spatial restriction of TN induction. 15 µm separate the section in A from the B section while B and C sections are separated by 35 µm. Following the section in C, which contains a portion of the implanting embryo, ten serial sections were probed. The seven sections following that in C contained embryo and were positive for TN as was the ninth section containing no embryo. Magnification is indicated in C.

Immunocytochemistry

Uteri were removed, embedded and immediately frozen in O.C.T. cryoprotectant, and stored at −70°C. 5 µm unfixed frozen sections were cut on a Reichert-Jung 2800 Frigocut cryostat and collected on glass slides presoaked in 99% (v/v) methanol:1% (v/v) HCl. Sections were briefly air dried and used immediately or stored frozen at −20°C. Implantation sites were located by visual inspection of serial sections by phase microscopy as they were generated. Unless otherwise indicated, uterine sections or stromal cultures were fixed 10 minutes at room temperature in 100% methanol followed by 10 minutes in phosphate-buffered saline (PBS). Primary antibody was applied for 1 hour at 37°C, followed by three rinses in PBS for 5 minutes each. Fluorescein-conjugated second antibody was applied for 40 minutes at 37°C, followed by three rinses in PBS for 10 minutes each. Sections were mounted in 90%(v/v) glycerol, 10%(v/v) PBS, 0.1%(w/v) p-phenylenediamine (Johnson and Nogueria Aravjo, 1981).

SDS-polyacrylamide gel electrophoresis and western blotting

Protein extraction, preparation of samples and western blotting were performed as described previously (Julian et al., 1992). Samples were separated on a 7.5% (w/v) polyacrylamide gel according to Laemmli (1970).

Cell adhesion assays

To determine the effect of TN on UE attachment and/or maintenance of adhesion, primary cultures of mouse UE cells were prepared as described previously (Jacobs et al., 1990). Cells were plated in serum-free medium on Matrigel-coated 24-well tissue culture plates at cell densities that produced >80% attachment efficiencies and subconfluent monolayers at 24 hours of culture. Where indicated, various ECM components, or TN fragments were mixed in with Matrigel while in liquid form prior to coating the tissue culture surface. Coating was performed by adding a sufficient amount of solution to completely cover the surface and aspirating the excess to leave a thin coating on the tissue culture surfaces which were incubated for 1 hour at 37°C and rinsed once with PBS prior to the addition of cells. Medium was changed at 24 hours of culture to remove unattached cells. At various times of culture, cell layers were rinsed with PBS to remove detached cells and a determination of relative cell number performed by hexosaminidase assay as described (Landegren, 1984). Such assays were performed in duplicate to quintuplicate cultures in all cases.

RESULTS

TN expression during the peri-implantation period

The polyclonal anti-TN used for most experiments specifically recognized components with the M_r expected for TN (Aufderheide and Ekblom, 1988; Aufderheide et al., 1987) in extracts of both mouse uterine endometrium as well as primary cultures of isolated mouse uterine stroma (Fig. 1). Immunohistochemical examination of uteri of pregnant mice during the preimplantation period, i.e., days 1 to 4 post coitum, revealed a similar invariant pattern of TN expression as was observed through the estrous cycle (data not shown). A consistent strong signal was detected in the myometrium at all stages of the cycle or preimplantation, while relatively low staining was observed in the endometrial stroma at all stages. At higher magnification, the stromal staining was resolved as fragmented fibrils occupying what appeared to be the extracellular space. No staining was detectable in either the glandular or lumenal epithelia or adventitial mesenchyme surrounding the bundles of myometrial smooth muscle. This pattern changed locally at implantation sites during the attachment period, i.e. day 4.5-5.0. Stroma subjacent to sites containing unhatched (surrounded by zona pellucida; Fig. 2B) blastocysts continued to

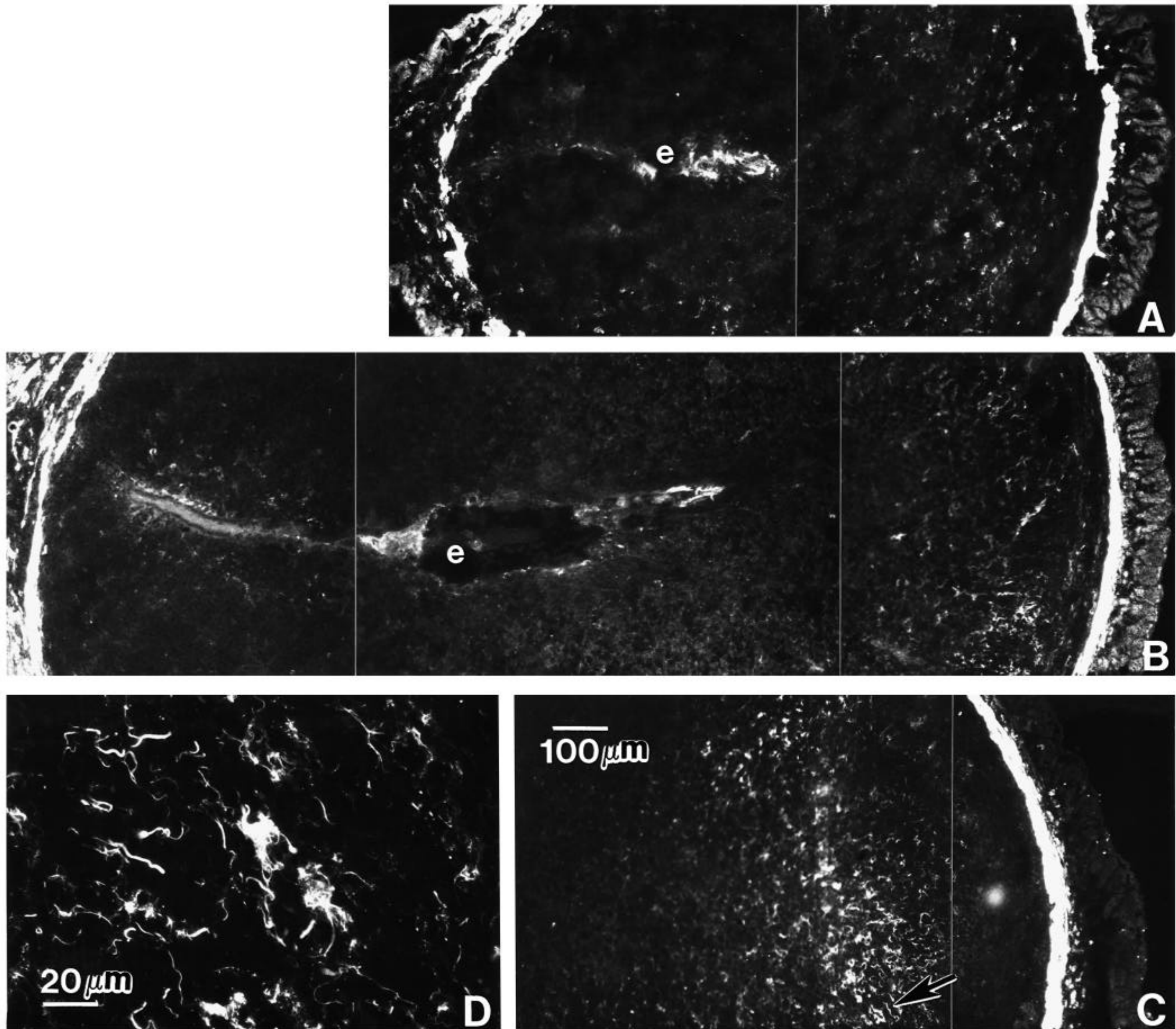

Fig. 5. Spatial and temporal restriction of elevated tenascin expression during implantation. Sections of implantation sites in uteri removed at day 5.5 (A), day 6.5 (B), and the antimesometrial aspect of the uterus on day 7.5 (C) post hCG injection were probed with antibody to TN. The staining of implanting embryo (e) is evident in A and B. Staining of the myometrial smooth muscle is evident to the right and left of A and B and the right of C. The intense signal in the stroma surrounding the implantation site on day 4.5 (see figures above) is largely diminished by day 5.5 and not observed in the primary decidual zone on day 6.5. In contrast, TN expression in smooth muscle of the myometrium remains constant throughout this period. The only stromal staining detected in sections of day 7.5 was in the deep antimesometrial stroma shown in C. Higher magnification of the deep stromal staining (arrow in C) indicates that TN is arranged in fibrillar arrays in this area (D). Magnification is indicated in C and D.

display low levels of fragmented fibrillar TN (Fig. 2A). In contrast, antimesometrial stroma subjacent to sites containing hatched (zona pellucida negative; Fig. 2D) blastocysts displayed a prominent increase in staining for TN organized into fibrillar arrays (Fig. 2C). A rat monoclonal antibody to mouse TN (TN12; Aufderheide and Ekblom, 1988) also labeled these fibrils (data not shown). Antibodies to several basal lamina components, i.e. laminin, collagen type IV and perlecan, or fibronectin failed to decorate these fibrils in serial sections, while basal lamina or interstitial matrix were labeled homogeneously by each antibody within the same sections (data not shown). Double staining of sections with antibodies to laminin B2 and TN demonstrated that TN fibrils did not extend beyond the basal lamina separating the epithelia and stroma (Fig. 3). Increased staining for TN was exclusively observed in areas containing hatched blastocysts and did not extend beyond sections containing a portion of the blastocyst as demonstrated in Fig. 4 where serial sections adjacent to the implantation chamber displayed a relatively low level of TN immunoreactivity (Fig. 4A). Central to the implantation chamber, strong induction of fibrillar TN was observed around stroma immediately subjacent to the UE surrounding the embryo, with fibrils extending approximately 10-30 µm from the basal lamina.

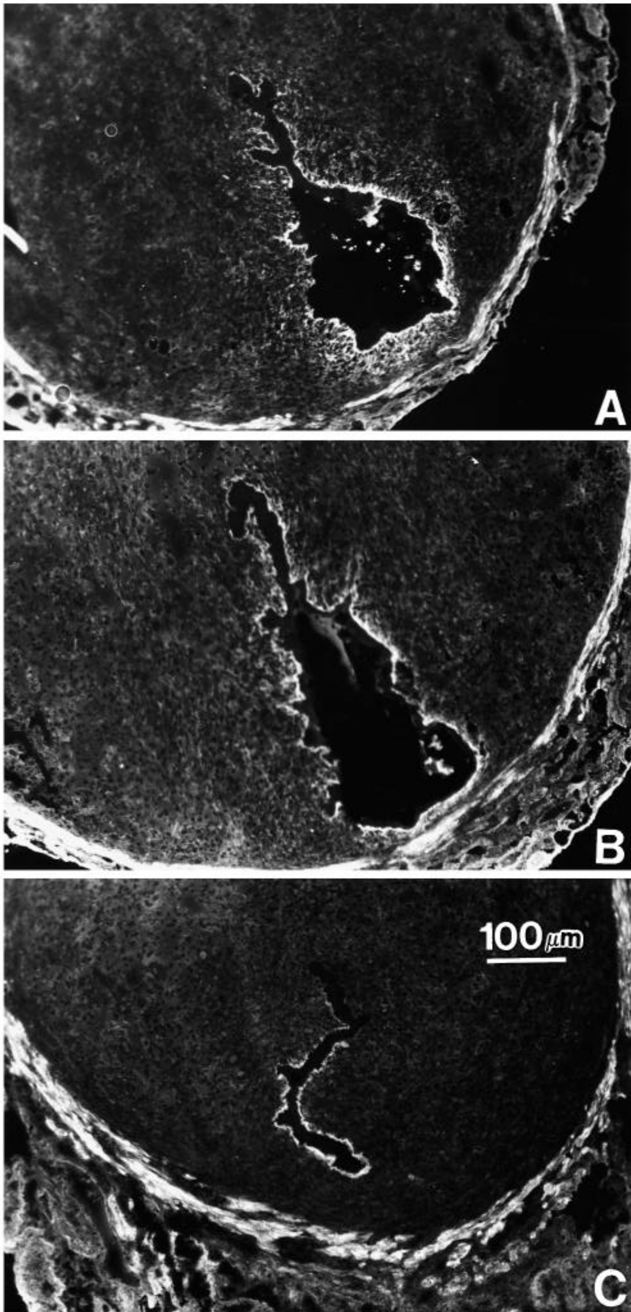

Fig. 6. Lack of spatial restriction for elevated tenascin during oil induced decidualization. Uteri from pregnant animals ligated on day 2 and injected intralumenally with oil on day 4.5 were removed 24 hours after injection. Sections collected at approximately 2 mm intervals display elevated TN expression in all areas where the oil distended the lumen. In A and B, the maximal distention and TN expression occurred at the mesometrial end of the lumen (to the lower right corner in all panels). The section in C displayed no distention but noticeable oil droplets were present at the mesometrial end of the lumen where TN was induced while TN was not elevated at the antimesometrial end of the lumen. Magnification is indicated in C.

TN induction at implantation sites was temporally as well as spatially restricted. Staining of implantation sites (Fig. 5), demonstrated that TN was induced only in subepithelial stroma at the antimesometrial end of the uterine lumen around the attaching embryo. The most intense staining was observed on day 4.5, i.e. the day upon which the attachment reaction is initiated, and prior to embryo invasion. By day 5.5, staining had diminished although it remained localized to the antimesometrial aspect of the implantation site. By day 6.5, the subepithelial staining for stromal TN was reduced, although embryonic tissues became positive. Neither decidual cells of the secondary decidual zone nor the mesometrial stroma displayed elevated levels of TN at this stage; however, fibrillar TN was observed in the deep antimesometrial stroma on day 7.5 (Fig. 5C,D). This staining was at the periphery of the stroma that displayed the decidualization markers laminin and desmin (data not shown).

TN induction in artificial deciduoma

To determine if TN induction required the presence of an embryo and/or an appropriate uterine hormonal milieu, an artificial decidual response model was employed. 2 days after mating, oviducts were ligated to prevent migration of embryos into the uterine lumen. Nonetheless, the uterus remained exposed to the same systemic hormonal influences as non-ligated animals would receive. On day 4.5, a small volume of oil was injected into the ligated horns to stimulate a decidual cell reaction. The distention of the lumen by the oil provided a generalized stimulus to both antimesometrial and mesometrial sides of the lumen along its entire length in contrast to the localized stimulus generated by embryos. Fig. 6 shows the results of TN staining of uteri subjected to this protocol. Sections taken at intervals along the length of the uterine horn demonstrated strong TN induction in stroma immediately subjacent to the lumenal epithelium. This staining was not restricted to the antimesometrial side, i.e. the side at which embryos normally attach. Nonetheless, the distribution of TN was similar to that observed in response to the blastocyst, i.e. in stroma immediately subjacent to the lumenal UE and emanating in an apparently fibrillar pattern from this area. The time course of the response to the oil stimulus was examined. Fig. 7A shows the staining of an implantation site for comparison. As shown in Fig. 7B, subepithelial TN staining was not observed 6 hours after oil induction; however, by 12 hours (Fig. 7C) intense staining was observed which persisted through 24 hours (Fig. 7D). By 48 hours after oil induction, subepithelial TN staining had declined and become fragmented, resembling that observed at implantation sites of day 5.5 pregnant mice while fibrillar TN appeared at the periphery of the antimesometrial deciduoma (data not shown). TN induction was not observed in unstimulated pregnant uteri. In addition, oil injection did not induce TN expression in uteri of cycling mice (data not shown). Collectively, these studies indicated that TN induction required both the presence of a decidual stimulus as well as an appropriate hormonal milieu.

Uterine stroma express TN in vitro

Positive staining for TN was observed in a very low percentage (1-2%) of the cells of primary cultures of mouse UE and was undetectable in extracts of UE cultures by western blotting (data not shown). Uterine stroma cultured in the absence of serum uniformly displayed low levels of TN (Fig. 8A) that appeared to be intracellular. Serum addition to the medium

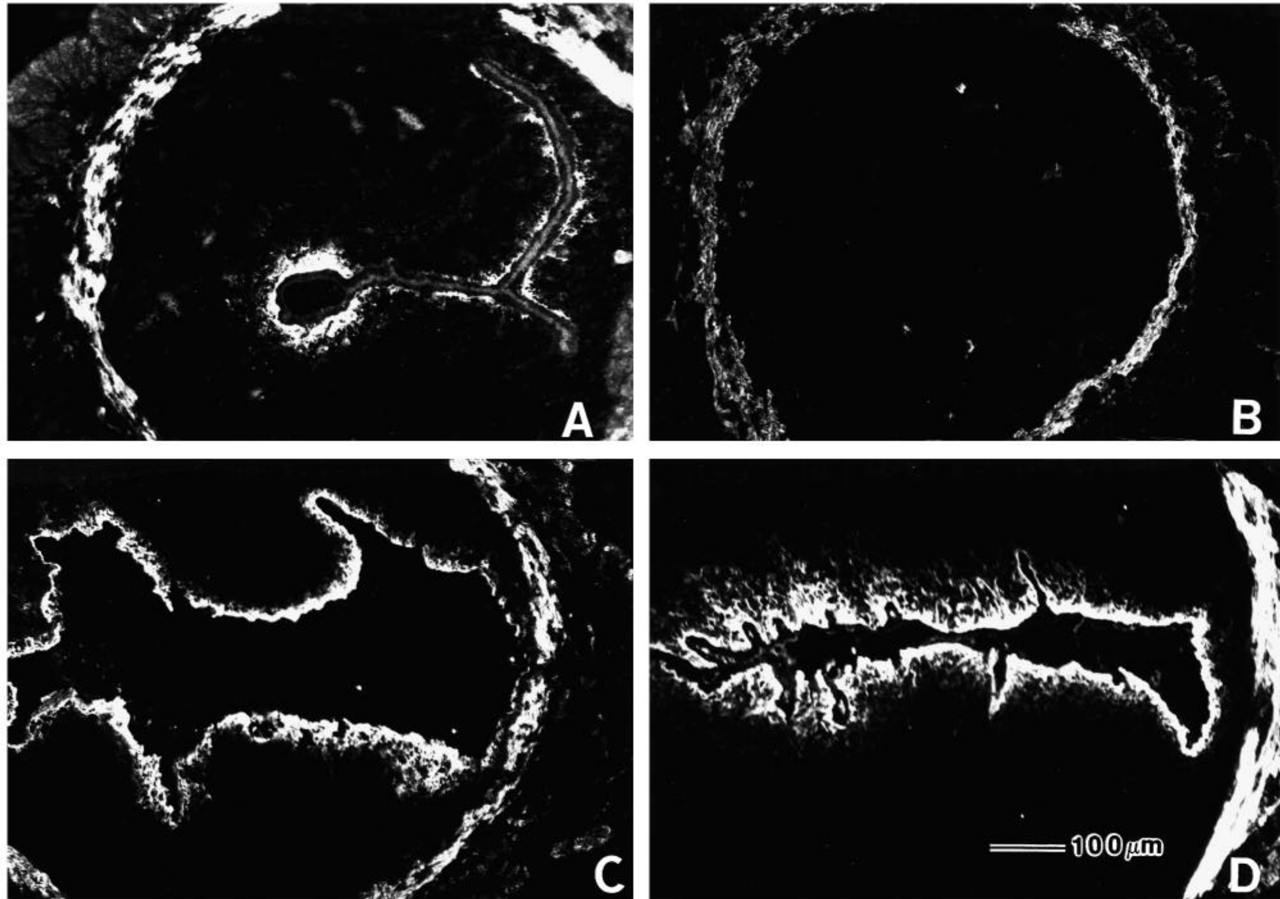

Fig. 7. Temporal progression of tenascin expression during oil induced decidualization. Uteri were collected from animals prepared as described in Fig. 6 at 6 hours (B), 12 hours (C), or 24 hours (D) after intralumenal oil injection. At 6 hours after oil instillation no TN had accumulated (B). Uteri from cycling females 24 hours after oil injection or uninjected, ligated uterine horns of pregnant mice were identical in terms of patterns of TN expression as observed in B. By 12 hours, an array of TN fibrils surrounded the lumen similar in intensity to that surrounding an implantation site on day 4.5 (A). A similar array surrounded the lumen at 24 hours after oil induction (D), but was reduced in the sublumenal stromal matrix at 48 hours (not shown). Antibody generated against human TN was used in this experimental series. Similar results were obtained with antisera directed against chicken TN. Magnification is indicated in D.

stimulated the expression of extracellular TN in uterine stroma (Fig. 8B) and was observed whether stroma were isolated from uteri of day 4.5 pregnant mice or mice chosen at random points in the estrous cycle. Therefore, the ability of serum to stimulate TN expression in uterine stroma cultured in vitro did not appear to depend upon the hormonal status of the animals from which the cells were derived. Furthermore, coculture with hatched blastocysts in the presence or absence of serum had no apparent influence on TN expression by stroma as determined by immunostaining (data not shown).

Modulation of TN expression in cultured stroma was examined. These results were based on cell-associated TN and are representative of relative concentrations of total TN since less than 5% of total TN was secreted to the medium (data not shown). The results are summarized in Table 1. Addition of serum increased overall TN production by 4.5-12.4-fold during the first 48 hours of culture relative to stroma cultured in the absence of serum. Incubation with UE-conditioned media substantially stimulated TN expression by uterine stroma in vitro. Apical secretions were more effective (4.3-fold) than basal secretions (2-fold). Collectively, these data confirmed that uterine stroma were capable of producing TN and that TN expression could be modulated by factors present in serum as well as factors secreted by UE.

TN effects on uterine cell adhesion

Experiments were performed to determine if TN interferes with adhesion of uterine cells. As shown in Fig. 9, UE cultured on TN-bearing substrata appeared to be less firmly attached to tissue culture surfaces than non-treated cells as evidenced by the frequency of rounded cells in TN-treated cultures. A similar response was observed whether UE were isolated from randomly cycling mice or from pregnant mice on the day of blastocyst attachment, i.e., day 4.5 post coitum. TN interfered with maintenance of UE adhesion regardless of whether UE were cultured on Matrigel-coated tissue culture dishes or semi-permeable filter supports, a condition supporting polarization of these cells (Jacobs et al., 1990). Tissue culture plastic precoated with Matrigel was used in almost all experiments for convenience. Matrigel was chosen for its compositional resemblance to UE basal lamina and was necessary to establish UE cultures in serum-free medium.

Dose dependence of the TN effect on UE is presented in Fig. 10. TN had minimal effects on UE adhesion during the first 24

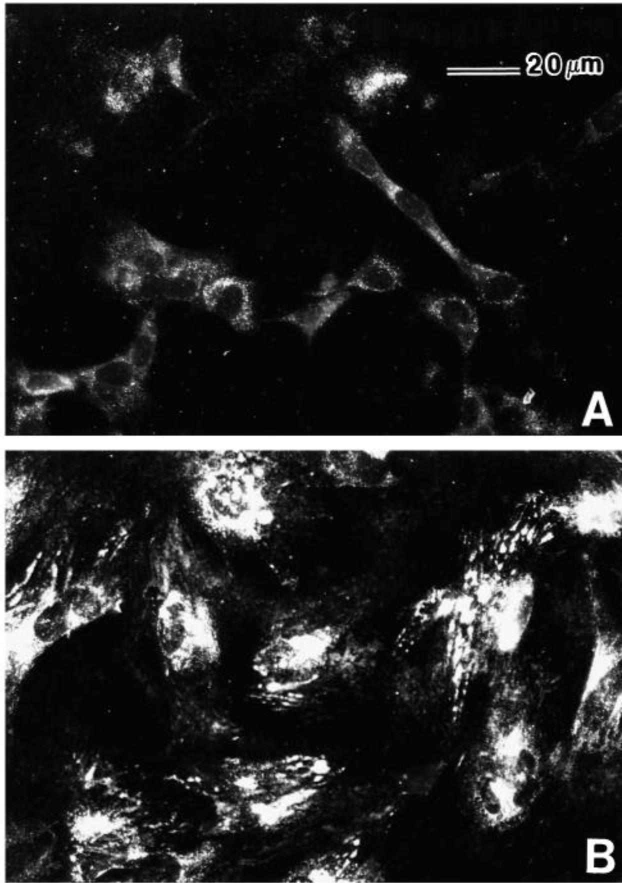

Fig. 8. Serum stimulates tenascin expression in primary cultures of uterine stroma. Stroma were isolated from day 4.5 pregnant uteri as described in Materials and Methods, plated on glass coverslips and cultured for 48 hours in medium without (A) or with 10% fetal calf serum (B). The low level of TN detected in stroma cultured in the absence of serum appeared to be primarily intracellular while, in the presence of serum, the external cell surface and the coverslip were decorated by a fibrillar array recognized by the antibody to human tenascin. Magnification is indicated in B.

hours of culture; however, at later times, e.g., 48-168 hours, up to 60% of the UE in these cultures had detached or could be dislodged from their substrata by gentle rinsing. Thus, while TN did not substantially inhibit the initial attachment of UE, it did interfere with maintenance of UE adhesion. This effect was observed with TN concentrations of 250 µg/ml Matrigel and was near maximal at 500 µg/ml. Several batches of TN were tested during the course of these studies and all interfered with UE adhesion at 500 µg/ml. Consequently, this concentration was used routinely. Under these conditions, the ratio of TN to Matrigel on a protein basis was 1:4 with an approximate 1:3 stoichiometry relative to the laminin content of Matrigel (Kleinman et al., 1987). TN had to be presented as a substratum component to exert its effect on UE adhesion. Addition of TN to the media only did not have this effect. Furthermore, combination of soluble TN with TN present in the substratum had no additional effect on UE adhesion beyond that observed with substratum-bound TN alone (data not shown).

Table 2 summarizes a series of studies using previously described recombinant fragments of TN domains (Aukhil et al., 1993). The locations of these fragments within the TN polypeptide are indicated in Fig. 11. Intact TN significantly interfered with maintenance of UE adhesion while fibronectin at a similar concentration had little effect in this regard. The only fragment of TN that significantly interfered with UE adhesion was TN(A-D), a fragment shown to have similar activity in other systems (Murphy-Ulrich et al., 1991). This effect was similar in degree to that observed with intact TN. It was concluded that the adhesion inhibitory activity of TN was specific and primarily localized to the A-D domain of the molecule.

TN effects on uterine stromal cell adhesion also were

Table 1. Modulation of tenascin expression by uterine stroma in vitro

Treatment	Duration of treatment	Relative TN level*
Experiment I		
None	24 hours	1.0
	48 hours	1.9
10% Serum	24 hours	8.7
	48 hours	8.6
Experiment II		
None	40 hours	1.0
UE - Basal CM_1	40 hours	2.0
UE - Basal CM_2	40 hours	1.9
UE - Apical CM	40 hours	4.3
10% Serum	40 hours	12.4

*Relative TN levels were determined by western blotting of stromal cell extracts as described in Materials and Methods. In all cases, autoradiograms were quantified by scanning densitometry and values used fell within the linear range of the instrument. The data are expressed as arbitrary integration units relative to 25 µg of cell-associated protein. Stroma were isolated from day 4.5 pregnant uteri (experiment I) or randomly cycling uteri (experiment II). UE were isolated from cycling uteri and cultured in serum-free medium on Matrigel-coated Anocell filters. UE-conditioned medium (CM) was collected between days 1 and 3 of culture. Subscripts 1 and 2 refer to duplicate cultures of stroma exposed to the same preparation of basal conditioned medium. Treatments were initiated after stroma were attached at 2 hours of culture. Western blots were probed with anti-chicken TN (experiment I) and anti-human TN (experiment II).

Table 2. Effects of TN fragments on maintenance of UE cell adhesion*

Protein added	% control UE attachment	*P* values
Intact TN	47±1	<0.001
Fibronectin	86±4	>0.05
TN (1-8)	89±5	>0.05
TN (1-5)	111±10	>0.05
TN (6-8)	87±1	>0.05
TN (fbg)	110±5	>0.05
TN (A-D)	46±1	<0.001

*UE were isolated, cultured and cell binding quantified by the hexosaminidase assay described in Materials and Methods. TN, fibronectin and TN fragments were mixed with Matrigel prior to coating. Concentrations were adjusted to be equivalent on a molar basis to that of 500 µg/ml intact TN subunit (approximately 500 nM). The data points represent the mean ± s.e.m. of 5 or 6 separate UE-containing wells. Control UE were UE cultured on surfaces coated with Matrigel only and the values for this group were 100±7% (mean ± s.e.m.). Statistical significance was determined for each experimental group relative to the control group by the Tukey-Kramer multiple comparisons test. Similar results were obtained in four individual culture series and throughout time in culture for the series described above.

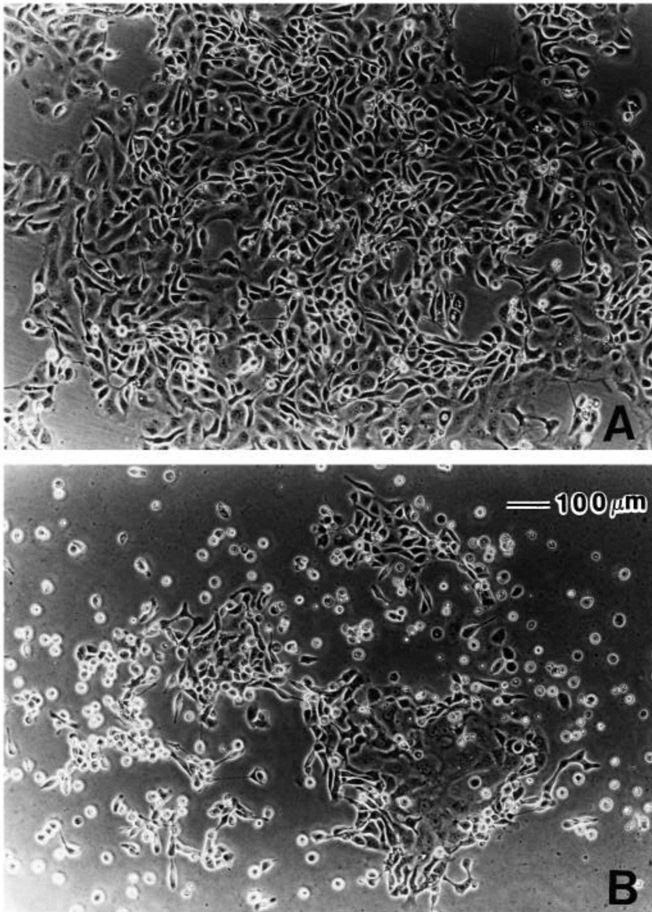

Fig. 9. Morphology of UE cultured on Matrigel with or without TN. UE were isolated and cultured in serum-free medium on Matrigel-coated surfaces as described in Materials and Methods. TN was dissolved in the Matrigel solution to a final concentration of 500 µg/ml prior to using this solution to coat the tissue culture surface. Photographs were taken of UE after 48 hours of culture on surfaces coated with Matrigel (A) or TN-containing Matrigel (B). Note the presence of many rounded and detaching cells in B. Magnification is indicated in B.

Table 3. Effects of TN and TN fragments on maintenance of uterine stromal cell adhesion*

Protein added	% Control stromal cell binding
Fibronectin	100±3
TN	90±1
TN (1-8)	98±4
TN (A-D)	101±2

*Assays were performed as described for UE in Materials and Methods and in the legend to Table II. The data points represent the mean ± s.e.m. of triplicate stromal cell cultures. Control stroma were cultured on surfaces coated with Matrigel only and the values for this group were 100±8% (mean ± s.e.m.).

examined and are summarized in Table 3. In these experiments, stroma were cultured in serum-free medium to minimize endogenous TN production. US attached and spread well on Matrigel-coated surfaces. As was the case with UE, inclusion of fibronectin in the Matrigel had no effect on stromal

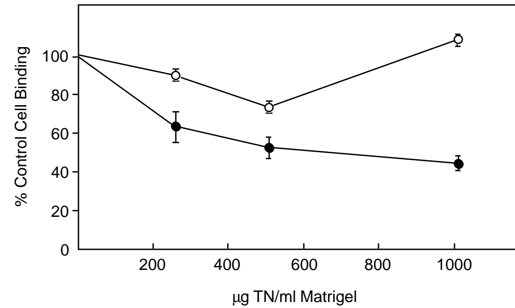

Fig. 10. Time and dose dependence of TN effect on UE detachment. UE were isolated and cultured in serum-free medium on Matrigel-coated surfaces as described in Materials and Methods. TN was dissolved in the Matrigel solution to the indicated final concentration prior to coating. Cell attachment was monitored after removing detached cells by gentle rinsing by the hexosaminidase assay described in Materials and Methods. Data is expressed relative to the value obtained for control, i.e., Matrigel lacking TN, UE cultures. Determinations were performed at two time points after plating, 24 hours (○) and 144 hours (●). The values represent the mean ± s.e.m. for triplicate determinations in each case.

cell adhesion. Inclusion of TN in Matrigel did not significantly affect stromal cell attachment and no obvious morphological differences were noticed between stroma cultured on TN-bearing substrata and controls (data not shown). Furthermore, no adhesion inhibitory activity was noted with either recombinant TN fragments 1-8 or A-D. This lack of response was maintained over several days of culture in vitro. It was concluded that UE were much more sensitive to the adhesion inhibitory effects of TN than uterine stroma.

DISCUSSION

With few exceptions, TN induction in developmental systems occurs in mesenchyme responding to molecular signals secreted from adjacent epithelium. Murine uterine stroma during the early phase of implantation communicate with two epithelia, i.e., UE and embryonic trophectoderm. The restricted induction in uterine stroma immediately subjacent to lumenal UE during implantation may reflect a response to a signal originating in the trophectoderm and transduced by UE or a molecular signal produced by UE in response to a stimulus from the blastocyst. Induction of TN in the stroma of the pseudopregnant uterus by oil injection demonstrates that molecular signals from trophectoderm are not essential; however, both oil injection and the blastocyst may provide mechanical stimuli. In normal pregnancy, blastocysts produce localized stimuli, molecular and mechanical, to the UE. Oil injection is expected to provide a stimulus throughout the uterine lumen. In either case, UE respond to the stimulus and release a signal. The broader distribution of TN expression in the oil-induced model demonstrates that the entire lumenal UE and adjacent stroma are capable of responding to the stimulus. Thus, the restricted region of induction observed during implantation appears to be due to the restricted disposition of the initial stimulus, i.e. the blastocyst, on a finite population of lumenal UE. It is suggested that in response to the apically applied stimulus, UE, in turn, release a molecular signal

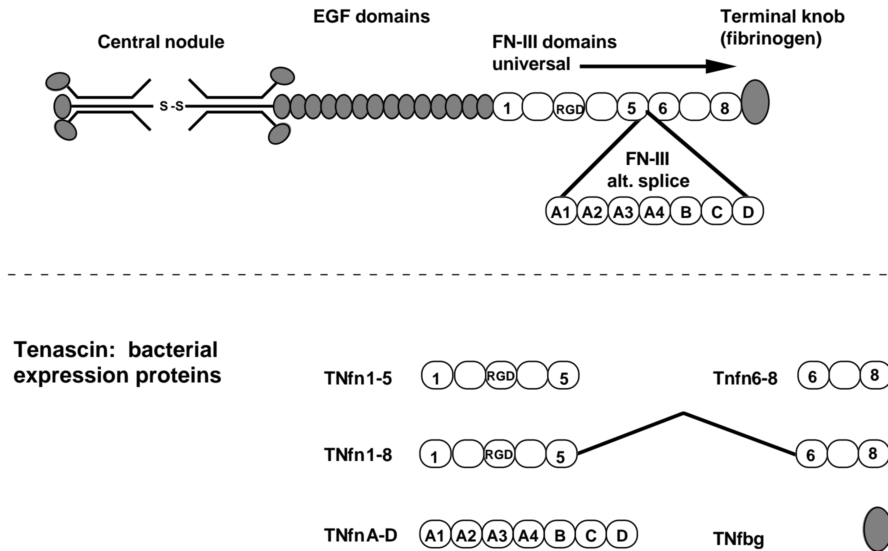

Fig. 11. Domain structure of human TN and TN expression proteins. In the central nodule region (upper left portion of the figure), three TN arms are joined at their N-termini forming a trimer. Two trimers are connected to form a hexamer in intact TN. Each arm contains 14 EGF-like domains of 31 amino acids each, 8-15 fibronectin type III repeat regions of 91 amino acids each and a C-terminal fibrinogen-like domain of 215 amino acids. Fibronectin type III repeats found in all forms of TN are designated 1-8. Seven additional fibronectin type III repeat units are introduced by alternative splicing and are designated A1-A4 and B-D. The bacterial expression proteins representing different TN domains used in this study are also indicated in the figure and have been described in detail previously (Aukhil et al., 1993).

capable of penetrating only to the adjacent stroma. The identity of this molecular signal is unknown at present. Several cytokines known to be produced by UE do not display the ability to stimulate TN expression by uterine stroma in serum-free medium (J. Julian and D.D. Carson, unpublished data). These cytokines include interleukin-1α (Jacobs and Carson, 1993), interleukin-6 (Jacobs et al., 1992), epidermal growth factor (Huet-Hudson et al., 1990) and transforming growth factor type-β1 (Tamada et al., 1990). Furthermore, neither prostaglandins $F_{2\alpha}$ nor E_2, lipid signals secreted by UE (Jacobs et al., 1990), display TN-inducing activity in vitro. Most of the TN-inducing activity is released from the apical aspect of polarized UE in parallel with the general pattern of protein secretion by these cells (Jacobs et al., 1990). It is possible that redirection of secretion of active factors occurs during the peri-implantation period (Denker, 1990). Moreover, these factors would not be diluted to the same extent in uterine tissue as they are in tissue culture. Therefore, a greater stimulation of TN expression by UE secretions may occur at the implantation site than is observed by UE basal secretions in vitro.

One function of TN at the implantation site may be to interfere with UE adhesion thereby promoting embryo penetration. The present studies demonstrate that TN interferes with UE adhesion to a basal lamina-like substratum, i.e., Matrigel, in a dose-dependent manner. Fibronectin does not have this activity. In addition, since Matrigel is largely composed of laminin and collagen type IV (Kleinman et al., 1987), it seems unlikely that these ECM components interfere with UE adhesion. Only one of the recombinant fragments of TN displayed this effect, namely region A-D. This portion of TN has been shown to inhibit cell adhesion in other systems as well (Aukhil et al., 1991; Murphy-Ulrich et al., 1991). Moreover, TN and TN fragments had little or no effect on uterine stromal cell adhesion. Thus, the effect on UE adhesion appears to be both protein- and cell type-specific. Several different preparations of intact TN were used during these studies and all displayed this activity. In addition, intact TN and recombinant fragment A-D are generated by independent procedures from very different sources (Aukhil et al., 1993). Therefore, it is unlikely that this activity is due to a common contaminant. The UE response also is not likely to be due to toxic effects of TN or recombinant TN fragments. No significant difference in cell viability or the presence of apoptotic figures in attached cells was observed between TN-treated and control UE (data not shown).

TN does not inhibit the initial adhesion of UE and so its effects are difficult to interpret in terms of a simple steric hindrance model. TN had to be presented in matrix, rather than soluble, form to exert its effect as reported previously (Lightner and Erickson, 1990). The observation that TN stimulates UE detachment is consistent with a physiological role in UE ‘loosening’. At implantation sites, UE are attached to basal lamina and each other. Biologically relevant anti-adhesion molecules would have to cause cell detachment from matrix components in this area. Moreover, such molecules would have to be expressed near the basal aspect of UE prior to embryo penetration and need not remain after this time. The in situ studies of the temporal and spatial patterns of TN distribution and the in vitro studies of TN function are consistent with such an activity.

Recent gene ‘knockout’ experiments indicate that the TN-C gene (nomenclature according to Bristow et al., 1993) is dispensible in mice (Saga et al., 1992). Normal offspring are produced from both male and female homozygous mutants; however, effects on other parameters of reproductive function, e.g., litter size, rate of formation, number and size of decidual swellings, etc., were not reported. Therefore, some aspects of implantation-related events may be compromised in these mice. It also is possible that in the absence of TN-C expression other genes are activated that compensate for this defect. TN-like proteins, TN-MHC (TN-X) and restrictin (TN-R), have been described (Matsumoto et al., 1992; Norenberg et al., 1992), but it is not known if these proteins are expressed in the uterus. Expression of these proteins needs to be examined not only in normal mice, but also in TN-C-deficient mice to address the possibility that they can replace or compensate for a lack of TN-C during early pregnancy.

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