

Endothelial cells adhere to the RGD domain and the fibrinogen-like terminal knob of tenascin

Paritosh Joshi^{1,*}, Chang-Y. Chung¹, Ikramuddin Aukhil² and Harold P. Erickson^{1,†}

¹Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA

²Department of Periodontics, University of North Carolina, Chapel Hill NC 27514, USA

*Present address: Department of Biochemistry, Indian Veterinary Research Institute, Isatnagar, U.P. 243122, India

†Author for correspondence

SUMMARY

We have found that endothelial cells adhere much more strongly than fibroblasts to domains of tenascin and fibronectin. Endothelial cells adhered weakly, without spreading, to bacterial expression proteins corresponding to the tenth fibronectin type III (FN-III) domain of fibronectin, which contains the RGD. A larger fibronectin protein, containing this domain and the three amino-terminal 'synergy' domains gave strong adhesion and spreading. Two widely separated domains of tenascin gave adhesion. The third FN-III domain, TNfn3, which contains an RGD sequence in human and chicken tenascin, gave very strong adhesion and spreading of endothelial cells when tested as an isolated domain. Larger segments containing TNfn3 and the adjacent TNfn2 gave weaker adhesion, probably because the RGD sequence is partially blocked. Adhesion to this domain required divalent cations, was exquisitely sensitive to soluble GRGDSP peptide, and was blocked by antisera to the integrin $\alpha_v\beta_3$. The second tenascin adhesion domain was the fibrinogen-like C-terminal knob, TNfbg. Cells adhered to but did not spread on this domain. This adhesion required divalent cations and was also sensitive to GRGDSP peptide, so it may be mediated by an integrin receptor.

We have explored a range of conditions for preparing the adhesion substratum, and our results may resolve the controversy over whether tenascin can act as a substratum adhesion molecule. When coated for short times (1-2 hours) on plastic, tenascin had no adhesion activity, in contrast to fibronectin and the expression proteins, which gave strong adhesion under these conditions. When coated for longer times (12-24 hours) on plastic, the tenascin substratum supported good adhesion, but not spreading, of endothelial cells. Tenascin coated on nitrocellulose gave substantially stronger adhesion than on plastic, but still required long coating times for maximal activity. Adhesion of endothelial cells to native TN was inhibited by GRDGSP peptide. The cell adhesion activity demonstrates the presence on endothelial cells of tenascin receptors, which may play a supportive role in angiogenesis, in the structure of blood vessels, or in binding tenascin to the cell surface to elicit or enhance a signalling function.

Key words: tenascin, endothelial cells, RGD, cell adhesion, extracellular matrix, fibronectin

INTRODUCTION

Tenascin (TN) is a large ECM protein prominent in restricted embryonic tissues, healing wounds and tumors (Erickson and Bourdon, 1989). Six elongated subunits are linked by disulfides into a 2,000 kDa molecule called the hexabrachion. Each arm of the hexabrachion is a tenascin subunit, and comprises 14 domains homologous to epidermal growth factor (EGF), 8-15 FN-III domains, and a fibrinogen-like domain (Fig. 1). The third FN-III domain (TNfn3) in human TN has an RGD on the loop connecting beta strands F and G (Leahy et al., 1992), which is the same location as the RGD in the fibronectin (FN) domain FNfn10 (Main et al., 1992). There has been considerable interest in testing TN for cell adhesion activity, with controversial

results reported by several laboratories (reviewed by Erickson and Bourdon, 1989).

More recently, attention has focused on possible anti-adhesion activities of TN. Chiquet-Ehrismann et al. (1988) reported that TN could inhibit adhesion of cells to a fibronectin (FN) substratum. Lightner and Erickson (1990) confirmed these observations and concluded that the mechanism was steric blocking of the substratum. The large hexabrachion molecules bound to the plastic, straddling the fibronectin molecules and efficiently covering up their cell adhesion sites.

Anti-adhesion mechanisms have been attributed to two specific domains of TN. Murphy-Ulrich et al. (1991) reported that the alternatively spliced segment of TN (TNfnA-D in Fig. 1) caused a loss of focal contacts when

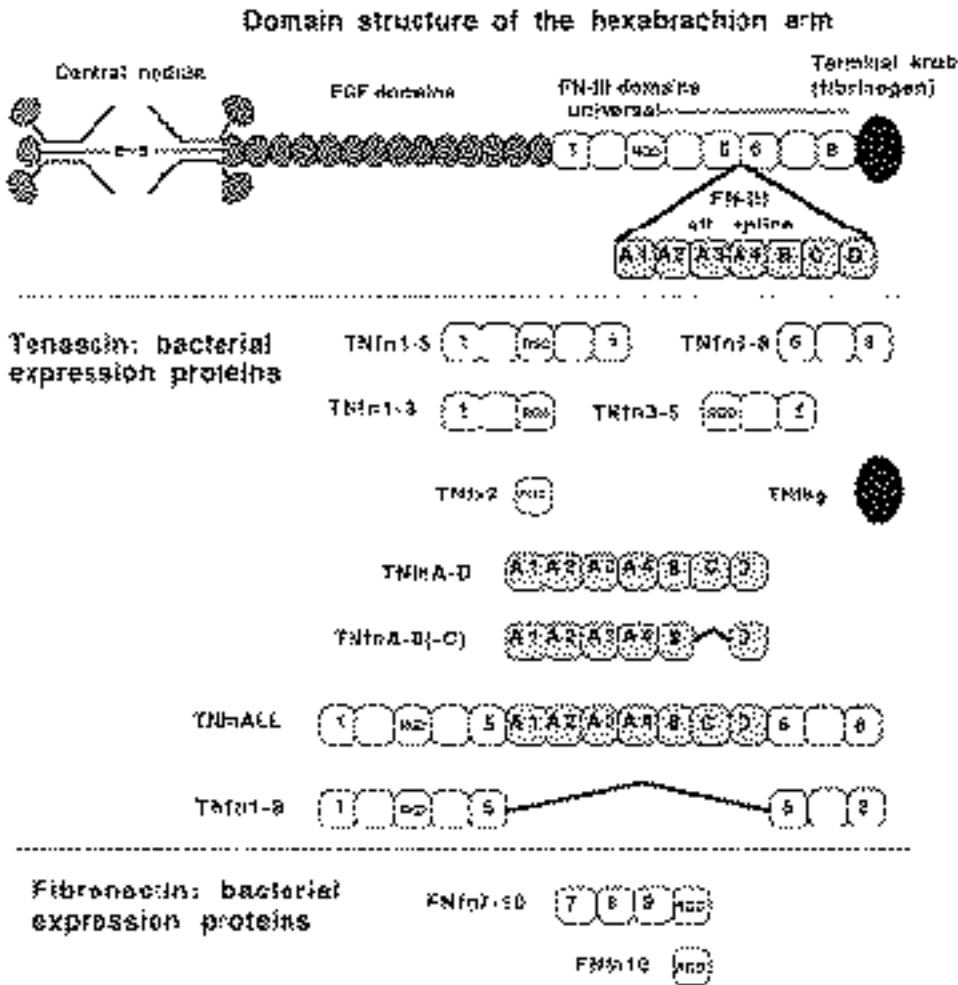


Fig. 1. Diagram of the domain structure of the TN subunit, and the expression proteins used in the present study. In addition to the TN proteins, two expression proteins for FN used in this study are indicated. (From Aukhil et al., 1993.)

added as a soluble protein to well-spread endothelial cells. Chiquet et al. (1991) reported that a proteolytic fragment comprising the last three FN-III repeats and the fibrinogen domain (TNfn6-8 plus TNfbg) inhibited spreading of fibroblasts on FN. Prieto et al. (1992) reported an anti-adhesion effect of a fusion protein corresponding approximately to TNfn7-8. Inhibition of adhesion and spreading was observed when this fusion protein was incorporated as a mixed substratum with FN, and also when pre-incubated with cells. These last two reports both indicate an anti-adhesion activity in TNfn7-8.

To provide tools for extended studies of TN, we have produced a library of bacterial and mammalian expression proteins that map the entire hexabrachion arm in defined segments (Aukhil et al., 1993; and Fig. 1). Each of the proteins corresponds to precisely defined boundaries of the FN-III domains and contains no fusion protein. The expression proteins are all soluble as monomeric molecules, and each has been highly purified. Of course these bacterial expression proteins are not glycosylated, but the biochemical analysis suggests that they are all correctly folded and otherwise identical to the domains in native TN (Aukhil et al., 1993). The set of bacterial proteins is complemented by mammalian expression proteins HxB.L, HxB.S and HxB.egf, corresponding to the large and small splice variants of human tenascin, and to a protein truncated at the

end of the EGF segments. These three proteins are secreted by transfected BHK cells and assembled into normal hexabrachion molecules (Aukhil et al., 1993). Finally, we have produced two bacterial expression proteins for FN domains.

Of particular interest is the domain TNfn3, which has an RGD sequence in human and chicken TN, and whose structure has recently been solved to atomic resolution (Leahy et al., 1992). In our initial studies we found that fibroblasts did not adhere to TNfn3 or to larger segments containing it. However, Prieto et al. (1992) demonstrated adhesion of fibroblasts and other cells to fusion proteins containing TNfn3, using an assay that was perhaps more sensitive than ours.

A key observation leading to our present analysis was that endothelial cells adhere very strongly to TNfn3. As well, endothelial cells adhere much more strongly than fibroblasts to FNfn10, the RGD-containing domain from FN. Because endothelial cells exhibited the strongest adhesion to several proteins, and because TN is frequently associated with blood vessels (Erickson and Bourdon, 1989), we explored in detail adhesion of endothelial cells to domains of TN and FN, and compared them to several other cell lines that have weaker or no adhesion. Finally, we have new experimental work that addresses the controversy over whether intact TN can act as a cell adhesion substratum. The adhesion activity depends on the nature of the sub-

stratum (plastic vs nitrocellulose) and the length of time the TN is incubated on the substratum.

MATERIALS AND METHODS

Cells and reagents

Non-transformed fetal bovine aortic endothelial cells (AG7680), and a transformed line derived from them (GM7373) (Grinspan et al., 1983; Maier et al., 1990) were obtained from Coriell Institute for Medical Research, Camden, NJ. Endothelial cells from mouse brain, heart and thorax were provided by Dr Robert Auerbach, Department of Zoology, University of Wisconsin, Madison. Cells were cultured in DMEM-H (Dulbecco's Modified Eagle's Medium, high glucose) with 10% fetal calf serum. NRK cells were obtained from the ATCC, and the hamster fibroblasts NIL.8M were obtained from Dr Richard Hynes, MIT. Anti- α 3 and anti- α 5 antisera were purchased from Telios Pharmaceuticals, Inc., San Diego, CA. Native FN was purified from human plasma by gelatin-agarose affinity chromatography, and native TN was purified from conditioned medium of U-251 MG glioma cells by gel filtration and anion exchange chromatography (Aukhil et al., 1990).

Bacterial expression proteins for defined domains of TN and FN were produced and purified as described by Aukhil et al. (1993). Tenascin and the TNfn3 domain were iodinated by the Chloramine T method (Greenwood et al., 1963) to a specific activity of 0.3-1 mCi per μ g of protein. The integrity of the labeled protein was determined by SDS-PAGE followed by autoradiography.

Cell adhesion assay

The assay was carried out as described by Lightner and Erickson (1990) and Aukhil et al. (1993). Falcon 3912 microtitre plates, which are 96-well polyvinylchloride plastic not treated for tissue culture, were used for quantitative assays. For experiments shown in Figs 2-8, substrata were coated with protein by adding 100 μ l protein solution in phosphate buffered saline (PBS) at 37°C for 60 min. Wells were emptied, washed twice with PBS, and free sites on plastic were blocked by adding 1 mg/ml heat-denatured BSA for 1 hour, followed by a PBS wash. (For the experiments in Figs 9-10 the substratum proteins were coated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ at the temperatures and times indicated, and then blocked with 5% non-fat dried milk. Also in these experiments, plastic substrata were compared with plastic over-coated with nitrocellulose, prepared as follows: 20 cm² nitrocellulose paper (Bio-Rad) was dissolved in

50 ml of methanol, 20 μ l of nitrocellulose solution was added to each well and dried under the laminar-flow hood.) Each well was loaded with 100 μ l PBS (sometimes containing an inhibitor such as RGD peptide) and 35,000 to 50,000 cells in 100 μ l DMEM medium. Prior to this assay the cells had been removed from their substratum by mild treatment with trypsin EDTA. The plates were incubated for 60 min at 37°C in an atmosphere of 5% CO₂, unbound cells were removed by gentle pipetting, wells were washed twice with PBS and cells were fixed with 3% paraformaldehyde.

Attached cells were quantitated by staining with 1% Toluidine blue and lysing the cells with 2% SDS. The intensity of the color was measured at 595 nm. Readings in wells precoated with BSA (typically $A_{595} \sim 0.04$) were considered as blank and subtracted from each value. Three duplicate wells were averaged for each point tested. The standard deviation of the three points was typically about 10% of the mean. Although this staining procedure does not permit an easy determination of the fraction of cells bound, we have previously determined (Lotz et al., 1989) that cell binding is saturated at 10 μ g/ml FN, with >80% of the cells bound. The A_{595} corresponding to this saturated binding of cells to FN varied from 0.5 to 0.6 in most experiments, so A_{595} values of 0.3 or 0.1 indicate 50% and 20% of added cells bound.

RESULTS

Endothelial cell adhesion to recombinant TN and fibronectin domains

We tested the cell adhesion activity of all 12 of our bacterial expression proteins and the three mammalian TN constructs, as well as purified native TN and fibronectin. Most of our work used the transformed endothelial cell line GM7373, but identical results were obtained with each of the endothelial cell lines tested. Table 1 summarizes the adhesion results for the GM7373 cells, their untransformed parent line AG7860, and three other cell types. The adhesion responses fell into three distinctly different categories: (1) adhesion and spreading; (2) adhesion but no spreading (cells remain round); and (3) no adhesion (Fig. 2 and Table 1).

An important preliminary discovery in these experiments was that endothelial cells adhere much more avidly than other cell types tested. This was particularly evident in adhesion to the single FN-III domains FNfn10 and TNfn3. All endothelial cells tested adhered to these domains,

Table 1. Cell adhesion to TN and FN expression proteins

Cell type	TNfn3	TNfn1-5	TNfnALL	TNfbg	FNfn10	FNfn7-10
GM7373 endothelial	Ad S	Ad R	Ad R	Ad R	Ad R	Ad S
AG7860 endothelial	Ad S	Ad R	Ad R	Ad R	Ad R	Ad S
NRK epithelial	Ad R	Ad R		Ad R		
RES fibroblasts	N Ad	N Ad	N Ad	Ad R	N Ad	Ad S
NIL fibroblasts	N Ad	N Ad		Ad R	N Ad	Ad S

Cell adhesion to the different substrata was classified into three categories: adherent and spread (Ad S), adherent and round (Ad R) and non-adherent (N Ad). These results are for proteins coated on plastic for 60 min. The expression proteins TNfnA-D, TNfn6-8 and HxB.egf gave no adhesion of any cell type tested.

Because our assay showed no adhesion of fibroblasts to domains containing TNfn3, while Prieto et al. (1992) did observe adhesion of fibroblasts to similar domains, we sent samples of our proteins to Prieto and Crossin to test in their assay. Proteins were coated at higher concentration than we use, i.e. 50-100 μ g/ml. Consistent with our results, they observed no adhesion to TNfnA-D or to TNfn6-8, but substantial adhesion to TNfbg. In addition, they observed adhesion of U251 MG glioma cells and 3T3 fibroblasts to TNfn3, TNfn1-5 and TNfn1-8. Apparently their assay is sufficiently sensitive to demonstrate adhesion of fibroblasts to segments containing TNfn3, which are negative in our assay (A. L. Prieto and K. L. Crossin, personal communication.)

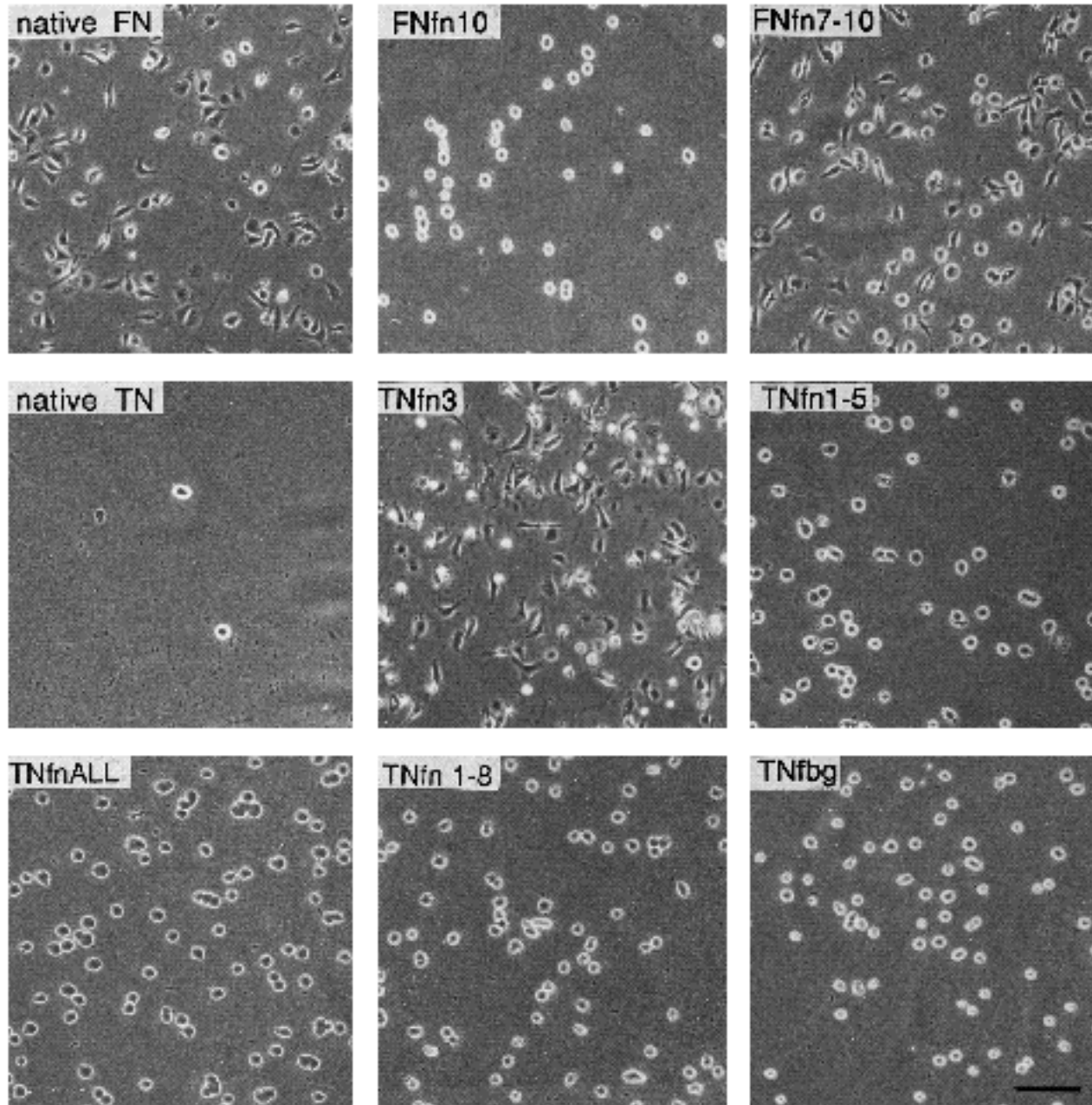


Fig. 2. Attachment and spreading of endothelial GM7373 cells on substrata coated with recombinant FN or TN fragments. For these microscope examinations, Nunc Lab-Tek polystyrene chamber slides were coated with native FN (5 $\mu\text{g/ml}$), FNfn10 (10 $\mu\text{g/ml}$), FNfn7-10 (5 $\mu\text{g/ml}$), native TN (purified from U-251 MG cell cultures, 25 $\mu\text{g/ml}$), TNfn3 (5 $\mu\text{g/ml}$), TNfn1-5 (10 $\mu\text{g/ml}$), TNfnALL (10 $\mu\text{g/ml}$), TNfn1-8 (10 $\mu\text{g/ml}$), and TNfbg (25 $\mu\text{g/ml}$). GM7373 endothelial cells were harvested with trypsin-EDTA and allowed to attach and spread on the substratum for 1 hour before fixation and photography. The fields shown here were selected to show a large number of cells of representative morphology, and do not always reflect the number of cells bound, e.g. TNfnALL gave the weakest adhesion in the quantitative assay. Quantitation of adhesion is given in Fig. 3. Bar, 100 μm .

whereas fibroblasts did not. It is well established from previous studies that fibroblasts adhere and spread on FNfn7-10, but adhere very weakly or not at all to the single domain FNfn10 (Kimizuka et al., 1991; Aota et al., 1991; Aukhil et al., 1993). In addition to the two lines of fetal bovine aortic endothelial cells listed in Table 1, endothelial cell lines 14077-(P9) from mouse heart, 1841-(P9) from mouse brain, and 1-C4 from mouse thoracic duct, all provided by Dr R. Auerbach, University of Wisconsin, attached and

spread on TNfn3. All of these endothelial cells also attached but remained rounded on TNfbg.

The only TN expression proteins that promoted cell adhesion were the domains TNfn3 (and larger segments that contain it) and TNfbg. No adhesion of any cell type was observed to TNfnA-D (the alternatively spliced segment), to TNfn6-8 (the last three FN-III domains), or to the mammalian expression protein HxB.egf, which comprises the central knob and the 14 EGF domains. Remarkably, prepa-

rations of native tenascin were completely negative for cell attachment in this assay condition (native TN in Fig. 2). Adhesion activity of native TN was later revealed by coating the protein on nitrocellulose or plastic for much longer times than the 60 min used for our routine assays (Figs 9-10).

Endothelial cell adhesion to TNfn3 is partially blocked by adjacent TNfn2 and is inhibited by RGD peptides and by antiserum to $\alpha_v\beta_3$

Fig. 3 shows the concentration dependence of adhesion to TNfn3 and to larger segments containing this domain. The single domain TNfn3 had the highest activity, and there was a clear trend toward reduced adhesion as more domains were added. The larger segments showed progressively less adhesion at a given coating concentration (e.g. 1-2 $\mu\text{g/ml}$), and the maximal adhesion obtained at any concentration was also reduced. Adhesion to the larger segments actually fell off at higher concentrations. This is seen in Fig. 3 for TNfn1-5, and was also observed for TNfn1-8 and TNfn-ALL, which gave reduced adhesion at 20 $\mu\text{g/ml}$ and no adhesion at all when coated at 100 $\mu\text{g/ml}$ (data not shown). The reduced adhesion activity of the larger fragments may be due to the increased steric blocking by the non-adhesive domains.

A second and very striking aspect of reduced adhesion was that the cells avidly spread on TNfn3, whereas on the larger domains they remained rounded (Fig. 2). Substrata giving the rounded phenotype did so at all coating concentrations, i.e. there was no indication that cell spreading could be obtained at higher substratum densities. It seems that TNfn3 has a very strong adhesion activity, sufficient to promote complete cell spreading, when presented on the substratum as an isolated domain, but this activity is severely reduced when the domain is incorporated into a larger segment. The explanation for this aspect of reduced adhesion was suggested by the X-ray crystal structure of TNfn3.

The crystal structure showed that the RGD sequence in TNfn3 is on a tight beta turn, exposed on the surface of the domain (Leahy et al., 1992). Although the RGD sequence is toward the C terminus of the linear sequence, in the 3-D structure it is seen to be very close to the N terminus. This suggested that the adjacent domain TNfn2 might partially block the RGD. To test this hypothesis we synthesized the segments TNfn1-3 and TNfn3-5 (Fig. 1). As seen in Figs 4 and 5, the adhesive activity of these segments is consistent with this prediction. Cells attached and spread on TNfn3 and TNfn3-5, both of which have the RGD exposed at the N terminus. Cell attachment to TNfn1-3 required higher substratum concentrations, and the cells remain rounded. The adhesion activity of TNfn1-3 was almost identical to that of TNfn1-5.

Since TNfn3 (from human and chicken) contains an RGD sequence, we tested whether RGD peptides would inhibit adhesion. Fig. 6 shows that adhesion to TNfn3 and to larger segments containing it is extremely sensitive to soluble RGD peptide. Adhesion to TNfn3 and TNfn3-5, on which cells spread, was inhibited 70% by 2.5 $\mu\text{g/ml}$ of soluble GRGDSP. Adhesion to TNfn1-3 and to FNfn10 was inhibited

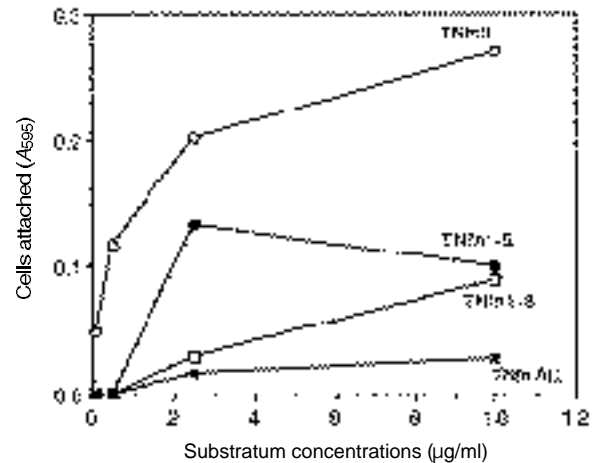


Fig. 3. Attachment of GM7373 cells to TNfn3 and to three larger expression proteins containing this domain. The concentration of the protein used to coat the plastic substratum is shown on the abscissa. Results are the mean of triplicate observations in an experiment. Similar results were obtained when the experiment was repeated twice. BSA served as a negative control, and its value (A_{595} 0.035) has been subtracted from all points.

ated at GRGDSP concentrations as low as 0.2-0.5 $\mu\text{g/ml}$. The control peptide GRGESP had no effect at 25 $\mu\text{g/ml}$. Adhesion to these domains is thus much more sensitive to soluble RGD peptides than is adhesion to intact fibronectin, which requires 350-1,000 $\mu\text{g/ml}$ for ~70% inhibition (Pierschbacher and Ruoslahti, 1984, and our unpublished data), or to vitronectin, which is inhibited by 35-100 $\mu\text{g/ml}$ RGD (Pierschbacher and Ruoslahti, 1987).

The sensitivity to RGD peptides strongly suggests that the adhesion is mediated by an integrin receptor. An integrin interaction was also suggested by the cation requirement for adhesion: EDTA or EGTA (2.5 mM) completely abolished cell attachment to TNfn3, TNfn1-5 and FNfn10 (data not shown).

To confirm the involvement of integrins and to identify the specific molecules, we obtained polyclonal antisera against $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, which block the activity of the specific integrin (see Massia and Hubbell, 1991, for a previous use of these antisera). Cell attachment to TNfn3 was inhibited by anti- $\alpha_v\beta_3$ antiserum (Fig. 7). This antiserum also inhibited cell attachment to TNfn1-5 and to FNfn10. Anti- $\alpha_5\beta_1$ antiserum gave no inhibition on any substrata. These results suggest that endothelial cell adhesion to TNfn3 and to FNfn10 is mediated by the integrin

$\alpha_v\beta_3$.

Endothelial cells adhere to TNfbg

Only one other TN domain, TNfbg, showed cell adhesion activity for endothelial cells. In a previous study we observed that fibroblasts attached to TNfbg, and that adhesion was mediated by a cell surface proteoglycan, not an integrin (Aukhil et al., 1993). Thus fibroblast cell attachment was inhibited by treating the substratum with heparin (which binds to TNfbg), or by treating the cells with heparinase (which cleaves cell surface heparan sulfate) or with chlorate ions (which inhibit sulfation of proteoglycans). We

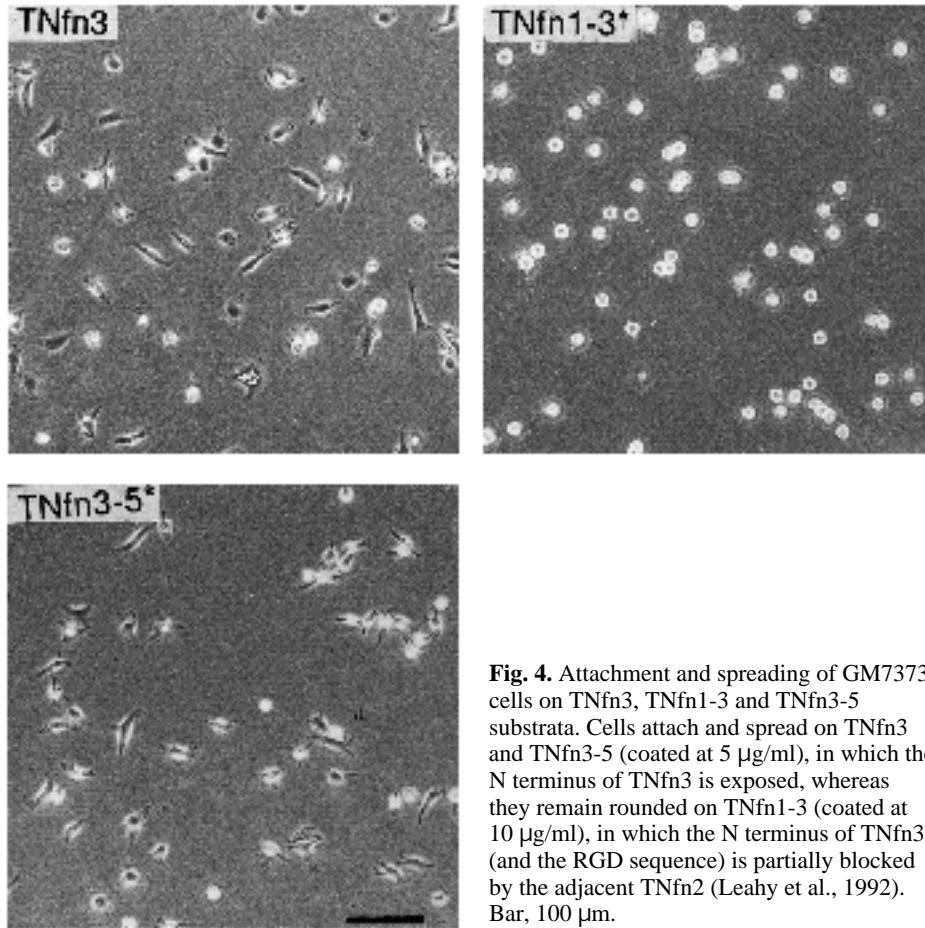


Fig. 4. Attachment and spreading of GM7373 cells on TNfn3, TNfn1-3 and TNfn3-5 substrata. Cells attach and spread on TNfn3 and TNfn3-5 (coated at 5 $\mu\text{g/ml}$), in which the N terminus of TNfn3 is exposed, whereas they remain rounded on TNfn1-3 (coated at 10 $\mu\text{g/ml}$), in which the N terminus of TNfn3 (and the RGD sequence) is partially blocked by the adjacent TNfn2 (Leahy et al., 1992). Bar, 100 μm .

expected the adhesion of endothelial cells to TNfbg also to be mediated by proteoglycans. However, several experiments (data not shown) suggest that endothelial cell adhesion to TNfbg is mediated by integrins, with little or no contribution from proteoglycans. (1) Adhesion was completely eliminated by adding 2.5 mM EDTA or EGTA to the medium. These chelators had no effect on fibroblast adhesion to TNfbg, but are known to inhibit integrin-mediated adhesion (Hynes, 1992). (2) Adhesion of endothelial cells to TNfbg was not inhibited by heparin, or by treating the cell cultures with chlorate. (3) Adhesion was inhibited by the peptide GRGDSP (Fig. 8) at peptide concentrations of 25–50 $\mu\text{g/ml}$, similar to the concentration that inhibits cell adhesion to vitronectin. Endothelial cell adhesion to TNfbg was not inhibited by the antisera to either the $\alpha_5\beta_3$ or the $\alpha_5\beta_1$ integrins.

A search for anti-adhesion domains

Since two domains of TN gave adhesion of endothelial cells when tested as isolated domains, we wanted to address the question: why do endothelial cells not adhere to native TN? One possibility was that an anti-adhesion domain might overwhelm the adhesion activity. Our interest focused on two segments: first, the segment TNfnA-D, which we have found to provoke dismantling of focal adhesions when added to endothelial cells spread on fibronectin (Murphy-Ullrich et al., 1991); second, the segment TNfn6-8, which

has been identified in two studies as inhibiting the adhesion and/or spreading of fibroblasts on FN (Chiquet et al., 1991; Prieto et al., 1992).

We first addressed this question by testing our mammalian expression proteins, HxB.L and HxB.S, corresponding to the two splice variants of native TN. Each of these is assembled by the cells and secreted as intact hexabrachions. Since HxB.S is missing the alternatively spliced segment (TNfnA-D), cells would be expected to adhere to it if this segment were responsible for a dominant anti-adhesion effect. However, we found that endothelial cells did not adhere to HxB.L, HxB.S (or to HxB.egf, a truncated hexabrachion containing only the EGF domains) at any substratum coating concentrations tested, up to 100 $\mu\text{g/ml}$. (Note that these experiments were all done by coating the proteins for 60 min on plastic; cf. Figs 9–10, below, for conditions giving positive adhesion to native TN.) Thus the alternatively spliced segment does not seem to be responsible for the lack of cell adhesion to native TN.

We next made an equimolar mixture of bacterial expression proteins TNfn1-5, TNfnA-D, TNfn6-8 and TNfbg, and the mammalian protein HxB.egf. This mixture should be equivalent to a complete TN subunit. This mixture was used to coat plastic at concentrations of 20, 100 and 200 nM (total protein concentration 5–50 $\mu\text{g/ml}$) and tested for cell adhesion. GM7373 cells adhered to this substratum at all three concentrations, the cells remaining

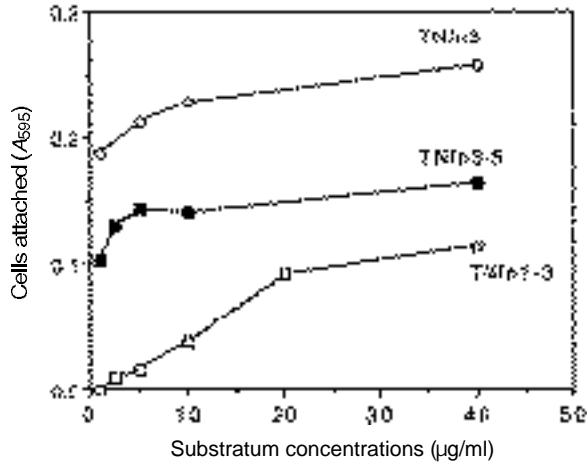


Fig. 5. Quantitative assay of adhesion to TNfn3, TNfn1-3 and TNfn3-5, as a function of substratum-coating concentration.

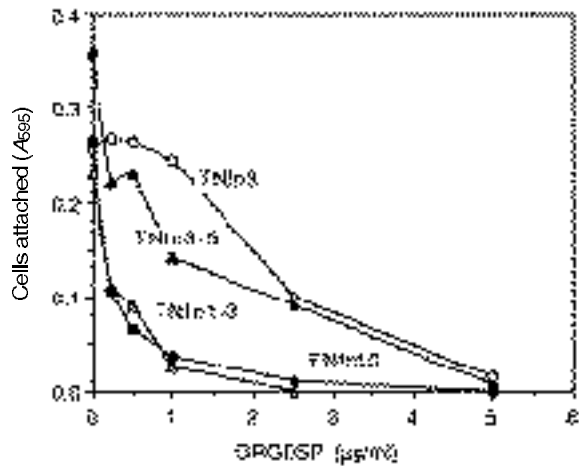


Fig. 6. Soluble GRGDSP inhibits cell attachment to the RGD-containing domains from TN and FN. The substratum-coating concentrations were 1 µg/ml for TNfn3; 2.5 µg/ml for TNfn3-5; 20 µg/ml for TNfn1-3; and 5 µg/ml for FNfn10.

rounded as on the separate segments TNfn1-5 and TNfn6-8. Thus the complete tenascin subunit can support adhesion when coated onto the plastic substratum as a mixture of smaller pieces.

Finally, we tested directly whether TNfnA-D or TNfn6-8 could inhibit adhesion to any defined adhesion substratum. Native FN, TNfn3 and TNfn6-8 were separately coated on plastic and tested for cell adhesion in the presence of TNfnA-D or TNfn6-8, added with the cells as soluble molecules. TNfnA-D had no effect on adhesion to any substratum at concentrations up to 500 µg/ml. TNfn6-8 gave mixed results: in some experiments it inhibited cell adhesion to FN by 30-50% when added with cells at 20-500 µg/ml, but in other experiments under apparently identical conditions it gave no inhibition at all. Even in experiments showing inhibition, the concentration dependence was frequently erratic. We therefore could not demonstrate a reproducible inhibitory effect of TNfn6-8, but further work is

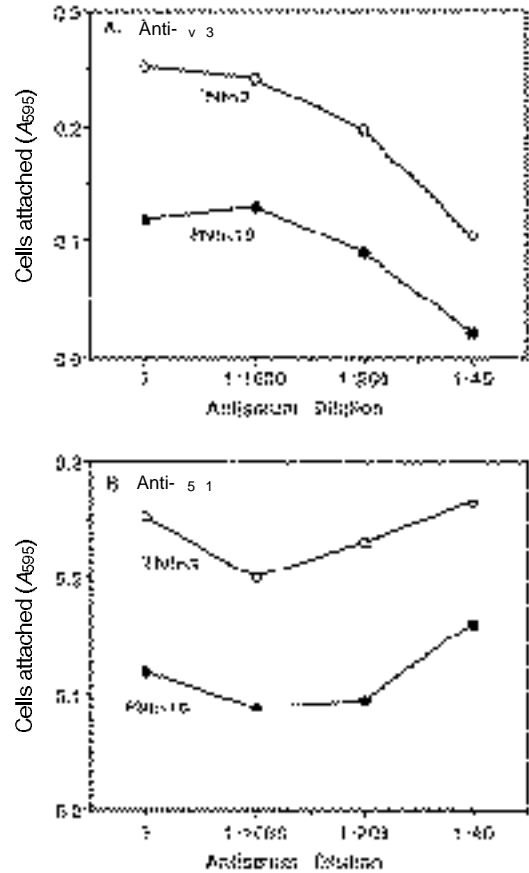


Fig. 7. Effects of integrin-specific antisera on GM7373 cell attachment to TNfn3 (open circles) or FNfn10 (filled circles). The coating concentrations of TNfn3 and FNfn10 were 1 and 3.6 µg/ml, respectively. (A) shows the effect of different dilutions of anti- v 3, and (B) shows anti- 5 1.

clearly indicated in view of the substantial inhibitions reported by Chiquet et al. (1991) and Prieto et al. (1992).

We also tested whether the adhesion domains of tenascin might be revealed by breaking the hexabrachion structure into single arms. We treated TN with 2 mM DTT for 30 min before coating on plastic, conditions that we have found sufficient to reduce the disulfide bonds in the central nodule and convert the hexabrachion into single subunits (Erickson and Briscoe, 1993). Substrata of reduced TN coated on plastic at concentrations up to 100 µg/ml were completely negative for adhesion activity in this assay. If there is a structural feature that blocks cell adhesion it appears to be inherent to each arm of the hexabrachion.

Cell adhesion to TN and recombinant domains depends on the substratum (plastic vs nitrocellulose) and on the coating time

Because nitrocellulose is known to bind proteins tightly and in a conformation suitable for binding antibodies and other ligands, we compared cell adhesion to proteins coated either on plastic or nitrocellulose. Fig. 9 shows that the chemical nature of the supporting substratum has a dramatic effect on adhesion activity. When the proteins were coated on

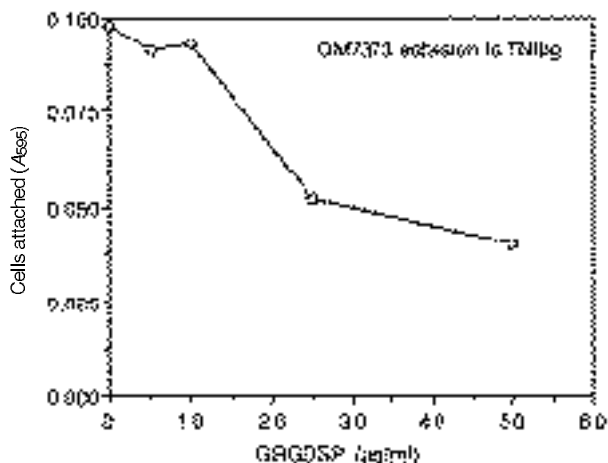


Fig. 8. Inhibition of GM7373 cell adhesion to TNfbg by GRGDSP peptide. The substratum coating concentration was 20 µg/ml.

plastic at 4°C for 12 hours, TNfn3 (20 µg/ml) gave strong adhesion, TNfbg (50 µg/ml) a weaker adhesion, while native TN (100 µg/ml) gave a weaker but still significant adhesion. The very weak adhesion to TNfnA-D and TNfn6-8 was not seen in most experiments. Cells spread on TNfn3 and FN, but remained rounded on TNfbg. On nitrocellulose, TNfn3 again gave strong adhesion, but TNfbg had no activity. Most remarkable, native TN on nitrocellulose demonstrated strong adhesion activity. This cell adhesion activity was not due to contamination by fibronectin or other adhesive proteins, since it could be blocked by a polyclonal antiserum to TN. Interestingly, TNfn3 on nitrocellulose was less active than TNfn3 on plastic. Furthermore, cell spreading was greatly reduced when TNfn3 was coated on nitrocellulose, relative to the very pronounced spreading on TNfn3 on plastic. Cells adhering to native TN remained rounded on both plastic and nitrocellulose. Fibronectin, as a positive control, supported strong cell adhesion and spreading on both plastic and nitrocellulose.

The cell adhesion activity of native TN required a remarkably long incubation time for coating the substratum. Fig. 10 shows the adhesion to native TN, TNfn3 and FN coated on plastic and nitrocellulose at 4°C for different times. While the cell adhesion activity of FN and TNfn3 reached a maximum after 1-3 hours coating time, the activity of native TN increased much more slowly out to 24 hours, the last time tested. At all time points tested, TN gave higher adhesion on nitrocellulose than on plastic. In contrast, FN had the same activity on both substrata, while TNfn3 had lower adhesion on nitrocellulose and, as noted above, cells only partially spread on this substratum.

One possibility for the differential effects was that adhesion activity might reflect different amounts of protein bound to the substrata. We therefore compared the amount of TN and TNfn3 domain bound to plastic and nitrocellulose by using radiolabeled proteins. As expected, both TN and TNfn3 bound more abundantly to nitrocellulose than to plastic. Thus, at least for TNfn3, adhesion is not simply correlated with the amount of protein bound: adhesion is weaker on nitrocellulose even though more protein is bound. For native TN on nitrocellulose the time course of

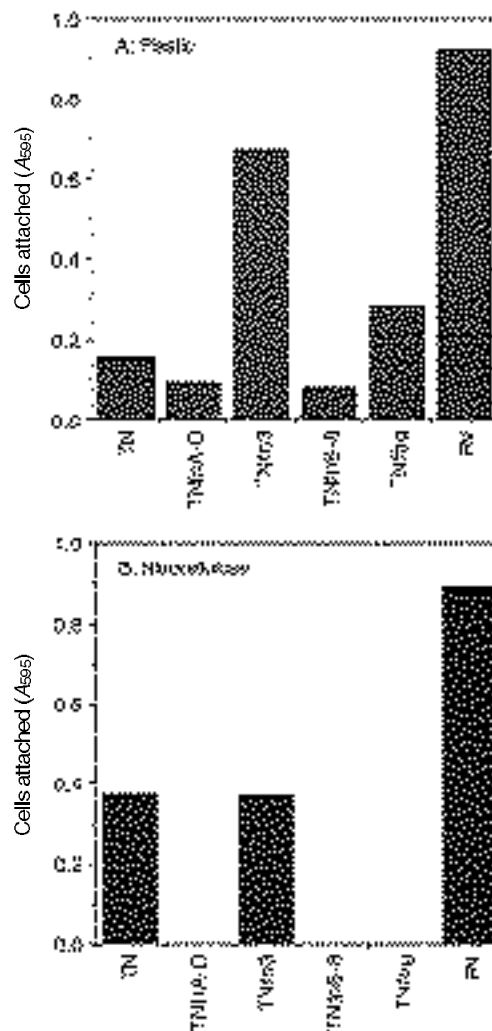


Fig. 9. Attachment of endothelial cells GM7373 on plastic (A) and nitrocellulose (B) coated with TN, TN fragments or FN. Each substratum was coated with native TN (100 µg/ml), TNfnA-D (50 µg/ml), TNfn3 (20 µg/ml), TNfn6-8 (50 µg/ml), TNfbg (50 µg/ml) or FN (50 µg/ml) for 12 hours at 4°C. GM7373 cells were harvested with trypsin-EDTA, washed, and allowed to attach on the substratum for 1 hour at 37°C. Attached cells were fixed, stained with toluidine blue, and read at 595 nm.

cell adhesion correlated reasonably well with that of protein binding. There was a 70% increase in both protein bound and adhesion at 24 hours compared to 6 hours. This correlation did not hold, however, for TN on plastic. Here protein binding was almost complete at 3-6 hours, increasing only about 20% from 6 to 24 hours. In contrast, cell adhesion doubled from 6 to 24 hours. These results suggest that the majority of TN molecules that bound to plastic in the first 3-6 hours of incubation are not active in cell adhesion, while a smaller fraction that bound over the next 18 hours are active.

We also tested whether the GRGDSP peptide would inhibit cell adhesion to native TN. The peptide GRGDSP completely inhibited adhesion of cells to TN coated on either plastic or nitrocellulose at a concentration of 20-50

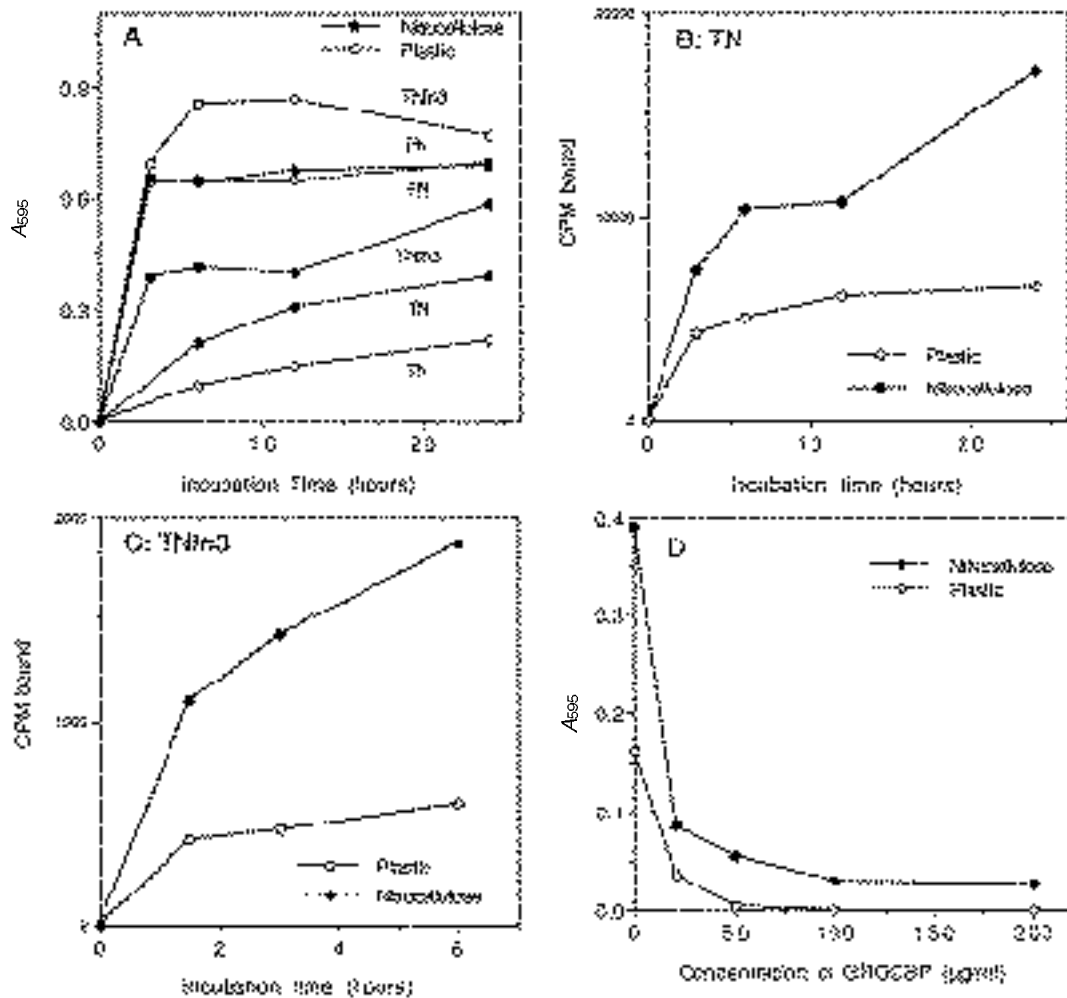


Fig. 10. (A) Attachment of GM7373 cells on plastic and nitrocellulose coated with native TN, native FN and TNfn3, as a function of the time the proteins were incubated with the substratum. Proteins were coated at 20 $\mu\text{g/ml}$ and 4°C. (B and C) Determination of the amount of substratum-bound TN (B) and TNfn3 (C), as a function of incubation time. Radiolabeled TN and TNfn3 (20 $\mu\text{g/ml}$ each) were incubated with plastic and nitrocellulose for various time at 4°C and radioactivity of substratum-bound material was determined. (D) GRGDSP peptide inhibits endothelial cell adhesion to native TN. Substrata were coated with TN for 24 hours at 4°C. Control peptide GRGESP had no effect on adhesion at a concentration of 500 $\mu\text{g/ml}$.

$\mu\text{g/ml}$ (Fig. 10D). The control peptide GRGESP had no effect on adhesion at concentrations up to 500 $\mu\text{g/ml}$. The GRGDSP peptide thus inhibits adhesion to native TN at low concentrations, similar to those effective on TNfn3 and TNfbg, and at least 10 times lower than the concentrations needed to inhibit adhesion to FN.

DISCUSSION

Our initial study of rat embryo skin fibroblasts (Aukhil et al., 1993), identified a single cell adhesion domain in TN, TNfbg. Prieto et al. (1992) observed adhesion of several cell types to their fusion proteins containing TNfn3, and they have also demonstrated adhesion of fibroblasts to our expression proteins containing TNfn3 (see note to Table 1). We conclude that their assay is more sensitive than ours in detecting the weak adhesion of fibroblasts to TNfn3.

We were surprised that fibroblasts did not adhere to either TNfn3 or FNfn10, because each of these domains contains the RGD sequence. We therefore expanded our study from fibroblasts to a variety of other cell types, and discovered that endothelial cells adhere strongly to both of these domains, as well as to larger segments that contain them. The vigorous adhesion of endothelial cells makes them an ideal candidate for identifying cell adhesion substrata. In the present study endothelial cells have been especially valuable in demonstrating the two cell adhesion domains of TN.

The first site, which mediates adhesion of fibroblasts (Aukhil et al., 1993) and all other cells tested, is in the terminal knob domain, TNfbg. This site is probably the one that Spring et al. (1989) attributed to the adjacent TNfn7-8, and it has been demonstrated independently by Prieto et al. (1992). In this latter study, as in ours, cells remain rounded on TNfbg. Surprisingly, the mechanism of adhesion to TNfbg seems to vary with cell type. Fibroblasts

adhere via a heparin sulfate proteoglycan (Aukhil et al., 1993), while the present study suggests that endothelial cells may adhere via an integrin. The inhibition of adhesion by RGD peptides is remarkable because TNfbg does not contain any RGD sequence, but this situation is not unique. The matrix Gla protein also contains no RGD sequence, but cell adhesion is strongly inhibited by 3 µg/ml of soluble RGD peptide (Loeser and Wallin, 1992).

The second cell adhesion site is TNfn3, which contains an RGD sequence in human and chick TN. Endothelial cells adhere strongly to and spread on TNfn3 and on TNfn3-5. All segments containing TNfn3 and having TNfn2 attached at the N terminus gave weaker adhesion and the cells remained rounded. The explanation for the reduced adhesion was suggested by the X-ray crystal structure (Leahy et al., 1992), which showed the RGD segment to be near the N terminus of TNfn3, where it can be partially blocked by TNfn2. Adhesion to TNfn3, and also to the single FN domain FNfn10, appears to be mediated by the α_3 integrin, because it is blocked by an antibody to that integrin, requires divalent cations, and is exquisitely sensitive to soluble RGD peptides.

The activities of TNfn6-8 remain controversial. Two laboratories have reported anti-adhesion activity for these domains (Chiquet et al., 1991; Prieto et al., 1992), but in our experiments TNfn6-8 gave equivocal results. We sometimes observed an inhibition of cell adhesion, but in most experiments the effect was not reproduced. Prieto et al. (1992) also reported that TNfn7-8 would support cell adhesion if the cells were treated with trypsin before the assay. Our cells are routinely harvested with trypsin before the assay and we found no adhesion to TNfn6-8.

Why do endothelial cells adhere more strongly than fibroblasts to the RGD-containing domains? One possibility is that endothelial cells may have substantially more α_3 integrin than fibroblasts. α_3 and β_1 both contribute to adhesion of microvascular endothelial cells to intact FN (Cheng et al., 1991), but HT-1080 fibrosarcoma cells use only β_1 to adhere to FN and to expression proteins similar to our FNfn7-10 (Aota et al., 1991).

We do not understand why adhesion to TNfn3 is stronger than to FNfn10. Both of these single FN-III domains contain an RGD sequence on a tight turn between the F and G beta strands (Leahy et al., 1992; Main et al., 1992). If anything, we would have expected stronger adhesion to FNfn10, in which the RGD loop extends outward about 7 Å further than in TNfn3. The strong adhesion to TNfn3 might involve other patches on the surface of this domain, but we have not initiated tests that might reveal the mechanism.

What is the biological significance of endothelial cell adhesion to TNfn3 and TNfbg? Two observations suggest that adhesion to TNfn3 may not be biologically important. First, this adhesion is dramatically reduced in the native molecule or in any protein that has TNfn2 linked to TNfn3. One might postulate a specific proteolytic cleavage, or a bending of the hexabrachion arm, that could expose the stronger adhesion site, but there is no evidence for such processing. Second, the RGD sequence that is found in human and chick TN is replaced by RVD in mouse (Weller et al., 1991; Saga et al., 1991), RAD in pig (Nishi et al.,

1991), and RGL in newt (Onda et al., 1991) TN. Both the cryptic location of the RGD in the intact TN molecule, and the species variation of the RGD sequence, suggest that this site may not be functional in cell adhesion.

Cell adhesion to TNfbg seems more likely to be biologically important. This site at the tip of each arm of the hexabrachion is ideally exposed for binding to cell surfaces, and indeed almost all cells tested bind to it (Prieto et al., 1992; Aukhil et al., 1993, and this paper). Although the cell adhesion to TNfbg is weaker than to FN or vitronectin, the large size and multivalent structure of the hexabrachion would make it an ideal bridge between cells. Alternatively, the physiological role of cells binding TNfbg might be something other than cell adhesion. The receptors for TNfbg might respond directly to the ligand binding by a signalling mechanism, or the binding might enhance interaction of TN with other receptors.

The experiments reported here may resolve the long controversy over the cell adhesion activity of native TN. Previous studies in our laboratory and several others reported no cell adhesion to TN-coated plastic substrata (Erickson and Taylor, 1987; Mackie et al., 1987; Lotz et al., 1989; Lightner and Erickson, 1990; Aukhil et al., 1990; Saginati et al., 1992), whereas other groups have reported a weak adhesion of cells with a rounded morphology (Friedlander et al., 1988; Bourdon and Ruoslahti, 1989; Chiquet-Ehrismann et al., 1988; Spring et al., 1989; Nörenberg et al., 1992; Prieto et al., 1992). Our present experiments suggest that the adhesion activity depends on how the substratum is prepared. If native TN is coated on plastic for 1-2 hours, the standard conditions for our assays over the years, it has no adhesion activity. We have now found two conditions that reveal a cell adhesion activity of TN: (1) coat the protein on plastic as separate domains or as a mixture of expression proteins, corresponding to tenascin subunits cut into pieces; or (2) coat the plastic with native TN for 12-24 hours, instead of the 1-2 hours that is sufficient for most other adhesion proteins (coating on nitrocellulose produces a stronger adhesion than on plastic). The mechanism for exposing or concealing the adhesion site(s) is not clear, but it may involve the orientation of the molecules when they are coated under different conditions. For example, it may be important that the terminal knobs, which we believe are the active adhesion domains in native TN, are pointing up toward the cells rather than bound to the plastic.

The results of the study by Sriramarao et al. (1993) agree with most of our findings and complements our observations in important aspects. They observed adhesion of endothelial cells to native TN coated on plastic (note that they coated the plastic overnight, 12-14 hours) and identified two integrins that are involved. Both their results and ours show that α_3 binds to the TN domain TNfn3. The second TN-binding integrin that Sriramarao et al. discovered, $\alpha_2\beta_1$, bound to an unknown domain of TN. In our work we concluded that the TN domain TNfbg bound an unknown integrin. We suggest that these results complement each other, and $\alpha_2\beta_1$ may be the integrin that binds TNfbg. Moreover, the peptide and antibody inhibition studies suggest that $\alpha_2\beta_1$ integrin binding to TNfbg may be the primary mechanism of adhesion of endothelial cells to native TN. The peptide GRGDSP inhibited adhesion to both TNfbg and

native TN (Figs 8, 10D; and Bourdon and Rouslahti, 1989), whereas the SRRGDMS peptide from TNfn3 only partially inhibited adhesion to native TN. Antibodies against $\alpha_2\beta_1$ blocked adhesion to native TN much more effectively than antibodies against $\alpha_v\beta_3$. As suggested above, the binding of $\alpha_v\beta_3$ to TNfn3 may not be a biologically significant mechanism, but binding of $\alpha_2\beta_1$ to TNfbg probably plays a role in binding native TN to endothelial cells.

Finally, we must recognize that the activities observed in these cell culture assays are apparently not vital for normal animal development, since complete deletion of TN in a mouse produced no detectable phenotype (Saga et al., 1992). TN is apparently not an essential component of the endothelium, in spite of the fact that it is localized around many blood vessels and mediates adhesion of endothelial cells. It is premature, however, to conclude that TN has no role in blood vessel structure. It is possible that TN contributes in a non-vital but supportive capacity to angiogenesis, to maintain the structure of formed blood vessels, or to modulate the function of endothelial and smooth muscle cells. The roles of TN may be subtle, contributing to survival under conditions of stress, yet not producing a gross phenotype that would be obvious from casual observation of mice in a laboratory environment. Biochemical and cell culture experiments may provide clues as to where to look for subtle roles in living animals. The present work suggests that the vasculature in the tenascin knockout mice should be examined closely for defects, perhaps under conditions of stress.

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Note added in proof

We have recently developed three polyclonal antibodies to separate recombinant proteins that allow us to probe the domains that promote adhesion to the native TN substrata-

tum. Antibodies to TNfn1-5 or TNfnA-D, applied to the TN substratum before the cells, inhibited adhesion of endothelial cells only slightly, while an antibody to TNfn6-8 plus TNfbg (the two proteins were combined for this immunization) inhibited adhesion 80%. Thus TNfbg appears to be the major cell adhesion site in substrata of native TN.