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Mitogenesis, Cell Migration, and Loss of Focal Adhesions Induced by Tenascin-C Interacting with Its Cell Surface Receptor, Annexin II

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In a previous study we demonstrated that the alternatively spliced region of tenascin-C, TNfnA-D, bound with high affinity to a cell surface receptor, annexin II. In the present study we demonstrate three changes in cellular activity that are produced by adding intact tenascin-C or TNfnA-D to cells, and we show that all three activities are blocked by antibodies against annexin II. 1) TNfnA-D added to confluent endothelial cells induced loss of focal adhesions. 2) TNfnA-D produced a mitogenic response of confluent, growth-arrested endothelial cells in 1% serum. TNfnA-D stimulated mitogenesis only when it was added to cells before or during exposure to other mitogens, such as basic fibroblast growth factor or serum. Thus the effect of TNfnA-D seems to be to facilitate the subsequent response to growth factors. 3) TNfnA-D enhanced cell migration in a cell culture wound assay. Antibodies to annexin II blocked all three cellular responses to TNfnA-D. These data show that annexin II receptors on endothelial cells mediate several cell regulatory functions attributed to tenascin-C, potentially through modulation of intracellular signalling pathways.

INTRODUCTION

Tenascin-C (TN-C) is a large extracellular matrix protein that is prominently expressed in many developing tissues but shows a restricted pattern of expression in adult tissues (Erickson and Bourdon, 1989). However, enhanced TN-C expression has been observed in areas where active cell proliferation and tissue reorganization occur, in wound healing (Mackie *et al.*, 1988; Whitby and Ferguson, 1991), in regenerating nerve (Daniloff *et al.*, 1989; Gatchalian *et al.*, 1989), and in a range of tumors (Chiquet-Ehrismann *et al.*, 1986; Koukoulis *et al.*, 1991). Angiogenesis, including proliferation of endothelial cells and differentiation of new vessels, is essential for tissue reorganization in healing wounds and growing tumors. In glioblastoma multiformes, TN-C is prominently associated with the hyperplastic blood vessels of the tumor, and much less with the adjacent normal blood vessels (Zagzag *et al.*, 1995). However, these immunohistochemical studies do not demonstrate any function for TN-C in the angiogenic process.

A previous study reported that endothelial cells lose focal adhesions in response to soluble TN-C (Murphy-Ullrich *et al.*, 1991). This rearrangement of the actin cytoskeleton is also seen when endothelial cells are treated with basic fibroblast growth factor (bFGF)[1] (Sato and Rifkin, 1988), or phorbol esters (Schliwa *et al.*, 1984), where the loss of focal adhesions is accompanied by a mitogenic response. Two recent studies report contradictory results on how TN-C can affect

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[1] Abbreviations used: BAE, bovine aortic endothelial; bFGF, basic fibroblast growth factor; BrdU, 5′-bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium with high glucose; D-TBS, 20 mM tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$; TN-C, tenascin-C.

the mitogenic response of cells. End *et al.* (1992) reported that TN-C was mitogenic for several cell types and that this activity was associated with a region in the fibronectin type III domains. In contrast, Crossin (1991) reported that TN-C inhibited the mitogenic response of 3T3 fibroblasts stimulated by growth factors.

TN-C expressed in healing wounds and in the stroma of malignant tumors usually includes the alternatively spliced segment, TNfnA-D, comprising fibronectin type III domains labeled A–D (Borsi *et al.*, 1992; Zagzag *et al.*, 1995). We have found that TNfnA-D is the primary site for the binding of soluble TN-C to cells and it binds with high affinity to cell surface annexin II (Chung and Erickson, 1994). The identification of annexin II as a receptor was surprising because annexin II has been well characterized as a cytoplasmic protein (Raynal and Pollard, 1994), and it does not have a hydrophobic signal sequence for secretion. However, a number of recent papers have reported that annexins can be secreted to the extracellular phase by an unknown mechanism, and can be found on the surface of cells as a receptor for various proteins (see Chung and Erickson, 1994, for references; also Hajjar *et al.*, 1994).

Does binding of TN-C to the annexin receptor induce any change in the activity of cells? In the present study, we studied three activities of cultured endothelial cells that are induced by addition of soluble TN-C: loss of focal adhesions, mitogenesis, and enhanced cell migration. Experiments demonstrate that all these activities are produced by the alternatively spliced segment TNfnA-D, and involve its interaction with the annexin II receptor.

MATERIALS AND METHODS

Cells and Cell Cultures

The bovine endothelial cell line GM7373 (from Coriell Institute for Medical Research, Camden, NJ; originally described by Grinspan *et al.*, 1983), was produced by treating a primary fetal aortic endothelial cell clone, BFA1c, with benzopyrene. The bovine aortic endothelial (BAE) cells were isolated from aortas obtained at local abattoirs using collagenase according to established protocol and routinely cultured in Dulbecco's modified Eagle's medium, high glucose (DMEM) supplemented with 10% heat-inactivated fetal calf serum. BAE cells used for focal adhesion experiments were cultured in DMEM supplemented with 20% fetal calf serum (HyClone, Logan, UT), and then in serum-free medium for treatment with TN-C.

Proteins and Antibodies

TN-C was purified from culture supernatant of U-251 MG human glioma cells by gel filtration and mono Q ion exchange chromatography as described by Aukhil *et al.* (1990). For the experiment in Figure 1B the large (HxB.L) and small (HxB.S) splice variants of native TN-C were purified from BHK cells transfected with the corresponding pNUT expression vectors (Aukhil *et al.*, 1993). Bacterial expression proteins were produced and purified as described by Aukhil *et al.* (1993). The polyclonal antibody against annexin II was described in Chung and Erickson (1994). Affinity-purified anti-annexin II antibody was prepared on a column of 0.8 mg annexin II (purified from bovine lung) coupled to cyanogen bromide-activated Sepharose. Bound antibody was eluted with 0.1 M glycine, pH 2.6, and neutralized. All proteins and antibodies were dialyzed against D-TBS (20 mM tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and sterilized by filtering.

[^{3}H]thymidine Incorporation Assay

Bovine aortic GM 7373 endothelial cells were grown to confluence and then labeled with 0.1 μCi/ml [^{14}C]thymidine for 24 h. After

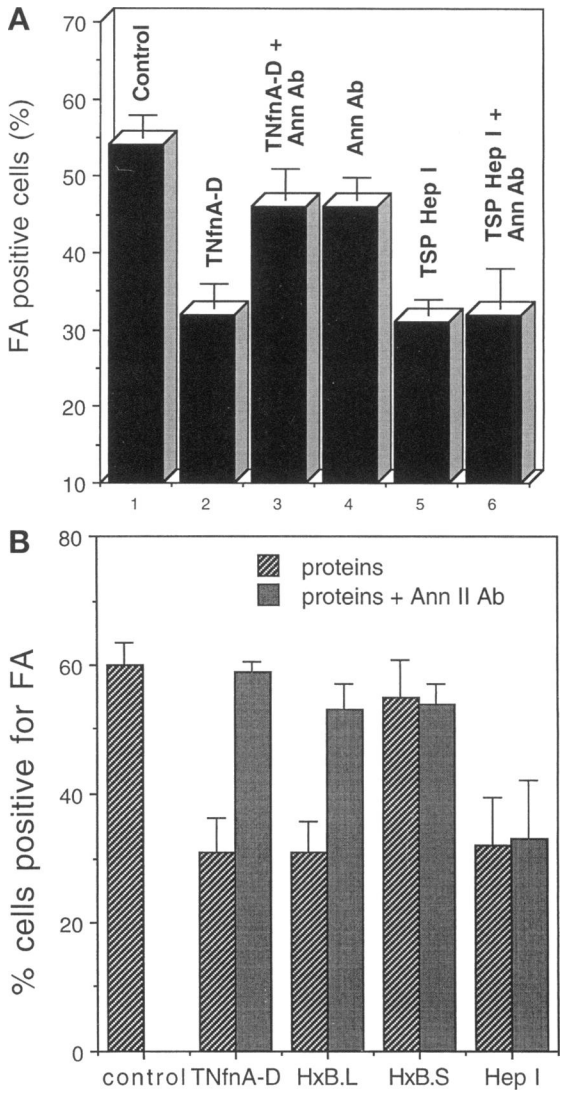

Figure 1. Disassembly of focal adhesions of BAE cells by TN-C or TNfnA-D is blocked by anti-annexin II antibody. (A) Affinity-purified anti-annexin II antibody (20 μg/ml) was added with TN-C (20 μg/ml), TNfnA-D (30 μg/ml), or Hep-1 peptide (1 μM) to confluent endothelial cells. The number of cells positive for focal adhesion was determined as described in Murphy-Ullrich and Hook (1989). The values plotted are the mean of three determinations. (B) The experiment was repeated using new preparations of all reagents. In this experiment we also tested the two splice variants of native TN-C (added at 30 μg/ml): HxB.L contains the domains A–D, while HxB.S is missing these domains.

labeling and washing, various amounts of TN-C and 1 μCi/ml of [^{3}H]thymidine were added to cultures in medium containing 1% fetal calf serum, and incubated for 24 h. After washing with phosphate-buffered saline, cells were harvested with 2% SDS and the radioactivity was determined by liquid scintillation counting. The ratio of ^{3}H to ^{14}C was used as an index for cell proliferation.

BrdU Incorporation Assay

Endothelial cells were grown to be confluent or subconfluent, washed, and incubated with TN-C or bacterial expression proteins in DMEM with 1% fetal bovine serum or 5 ng/ml of human recombinant bFGF. After 12 h incubation, cells were washed and labeled with 8-bromo deoxyuridine (BrdU; 10 mM) for 2 h. Based on the length of the endothelial cell cycle (26–29 h), this incubation time should label cells reentering the cell cycle from a quiescent stage. Cells were fixed with 70% ethanol in 50 mM glycine buffer (pH 2.0) at −20°C for 30 min. Labeled nuclei were detected with a monoclonal antibody against BrdU (from Behringer Mannheim, Indianapolis, IN) and secondary anti-mouse IgG antibody conjugated with fluorescein. The number of total nuclei was determined by 4′,6-diamidino-2-phenylindole staining, and the ratio of labeled nuclei to total nuclei was used as a cell proliferation index.

Cell Culture Wound Closure Assay

Wound migration assays were done as described by Sato and Rifkin (1988) with minor modifications. A confluent monolayer of GM7373 endothelial cells was scraped with a micropipette tip. After wounding, original edges were marked by drawing lines underneath the slides. The cultures were washed with DMEM and further incubated at 37°C in DMEM containing 1% fetal calf serum plus TN-C, TN-C expression proteins, or antibodies. Pictures of the wound edge were taken at 0, 24, and 48 h after incubation (100× or 40× magnification) and cell migration was determined by measuring the distance that the monolayer advanced at the wound edge. Cells located at the advancing wound edge and retaining contact with neighboring cells were considered as the forefront of translocating cells. The values represent the mean of 15 or more fields from five independent cultures.

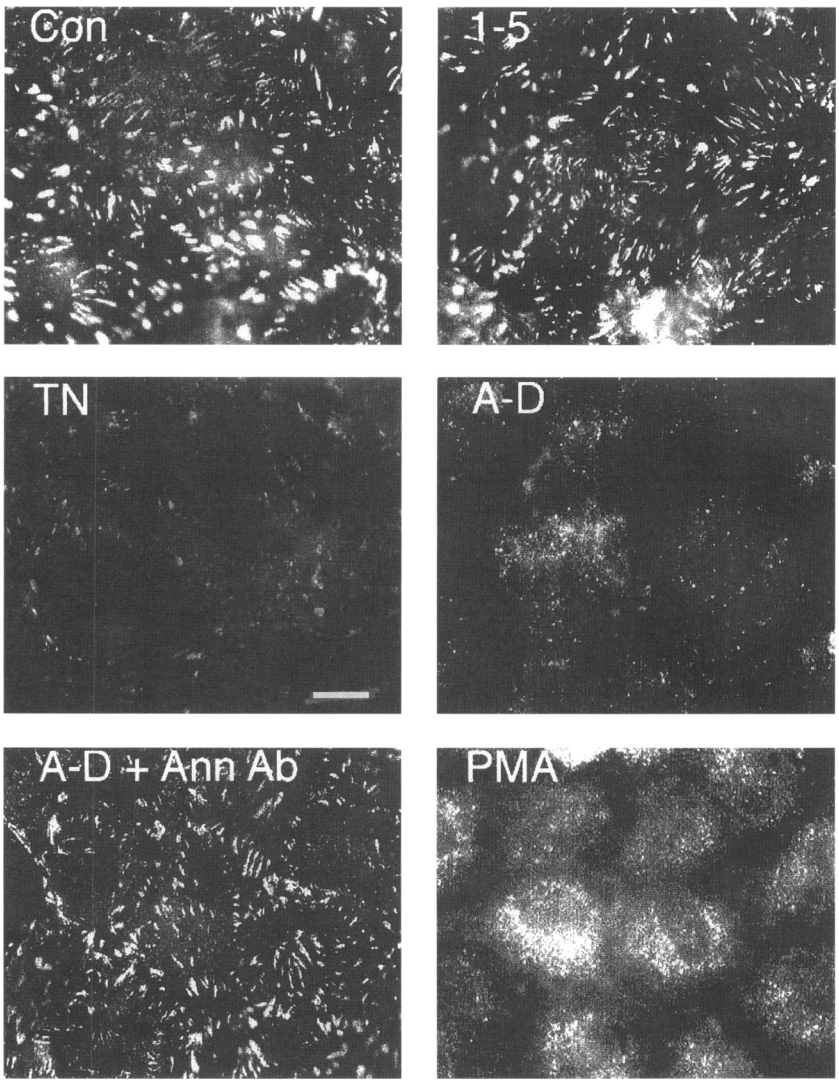

Figure 2. Distribution of vinculin in BAE cells treated with TN-C, recombinant proteins, and phorbol myristate acetate. Cells were treated with TN-C (30 μg/ml), recombinant proteins (50 μg/ml), affinity-purified annexin II antibody (20 μg/ml), or phorbol myristate acetate (1 mM), fixed, and permeabilized. Cells were then stained with a monoclonal antibody against vinculin. Control cells and cells treated with TNfn1–5 or TNfn6–8 have prominent vinculin plaques, but TN-C– or TNfnA-D–treated cells lack vinculin plaques. Bar, 20 μm.

Focal Adhesion Assays

Focal adhesion assays were performed as described (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich *et al.*, 1991). Briefly, BAE cells were grown for 20–24 h on 12-mm glass coverslips in DMEM with 20% fetal calf serum. After cells were nearly confluent, cycloheximide (10 μg/ml) was added to the cultures 1 h before beginning the assay (and it was present during the remainder of the experiment) to minimize interference from proteins synthesized during the course of the assays. Cells were rinsed and then treated for 1–2 h at 37°C with annexin II antibody, TNfnA-D, or Hep I peptide (a synthetic peptide from thrombospondin; Murphy-Ullrich *et al.*, 1993). Bovine serum albumin or protein-free DMEM were used as controls. Cells were then fixed with 3% warmed glutaraldehyde and examined for the presence of focal adhesions by interference reflection microscopy. A minimum of 300 cells/condition were examined. Cells were scored as positive if they had at least three to five focal adhesions.

RESULTS

Anti-annexin II Antibody Can Block the Focal Adhesion Dismantling Activity of TN-C

The TNfnA-D segment of TN-C was previously shown to induce the loss of focal adhesions in nearly confluent endothelial cells (Murphy-Ullrich *et al.*, 1991). The effect is best observed at ~80% confluence. In sparse cultures the cells are more migratory and have fewer focal adhesions, while in denser cultures cell-cell junctions predominate. Since we have identified annexin II as a high affinity receptor for TNfnA-D (Chung and Erickson, 1994), it seemed likely that this cellular response might be mediated by cell surface annexin II. The experiment shown in Figure 1 confirms this hypothesis: TNfnA-D added to confluent endothelial cells reduced the number of focal-adhesion positive cells by 50%, but this activity was substantially blocked by an affinity-purified annexin II antibody. A peptide from the heparin-binding domain of thrombospondin, called Hep-1, has also been shown to reduce focal adhesions (Murphy-Ullrich *et al.*, 1993), but the activity of Hep-1 was not affected by the annexin II antibody (Figure 1). Thus TNfnA-D initiates its response by binding to the annexin II receptor, but the Hep-I peptide appears to interact with a different receptor. Figure 1B shows a repeat of this experiment using different preparations of all reagents, to demonstrate the reproducibility. In this experiment the annexin II antibody itself had no effect on focal adhesions (58% ± 1.5). We also tested separately the two splice variants of native TN-C. HxB.S, which is missing the alternatively spliced A-D domains, had no activity, and the activity of HxB.L was completely blocked by the annexin II antibody. Thus the effect of TN-C on focal adhesions is generated completely by the interaction of the TNfnA-D segment with the annexin II receptor.

The loss of focal adhesions was also assayed by immunohistochemical staining for vinculin plaques (Figure 2). In confluent endothelial cell cultures, about 40–50% of total cells showed prominent vinculin plaques at the periphery of the cell. Cells treated with TNfn1–5 or TNfn6–8 showed vinculin plaques identical to those of untreated cells, but in cultures treated with TN-C or TNfnA-D the number of cells with prominent vinculin plaques was greatly reduced. Phorbol myristate acetate induced a similar but stronger response, leading to a complete loss of vinculin plaques in all cells. Cells treated with TNfnA-D plus the affinity-purified anti-annexin II antibody showed vinculin plaques as prominent as the control cells.

A Mitogenic Activity of TN-C Mediated by Annexin II

Because several growth factors produce a mitogenic response in addition to loss of focal adhesions, we tested TN-C for mitogenic activity on GM 7373, a transformed fetal aortic endothelial cell line. When confluent GM 7373 endothelial cells were incubated with various amounts of TN-C for 24 h, the incorporation of [^{3}H]thymidine was stimulated in a dose-dependent manner (Figure 3). Although 20 μg/ml TN-C (which caused almost maximal loss of focal adhesions) gave only a small stimulation in this experiment, it induced significant mitogenesis in others. Soluble TN-C (40–75 μg/ml) reproducibly caused an

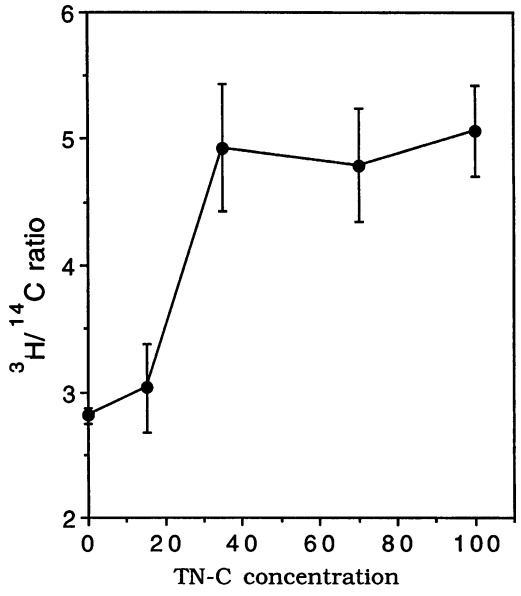

Figure 3. [^{3}H]thymidine incorporation assay of the mitogenic activity of TN-C. BAE cells were grown to confluence, and labeled with 0.1 μCi/ml [^{14}C]thymidine for 24 h. After labeling and washing, various amounts of TN-C and 1 μCi/ml of [^{3}H]thymidine were added to the cultures and incubated for 24 h. One percent fetal bovine serum was present throughout the experiment. After washing with phosphate-buffered saline, cells were harvested with 2% SDS and the radioactivity was determined. The ratio of ^{3}H to ^{14}C was used as an index for cell proliferation.

80–130% increase in growth when added to confluent GM 7373 endothelial cells in DMEM containing 1% fetal calf serum. The dose required for stimulation of mitogenesis is thus similar to or perhaps a bit larger than that inducing loss of focal adhesions. One should be cautious, however, in correlating the two responses, because the focal adhesion response was determined in serum-free medium, while the mitogenic response was measured in 1% serum, which partly inhibits the focal adhesion response.

A BrdU incorporation assay gave much more rapid labeling of proliferating cells, so it was used for the rest of our experiments. In this assay, cells were incubated with TN-C in the presence of 1% fetal calf serum for 12 h, and then labeled with BrdU for 2 h in the continued presence of TN-C. The number of nuclei labeled during a 2-h incubation with BrdU was around 10–15% of total nuclei visualized by 4′,6-diamidino-2-phenylindole staining (Figure 4). As in the [^{3}H]thymidine incorporation assay, TN-C doubled the number of labeled nuclei (Figures 4 and 5). Labeled nuclei seemed to be distributed evenly. The mitogenic activity of whole TN-C was duplicated by TNfnA-D, but TNfn1–5, TNfn6–8, and TNfbg were inactive (Figures 4 and 5).

The mitogenic responses of cells to TN-C and recombinant proteins was also examined in (nontransformed) BAE cells in which the focal adhesion disassembly activity of TN-C was originally described. As shown in Figure 5, the basal level of labeling of BAE cells was only about half that of GM7373, but TN-C and TNfnA-D increased the level about 80–120%, which is very similar to the response of GM7373 cells. Thus, the mitogenic activity is not dependent on the transformed phenotype of GM 7373 endothelial cells.

The same concentrations of TN-C or TNfnA-D added to subconfluent GM 7373 endothelial cells showed no effect on cell growth (Figure 6). This result was interesting because most growth factors stimulate subconfluent cells more strongly than confluent ones. However, these cells are growing in the presence of

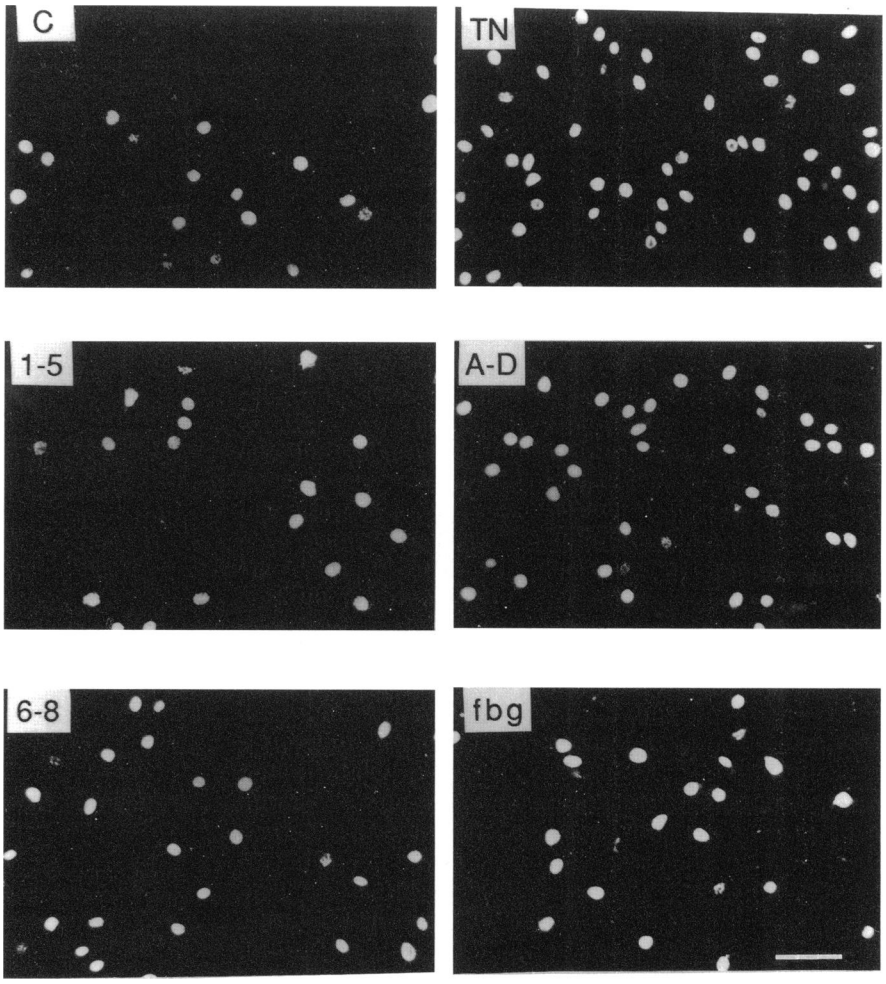

Figure 4. Immunostaining with anti-BrdU antibody. Confluent GM7373 endothelial cells were washed with DMEM, and incubated with TN-C (100 μg/ml) or bacterial expression domains (50 μg/ml for TNfnA-D and TNfn1–5 and 30 μg/ml for TNfn6–8 and TNfbg) in medium with 1% fetal calf serum. After 12 h of incubation, cells were washed and labeled with BrdU (10) for 2 h. Labeled nuclei were detected by immunostaining with a monoclonal antibody against BrdU. The total number of nuclei was 120–140 in each photograph. Bar, 50 μm.

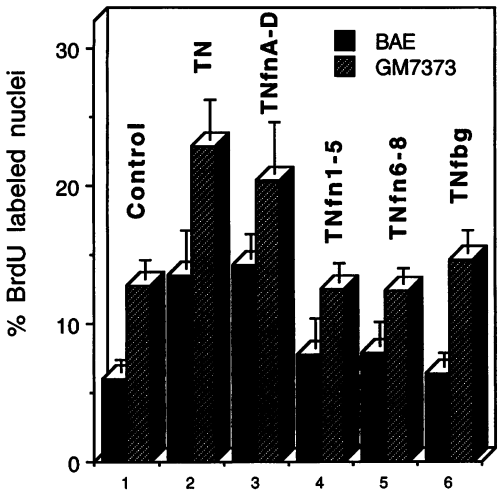

Figure 5. Mitogenic activity of TN-C and expression proteins on confluent BAE or GM 7373 endothelial cells assayed by BrdU incorporation. The concentrations of added proteins were the same as in Figure 4. The values plotted represent the mean of at least 20 microscopic fields from three or more independent experiments, and the error bars indicate standard error of the mean. The number of labeled nuclei in TN-C– and TNfnA-D–treated cultures was significantly increased relative to the control culture ($p < 0.01$, calculated by Student's t test).

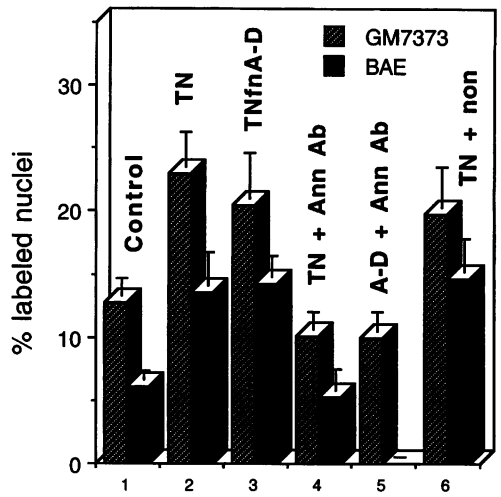

Figure 7. Inhibition of the mitogenic activity of TNfnA-D by anti-annexin II antibody in GM7373 endothelial cells or BAE cells. Affinity-purified annexin II antibody (15 μg/ml) was added to confluent cells with TN (30 μg/ml or TNfnA-D (50 μg/ml), and cellular proliferation was determined by the BrdU assay. As a negative control, 30 μg/ml nonimmune rabbit IgG (labeled "non") was also tested. The values represent the mean and standard error of the mean of 16 determinations from three experiments.

1% fetal calf serum, and 45% of the cells are labeled during the brief 2-h labeling period. They may therefore be maximally stimulated so that no growth factor or TN-C could further enhance their proliferation.

We tested whether the mitogenic activity of TN-C might be mediated by the annexin II receptor. With both BAE cells and GM 7373 endothelial cells, the affinity-purified antibody against annexin II completely neutralized the activity of TN-C and TNfnA-D to stimulate the proliferation, whereas nonimmune IgG had no effect (Figure 7).

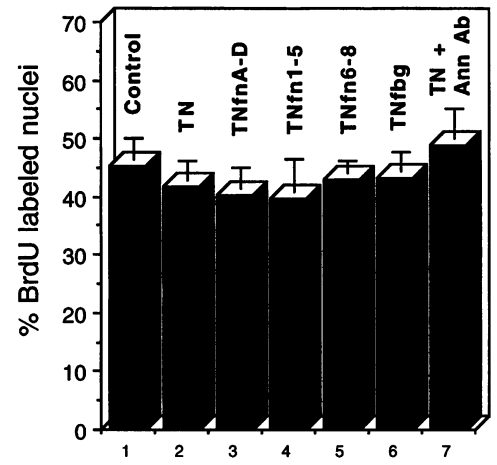

Figure 6. Mitogenic activity of TN-C and expression proteins on subconfluent GM7373 endothelial cells. TN-C (30 μg/ml), TNfnA-D (50 μg/ml), TNfn1–5 (50 μg/ml), TNfn6–8 (30 μg/ml), TNfbg (30 μg/ml), and antibody (20 μg/ml) were incubated with cells. The cultures all contained 1% fetal calf serum. The mean and standard error of the mean of 8–14 fields, each containing 50–200 nuclei, is shown.

The Mitogenic Effect of TN-C Is Cooperative with a Growth Factor

Since the mitogenic activity of TN-C was always observed in the presence of serum, this raised the question of whether TN-C had its own mitogenic activity or was acting cooperatively with growth factors. When TN-C or TNfnA-D was added to confluent endothelial cells in the absence of serum or growth factor, there was no stimulation of cell proliferation (Figure 8). Similarly, bFGF did not stimulate the confluent cells in the absence of TN-C or TNfnA-D (Figure 8). In contrast, when BAE or GM 7373 cells were pre-treated with TN-C or TNfnA-D for 12 h, they showed a proliferative response when subsequently treated with bFGF. The lack of response to bFGF alone is probably due to the very short exposure time in this assay, only 2 h. Treatment of BAE cells with bFGF for 6 h resulted in a significant increase in proliferation, the rates of cell proliferation being similar between control cells and TN-treated cells (our unpublished observations). The stimulation of cell proliferation by bFGF did not require the continuous presence of TN-C because BAE cells treated with TN-C for 12 h (Figure 8) or 4 h and washed, still responded to bFGF. Thus, N-C seems to

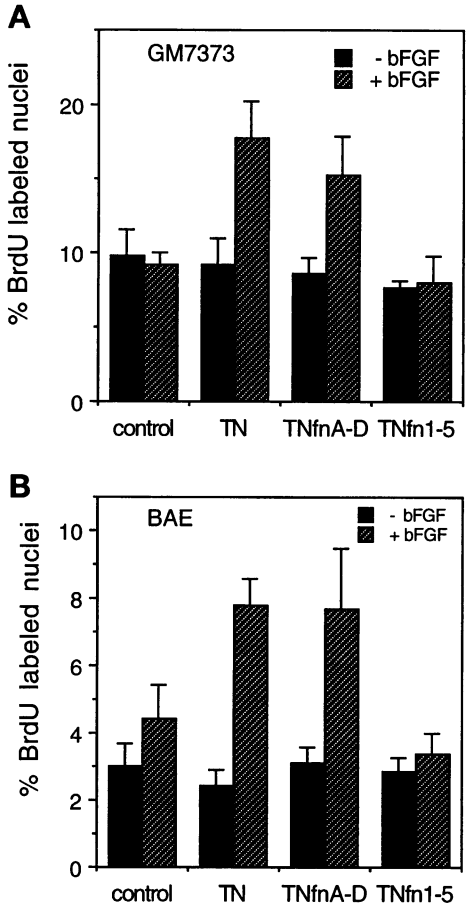

Figure 8. Cooperativity of bFGF and TNfnA-D for stimulating proliferation of GM7373 (A) and BAE (B) cells. Cells were grown to confluence, washed, and incubated in DMEM (no serum) including 70 μg/ml TN-C, or 50 μg/ml TNfnA-D or TNfn1–5. After 12 h incubation, BrdU and 5 ng/ml of recombinant human bFGF were added without tenascin proteins, and incubated an additional 2 h. The values represent the mean of 12 determinations from two experiments. The number of labeled nuclei in TN-C– and TNfnA-D–treated cultures in the presence of bFGF was significantly increased ($p < 0.01$, Student's t test) compared with control culture.

facilitate the rapid response of cells to growth factors, rather than having a mitogenic activity of its own.

TN-C Enhances Cell Migration

We used an in vitro wound closure assay to test whether TN-C can affect the migration of GM 7373 cells. TN-C increased the migration of GM7373 endothelial cells into the denuded area at the wound edge about 80% (Figures 9 and 10). The active site was mapped to TNfnA-D by testing recombinant expression proteins (Figure 10). TNfnA-D stimulated the migration similarly to native TN-C, whereas cells treated with TNfn1–5 and TNfn6–8 showed basal level migration. TNfbg showed a moderate stimulation. The morphology of cells at the wound edge and in the monolayer of TN-treated culture was different from control cells. A large portion of cells in TN-C and TNfnA-D–treated cultures showed a more elongated morphology and appeared to be less flattened than the polygonal, cobblestone-like control cells (Figure 9). Cells treated with TNfn1–5, TNfn6–8, or TNfbg showed the same morphology as control cells.

In previous studies of primary cultures of endothelial cells (Sato and Rifkin, 1988; Joyce and Meklir, 1992), migrating cells moved ahead of the advancing wound edge as individuals, and retained minimal contacts with neighboring cells. Interestingly, migrating GM7373 cells at the wound edge maintained contacts with each other and we did not observe many individual cells ahead of the advancing monolayer. The contacts were generally looser in TN-C treated cultures, but even these cells retained contacts, and a migration edge was easily defined. Thus, the transformed GM7373 cells behave somewhat differently in this wound/migration assay, and their tendency to retain cell-cell contacts facilitated measurement of the migration rate.

The anti-annexin II antibody abolished the stimulatory effect of native TN-C and TNfnA-D on cell migration, but nonimmune IgG did not (Figure 10). Thus, the increased migration of endothelial cells appears to result from the interaction of TNfnA-D with cell surface annexin II.

DISCUSSION

The three cellular responses to TN-C explored here were all mapped to the alternatively spliced segment, TNfnA-D, and we have now shown that all of these responses are mediated by the cell surface receptor, annexin II. At least three additional cellular responses to soluble TN-C have been mapped to TNfnA-D: inhibition of adhesion of uterine epithelial cells to Matrigel (Julian *et al.*, 1994); inhibition of milk production by mammary epithelial cells (Jones *et al.*, 1995); and inhibition of branching morphology of embryonic lung explants (Young *et al.*, 1994). It seems likely that these activities may also be mediated by TNfnA-D binding to annexin II, although it should be noted that the last two responses were also generated by other FN-III domains of TN-C.

Our work generally confirms and extends the study of End *et al.* (1992), but with some important differences. End *et al.* found three different proliferative responses of cells to soluble TN-C. 1) The epithelial cell line Pam 212 was inhibited by TN-C, and the NR6 cell line showed no response to TN-C. 2) Arterial smooth muscle cells and NIH 3T3 cells showed minimal stimulation of proliferation when TN-C was added in serum-free medium; however, when added in combination with EGF, TN-C strongly stimulated

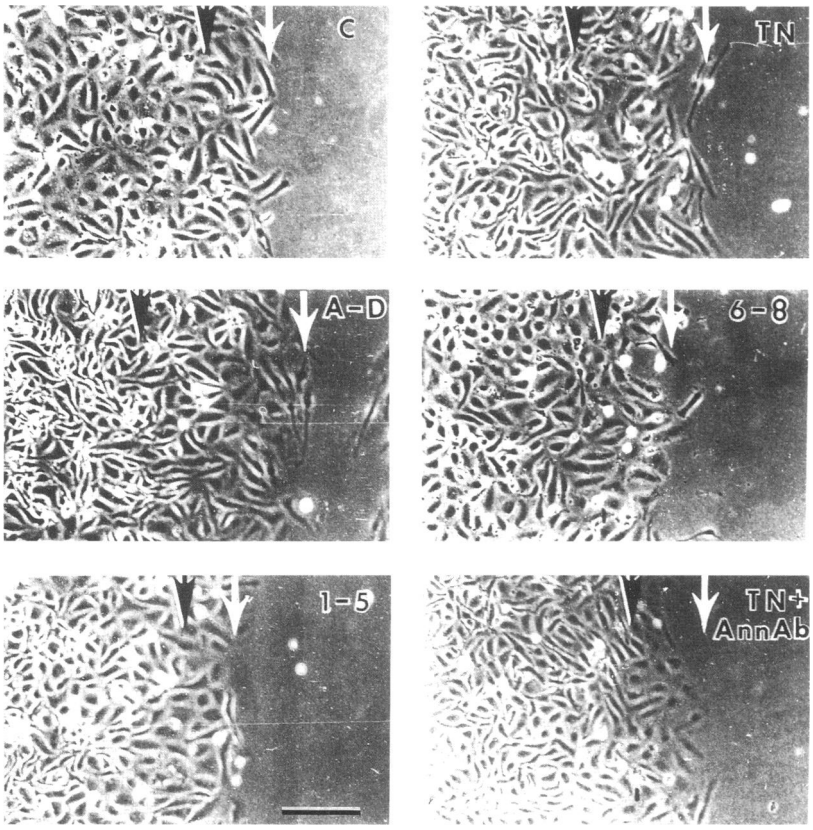

Figure 9. The effect of TN-C on GM7373 cell migration tested in an in vitro wound closure assay. One percent fetal calf serum was present before and after the wounding. TN-C (30 μg/ml), bacterial expression proteins (TNfnA-D and TNfn1–5, 50 μg/ml; TNfn6–8, 30 μg/ml), and antibody (20 μg/ml) were added after wounding. The edge of the cell sheet was marked immediately after wounding (black arrows) and 48 h later (white arrows). Bar, 100 μm.

proliferation. This is very similar to our results with endothelial cells, where the mitogenic response required both TN-C and growth factor. Note that both studies were done with nearly confluent cultures. 3) Swiss 3T3 cells were stimulated by TN-C in the absence of growth factor. The synergetic effects of growth factors and TN-C are thus complex and depend on the cell type. An unresolved contradiction in the two studies is the location of the active domains in TN-C. End *et al.* did not have a range of expression proteins, but they found that all three splice variants of TN-C could stimulate the proliferation of both Swiss and NIH 3T3 cells. This would seem to exclude the alternative splice segment, which was the single active domain in our experiments. It may be that other regions of TN-C have mitogenic activity for at least some cell types.

Crossin (1991) reported a seemingly contradictory result, that TN-C inhibited the cell division of NIH 3T3 cells. Inhibition was observed when TN-C was added to sub-confluent cultures, which had a high level of cell division, and when TN-C was added with various growth factors to quiescent confluent cultures. This is clearly different from our results with endothelial cells, and is the opposite of the results of End *et al.*, with the same NIH 3T3 cells. We do not have any explanation for these seemingly contradictory results.

The primary response of cells to TN-C may be the down-regulation of focal adhesions and associated cytoskeleton. This response was first demonstrated with endothelial cells (Murphy-Ullrich *et al.*, 1991), and has been confirmed with both endothelial and smooth muscle cells (Hahn *et al.*, 1995). Growth factors alone also cause loss of focal adhesions, and this may be necessary to permit subsequent events of cell division, or cell migration. The role of TN-C in mitogenesis might be to facilitate this initial disruption of focal adhesions and associated cytoskeleton, thereby augmenting the effectiveness of growth factors. Since focal adhesions need to be broken to permit cell migration, the ability of TN-C to augment migration might also be due to its effect on focal adhesions. However, we have not yet determined the cell cycle point affected by TN-C and subsequently by bFGF. Preliminary study shows that our newly confluent endothelial cell cultures are not completely quiescent (G0/G1); some cells are still cycling and in G2/M. A more extensive analysis will be needed to determine cell cycle points affected by TN-C, and whether the modulation of cytoskeleton is a primary factor in facilitating growth factor response.

Pretreatment of cells with TN-C permitted a rapid (2 h) response to bFGF. A rapid response to bFGF can also be obtained by pre-activating protein kinase C

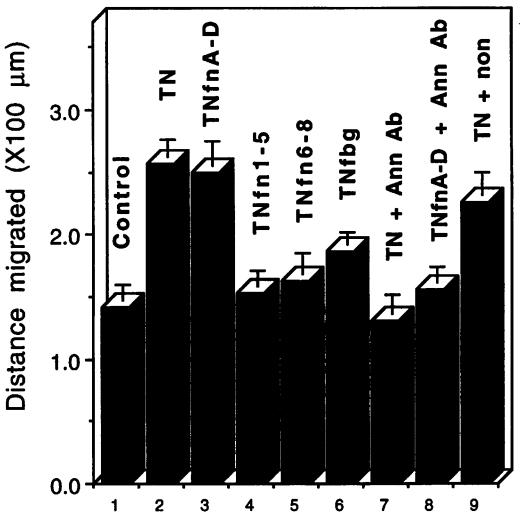

Figure 10. Quantitative analysis of the wound closure assay. Confluent GM7373 cell monolayers were lesioned by scraping with a micropipette tip and incubated with TN (70 μg/ml) or expression proteins (50 μg/ml for TNfnA-D and TNfn1–5 and 30 μg/ml for TNfn6–8 and TNfbg) in 1% fetal calf serum medium for 48 h. Cells located at the advancing wound edge but retaining contact with neighboring cells were considered as the forefront of migrating cells. The distance of these forefront cells to the original wound edge was measured. The values represent the mean and standard error of 15 determinations from seven experiments.

with phorbol ester, which also causes complete loss of focal adhesions and alterations in actin fibers (Schliwa *et al.*, 1984; Hedberg *et al.*, 1991). KT 5823, a selective inhibitor of cGMP-dependent kinase, inhibited the focal adhesion disassembling activity of TNfnA-D (Murphy-Ullrich, Pallero, Boerth, Greenwood, Lincoln, and Cornwell, unpublished observations). This suggests that cGMP-dependent kinase rather than protein kinase C may be the primary signal induced by TNfnA-D. The common feature in treatment with phorbol ester and TNfnA-D is the response of focal adhesions, but this response may be elicited by different kinase pathways.

How does binding of TNfnA-D to annexin II initiate a signaling response? Calcium channel activities of membrane bound annexins have been demonstrated in vitro and analyzed in terms of the atomic structure (Chen *et al.*, 1993; Demange *et al.*, 1994); it is possible that binding of TNfnA-D modulates a calcium channel activity. Another possibility is that cell surface annexin II is bound to a transmembrane protein, and it is this protein that initiates the cytoplasmic signaling.

Capillary endothelial cells are normally comparable to a quiescent monolayer. Upon injury or stimuli from tumor cells, quiescent endothelial cells escape from the nonproliferative state and undergo morphological change, migration, and proliferation, and eventually form the new capillary network. An initial response of endothelial cells may involve local breakdown of the basement membrane, which probably results in down-regulation of cell adhesion and/or cell-cell contact (Folkman *et al.*, 1989). It is primarily the large splice variant of TN-C that is expressed in wounds and tumors (Borsi *et al.*, 1992), which allows the possibility that TN-C may play a role in the initial stages of angiogenesis. It must be noted that wound healing was apparently normal in mice lacking TN-C (Saga *et al.*, 1992). However, a detailed study of wound healing in these mice has not been reported, nor has any study of tumor development. Changes in angiogenesis that alter but do not prevent wound healing, as well as alterations in tumor growth, need to be carefully examined in these mutant mice without tenascin.

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