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The Fibrinogen Globe of Tenascin-C Promotes Basic Fibroblast Growth Factor-induced Endothelial Cell Elongation

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To investigate the potential role of tenascin-C (TN-C) on endothelial sprouting we used bovine aortic endothelial cells (BAECs) as an in vitro model of angiogenesis. We found that TN-C is specifically expressed by sprouting and cord-forming BAECs but not by nonsprouting BAECs. To test whether TN-C alone or in combination with basic fibroblast growth factor (bFGF) can enhance endothelial sprouting or cord formation, we used BAECs that normally do not sprout and, fittingly, do not express TN-C. In the presence of bFGF, exogenous TN-C but not fibronectin induced an elongated phenotype in nonsprouting BAECs. This phenotype was due to altered actin cytoskeleton organization. The fibrinogen globe of the TN-C molecule was the active domain promoting the elongated phenotype in response to bFGF. Furthermore, we found that the fibrinogen globe was responsible for reduced cell adhesion of BAECs on TN-C substrates. We conclude that bFGF-stimulated endothelial cells can be switched to a sprouting phenotype by the decreased adhesive strength of TN-C, mediated by the fibrinogen globe.

INTRODUCTION

Adhesive interactions of cells with the extracellular matrices (ECMs) play an important role in the dynamic changes involved in morphogenetic processes such as neurite outgrowth, branching morphogenesis of epithelium, and also angiogenesis (Bischoff, 1995; Gumbiner, 1996). During angiogenesis new blood vessels are formed by sprouting of capillaries from preexisting vessels. This requires the breakdown and reassembly of the ECM, migration and proliferation of endothelial cells, and endothelial tube formation. To switch from a quiescent to a sprouting phenotype, endothelial cells require angiogenic growth factors, such as basic fibroblast growth factor (bFGF), as well as interactions with ECM molecules (Battegay, 1995; Bischoff, 1995; Folkman and Shing, 1992).

Interactions between cells and ECM molecules are mediated by cellular receptors, mostly integrins (Hynes, 1992), that link the ECM to intracellular cytoskeletal complexes (Yamada and Geiger, 1997). Integrin binding leads to cytoskeletal rearrangement and activates intracellular signals that overlap the signaling pathways usually stimulated by growth factors (Juliano and Haskill, 1993; Clark and Brugge, 1995). Thus, the ECM (via integrins) might act cooperatively with growth factors with respect to cell proliferation, migration, and differentiation during angiogenesis.

The ECM molecule tenascin-C (TN-C) is of particular interest because it binds to several integrins, including $\alpha_2\beta_1$ and $\alpha_v\beta_3$ (Joshi *et al.*, 1993; Sririmarao *et al.*, 1993), both of which have been demonstrated to be involved in angiogenesis (Strömblad and Cheresh, 1996). TN-C is a modular hexameric ECM glycoprotein (see Figure 1). It is composed of six identical subunits that are made up of repeated sequence motifs that fold independently into small globular domains. The most prominent structural domains are the tenascin-type EGF-like repeats, the fibronectin type III repeats, and the fibrinogen globe. Various variants of TN-C have been described that are generated by alternative mRNA splicing of the fibronectin type III repeats (Chiquet-Ehrismann, 1995).

Many functional properties have been ascribed to TN-C. TN-C stimulates neurite outgrowth (Wehrle-Haller and Chiquet, 1993) and promotes osteoblastic differentiation (Mackie and Ramsey, 1996). On the other hand, TN-C inhibits milk protein synthesis by mammary epithelial cells (Jones *et al.*, 1995) and T-lymphocyte activation (Rüegg *et al.*, 1989). TN-C can stimulate or inhibit cell proliferation, depending on cell type (End *et al.*, 1992). The effects of TN-C on cell adhesion are complex in that TN-C supports attachment of some cell types but is nonadhesive or even repulsive for other cell types (Erickson and Bourdon, 1989; Faissner and

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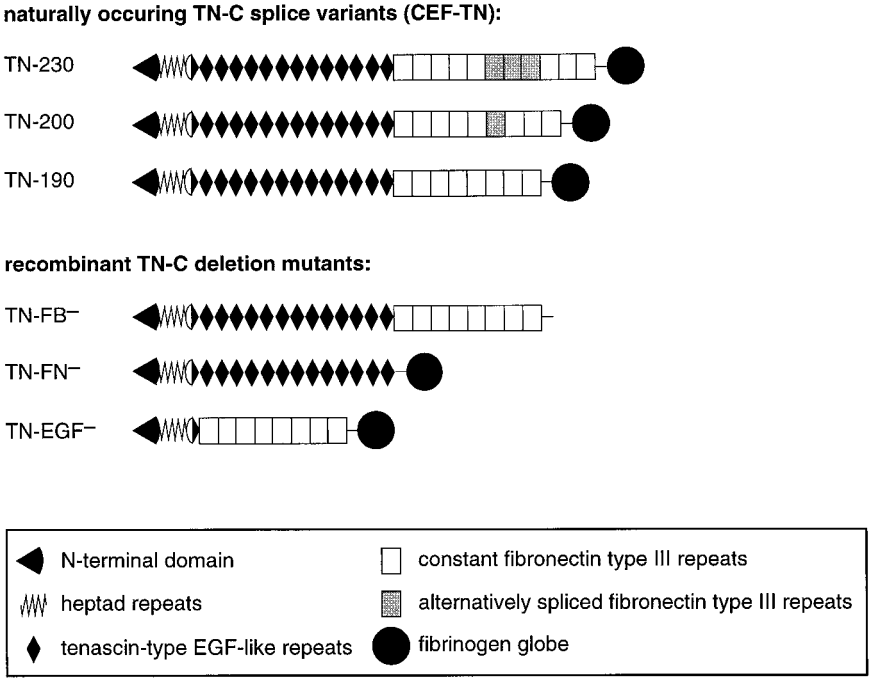


Figure 1. Models of one subunit of naturally occuring TN-C variants as isolated from chick embryo fibroblasts (CEF-TN) and of the deletion mutants used. The subunits consist of an N-terminal part involved in the oligomerization to hexamers (N-terminal domain and heptad repeats), followed by tenascin-type EGF-like repeats, FN type III repeats, and a domain homologous to the globular part of b- and g-fibrinogen. Alternatively spliced FN type III repeats are shown as gray rectangles. Each of the deletion mutants lacks one type of domains: TN-FB$^-$ lacks the fibrinogen globe, TN-FN$^-$ lacks all FN type III repeats, and TN-EGF$^-$ lacks all EGF-like repeats. All recombinant TN-C mutants were proven to occur as hexamers and to show expected dimensions and structural features (Fischer *et al.*, 1997).

Kruse, 1990; Prieto *et al.*, 1992). Because some fragments of TN-C are more adhesive than the intact molecule, it appears that the latter contains both adhesive and counteradhesive domains (Prieto *et al.*, 1992; Fischer *et al.*, 1997).

During embryonic development TN-C is detected at high levels in many organs and tissues in a changing spatiotemporal pattern (reviewed by Erickson and Bourdon, 1989). In contrast, low levels of TN-C are found in normal adult tissue (Oike *et al.*, 1990; Natali *et al.*, 1991); however, TN-C expression is up-regulated in many conditions associated with angiogenesis, such as wound healing (Mackie *et al.*, 1988), arthritis (Cutolo *et al.*, 1992; Salter, 1993), and tumor formation (reviewed by Chiquet-Ehrismann, 1993). In human gliomas TN-C accumulation was found to correlate well with the degree of histological malignancy (Zagzag *et al.*, 1995) and with tumor neovascularization (Higuchi *et al.*, 1993). TN-C immunostaining was consistently stronger around and within walls of hyperplastic blood vessels than in non-hyperplastic vessels, suggesting that TN-C might play a role in tumor angiogenesis.

Soluble TN-C was found to reduce focal adhesions in endothelial cells (Murphy-Ullrich *et al.*, 1991) and to enhance endothelial cell migration (Chung *et al.*, 1996). Furthermore, soluble TN-C was found to facilitate the mitogenic response of endothelial cells to growth factors such as bFGF. These effects were assigned to the alternatively spliced region of TN-C (Murphy-Ullrich *et al.*, 1991; Chung *et al.*, 1996). TN-C expression was found to be associated with the sprouting (angiogenic) but not with the resting (nonangiogenic) phenotype of aortic endothelial cells in vitro. Interestingly, the angiogenic phenotype was inhibited when cells were grown in the presence of anti–TN-C antibodies, suggesting that the transition from a resting to a sprouting phenotype may be promoted by TN-C (Canfield and Schor, 1995).

In the present study, we investigated the pattern of TN-C expression during different stages of angiogenesis in vitro. Furthermore, the mechanistic role of TN-C in endothelial sprouting was examined. In particular we investigated whether 1) substrate-bound TN-C had any effect on the sprouting of the endothelial cells in the presence or absence of bFGF and 2) which of the TN-C domains were required for its activities.

MATERIALS AND METHODS

Proteins and Antibodies

Chick tenascin-C (CEF-TN) was purified from conditioned medium of confluent cultures of primary chick embryo fibroblasts using immunoaffinity chromatography with mAb TnM1 (Chiquet *et al.*, 1991). Recombinant chick TN-C variants (TN-230, TN-190) and deletion mutants lacking specific domains within the TN-C molecule (TN-FB$^-$, TN-FN$^-$, TN-EGF$^-$) were constructed, expressed, and isolated as described (Fischer *et al.*, 1995, 1997). Schematic models of the recombinant proteins are shown in Figure 1. All TN-C preparations were dialyzed against PBS containing 0.01% Tween 20 to prevent sticking of TN-C to plastic tubes during storage at −70°C.

Fibronectin (FN) was isolated from horse serum (Life Technologies, Basel, Switzerland) by affinity chromatography using a gelatin-agarose column (Sigma, Buchs, Switzerland). After the column was washed with PBS, bound horse FN (hFN) was eluted with 4 M urea in PBS. hFN-containing fractions were dialyzed against PBS containing 0.01% Tween 20 and then stored at −70°C. Human recombinant bFGF was from Sigma.

Rabbit anti-human TN-C antibodies were purchased from Life Technologies, and Cy3-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The specificity of rabbit anti-human FN antibodies has been described previously (Chiquet-Ehrismann *et al.*, 1986).

Cell and Cell Cultures

DMEM, FCS, nonessential amino acids, Na-pyruvate, and trypsin/EDTA were obtained from Seromed (Berlin, Germany), and antibiotic mix (penicillin, streptomycin, amphotericin B) was purchased from Life Technologies.

Bovine aortic endothelial cells (BAECs) were isolated, cloned, and characterized as described previously (Cotta-Pereira *et al.*, 1980; Iruela-Arispe *et al.*, 1991). When cloned, isolates that either exhibited spontaneous organization of cord-like structures (cord-forming BAECs) or that grew in monolayers without sprouts (nonsprouting BAECs) were established. Stock cultures were maintained in DMEM/10% FCS, supplemented with nonessential amino acids, Na-pyruvate, and antibiotics. Two strains of sprouting and nonsprouting BAECs were used between passage 10 and 17.

Long-Term Cell Assay

Assays were performed in 60-well tissue-culture dishes. For coating, hFN and CEF-TN as well as recombinant TN-Cs were diluted to 50 nM in PBS containing 0.01% Tween 20. The proteins were allowed to adsorb overnight to the tissue culture dishes at 4°C. The wells were then blocked with 0.1% heat-denatured BSA in PBS for 1 h at room temperature and finally washed three times with sterile PBS.

Nonsprouting BAECs were trypsinized from stock cultures, washed once in DMEM containing 10% FCS and once in serum-free DMEM, and resuspended in serum-free DMEM at a concentration of 5×10^5 cells/ml. The cells were further diluted to 1.25×10^4 cells/ml in DMEM containing 5% FCS ± 10 ng/ml bFGF; 250 cells were added to each well of the 60-well plate and incubated at 37°C for 24–48 h. Cells were fixed with 4% formaldehyde in PBS, stained with Crystal Violet (0.1% in H_2O), photographed, and counted.

For actin staining the assay was performed for 24 h in 24-well plates on round glass coverslips (diameter 12 mm) with 37,500 cells/well.

Short-Term Cell Adhesion Assay

Sixty-well tissue-culture dishes were coated with protein as described for long-term assays. Cells were trypsinized from stock cultures, washed once in DMEM containing 10% FCS and once in serum-free DMEM, and resuspended in serum-free DMEM at a concentration of 2×10^5 cells/ml; 4000 cells were added to each well of the 60-well plate. After incubation at 37°C for 45 min, cells were fixed with 4% formaldehyde in PBS, stained with Crystal Violet (0.1% in H_2O), photographed, and counted.

Immunofluorescent Staining of Cell Cultures and Actin Staining

Cells were plated at high density on round glass coverslips (diameter 12 mm) in 24-well plates and cultured for various periods of time. Cells were fixed in 4% formaldehyde in PBS for 20 min. The cell membrane was then permeabilized by incubating in 0.2% Triton X-100 in PBS for 10 min. After they were washed three times with 0.1% BSA in PBS (washing solution), nonspecific binding sites were blocked with the same solution for 20 min at room temperature. Polyclonal rabbit anti-human FN (diluted 1:500 in blocking solution) or polyclonal rabbit anti-human TN-C (diluted 1:500 in blocking solution containing 5% horse serum) was added for 1 h at room temperature. After three washes a Cy3-conjugated secondary antibody (diluted 1:500 in blocking solution) was added for 1 h at room temperature. The coverslips were washed twice with blocking solution and once with PBS and mounted upside-down in Mowiol (Calbiochem, La Jolla, CA).

For actin staining, cells were fixed in 4% formaldehyde in PBS for 20 min and permeabilized by incubating for 5 min with 0.5% Triton X-100. After they were washed three times with PBS, cells were incubated with 0.5 μg/ml TRITC-conjugated phalloidin (Sigma) for 20 min at room temperature. Coverslips were washed three times with PBS and once with H_2O and mounted upside-down in Mowiol.

Samples were examined under epifluorescence using a Zeiss Axiophot microscope (Carl Zeiss, Feldbach, Switzerland), and photos were taken using Ilford HP5 film (400 ASA).

Statistical Analysis of Data

All values are given as mean ± SD. Global hypothesis was tested by ANOVAs using the Primer of Biostatistics 3.01 program (written by Stanton Glantz, McGraw-Hill, New York) on a Macintosh personal computer. To identify differences between groups in ANOVA, multiple-comparison procedures such as the Bonferroni t test and the Student–Neuman–Keuls test were applied (Primer of Biostatistics 3.01 program). A P value < 0.05 was considered to indicate significant differences between the tested samples (Glantz, 1992).

RESULTS

During angiogenesis, endothelial cells in mature blood vessels are activated to form new capillaries by sprouting from a preexisting blood vessel. In this study we used two phenotypically distinct clones of BAECs: nonsprouting BAECs (nonangiogenic phenotype) grow only in monolayers, whereas cord-forming BAECs (angiogenic phenotype) spontaneously sprout and form networks of cords and tubes (Cotta-Pereira *et al.*, 1980; Iruela-Arispe *et al.*, 1991; Battegay *et al.*, 1994).

TN-C Is Specifically Expressed by Sprouting and Cord-forming Endothelial Cells but Not by Nonsprouting Endothelial Cells

To determine the pattern of TN-C expression in sprouting and nonsprouting BAECs we used immunocytochemical staining. BAECs were grown to confluence or postconfluence and stained for TN-C using a polyclonal antiserum raised against human TN-C (Figure 2). At confluence (day 1) no TN-C staining could be detected in nonsprouting BAECs strains (Figure 2a). Only few "sprouting" cells were found to express TN-C in cord-forming BAEC isolates at confluence (Figure 2c); however, during the next 3 d of culture, TN-C–positive cells from the cord-forming isolate continued to elongate and started to make contact with other TN-C–expressing cells (Figure 2e, day 4). TN-C staining was still apparent at day 8 postconfluence when cord-forming cells have formed a dense network of capillary-like tubes (Figure 2g). No TN-C staining was detected in the nonsprouting cells throughout the 8-d observation (Figure 2a).

TN-C often colocalizes with FN but exhibits a much more restricted tissue distribution (Crossin *et al.*, 1986). To assess whether TN-C was more specifically associated with sprouting endothelial cells than with FN, FN expression was investigated in nonsprouting and cord-forming BAECs using a polyclonal antibody raised against human FN. In contrast to TN-C, FN was expressed by both endothelial cell types. In confluent nonsprouting BAECs, prominent FN expression could be detected in most or all cells. Furthermore, FN was found to be deposited in the ECM surrounding these cells. In cord-forming BAECs, the most prominent FN staining was found in elongated cells involved in sprouting and cord formation, but FN was also expressed by cells of the monolayer underlying developing cords. FN immunoreactivity was not restricted to single cells but accumulated in the

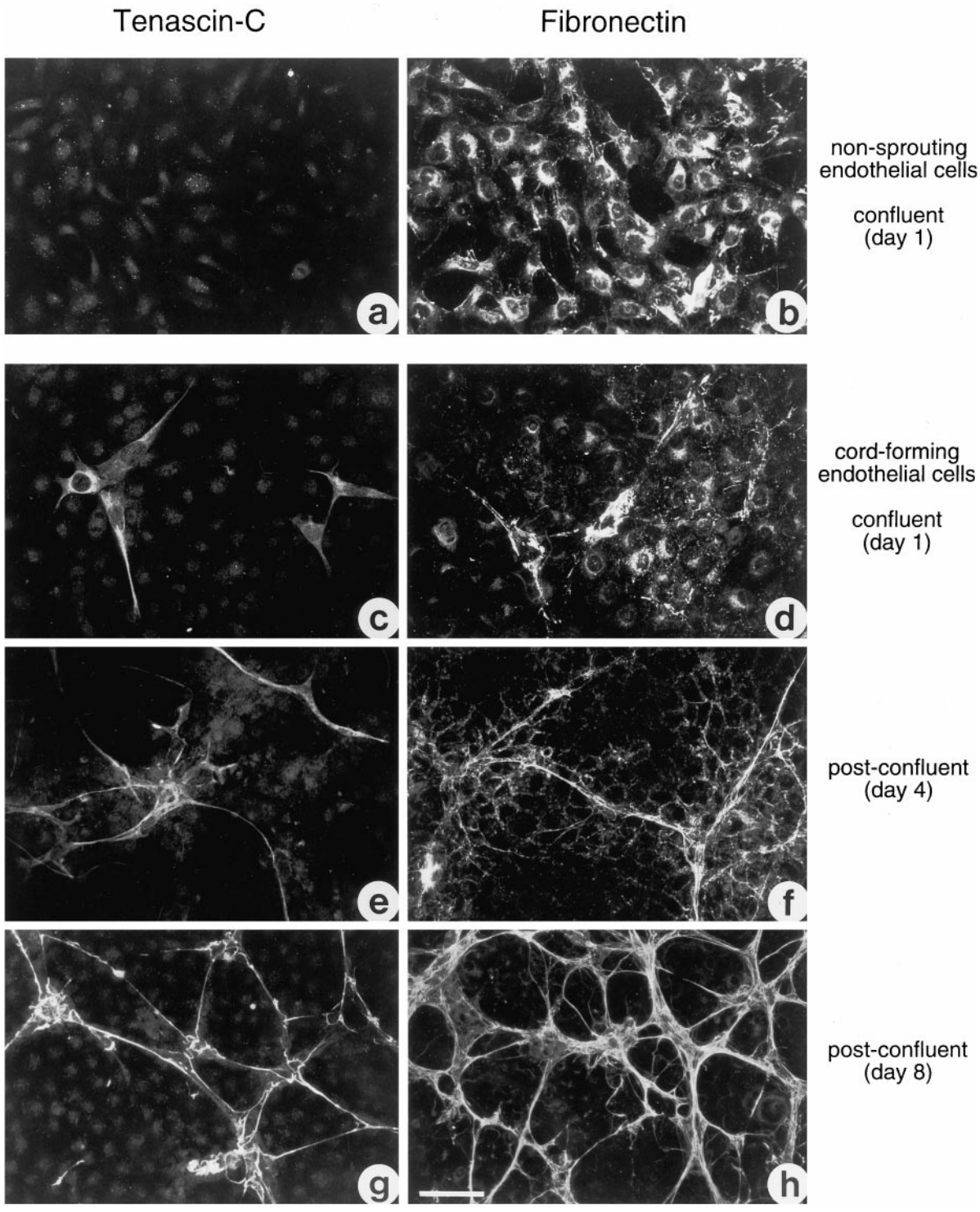


Figure 2. TN-C but not FN expression is specifically associated with cord formation of endothelial cells in vitro. Nonsprouting (a, b) and cord-forming BAECs (c–h) were cultured to confluence (day 1) or postconfluence (days 4 + 8) and immunostained for TN-C (a, c, e, g) or FN (b, d, f, h). TN-C staining was only found in sprouting or cord-forming cells but never in the endothelial monolayer. Bar, 50 μm.

extracellular network around cells. In contrast, TN-C specifically colocalized with sprouts and cords in cord-forming BAECs.

TN-C in Combination with bFGF Promotes an Elongated Phenotype in Nonsprouting BAECs

Because we found specific expression of TN-C only by cord-forming BAECs, we tested whether exogenous TN-C could enhance endothelial sprouting or cord formation in BAECs that normally do not sprout or form cords. Nonsprouting BAECs were plated onto TN-C–coated tissue-culture plates and cultured to postconfluence; however, CEF-TN (TN-C isolated from chick embryo fibroblasts) alone did not induce any sprouting in these cells. Because Chung *et al.* (1996) showed a synergistic effect of TN-C on the mitogenic response induced by bFGF, we hypthesized that TN-C and bFGF might cooperate in inducing sprouting of BAECs. Nonsprouting BAECs were cultured on CEF-TN– or hFN–coated 60-microwell plates in medium containing 5% FCS with or without bFGF (10 ng/ml). Under these conditions BAEC adhered and spread equally well on all substrates within 2 h after plating (our unpublished results). Cells were kept in culture for up to 72 h when they reached confluence. Only when plated on TN-C and in the presence of bFGF, nonsprouting BAECs adopted a bipolar, elongated, and sometimes branched cell shape within 24–48 h (Figure 3, a and b). This phenotype was lost when cells reached confluence (our unpublished results). In contrast, the majority of the nonsprouting BAECs plated on hFN or plastic remained well spread even in the presence of bFGF. This suggests specific effects of TN-C together with bFGF on endothelial sprouting.

To quantify the observed phenotypic changes of nonsprouting BAECs in response to TN-C and bFGF, elongated cells were counted, and the percentage of elongated cells from the total cell number was calculated. A significantly higher number of elongated cells on CEF-TN were counted in the presence of bFGF versus CEF-TN in the absence of bFGF ($P < 0.05$). No difference was found for cells plated with or without bFGF on hFN or plastic. Figure 3b shows mean values ± SD from a representative experiment (triplicates).

TN-C Together with bFGF Alters the Cytoskeletal Organization

Growth factors as well as ECM molecules transmit signals to a cell, which often results in reorganization of the actin cytoskeleton (Zigmond, 1996). To investigate the specific consequences of TN-C and bFGF on cytoskeletal reorganization and to investigate whether and how the observed effects of TN-C and bFGF on endothelial sprouting translated in cytoskeletal reorganization, nonsprouting BAECs were plated on different matrices, and actin microfilament organization was examined (Figure 4). In the absence of bFGF, cells assembled prominent microfilament bundles that were located at the cell margins or ran longitudinally across the cell body. There were no detectable differences of the actin filament pattern on the substrates tested (CEF-TN, hFN, glass). Cells adherent to hFN or plastic in the presence of bFGF showed a reduced number of organized microfilament bundles compared with their counterparts in the absence of bFGF.

In contrast, in the presence of bFGF the majority of the cells adherent to CEF-TN displayed a diffuse phalloidin staining within the cell body and intense staining within a broad area of membrane ruffles. Some cells grown on hFN with bFGF also displayed thin, actin-rich ruffles at the periphery; however, the cell bodies on hFN with bFGF remained well spread, whereas on CEF-TN in the presence of bFGF the cells were more compact, and the membrane ruffling was more pronounced. The cytoskeletal changes seen in the presence of bFGF suggest specific effects of TN-C but not FN on the architecture of the cytoskeleton.

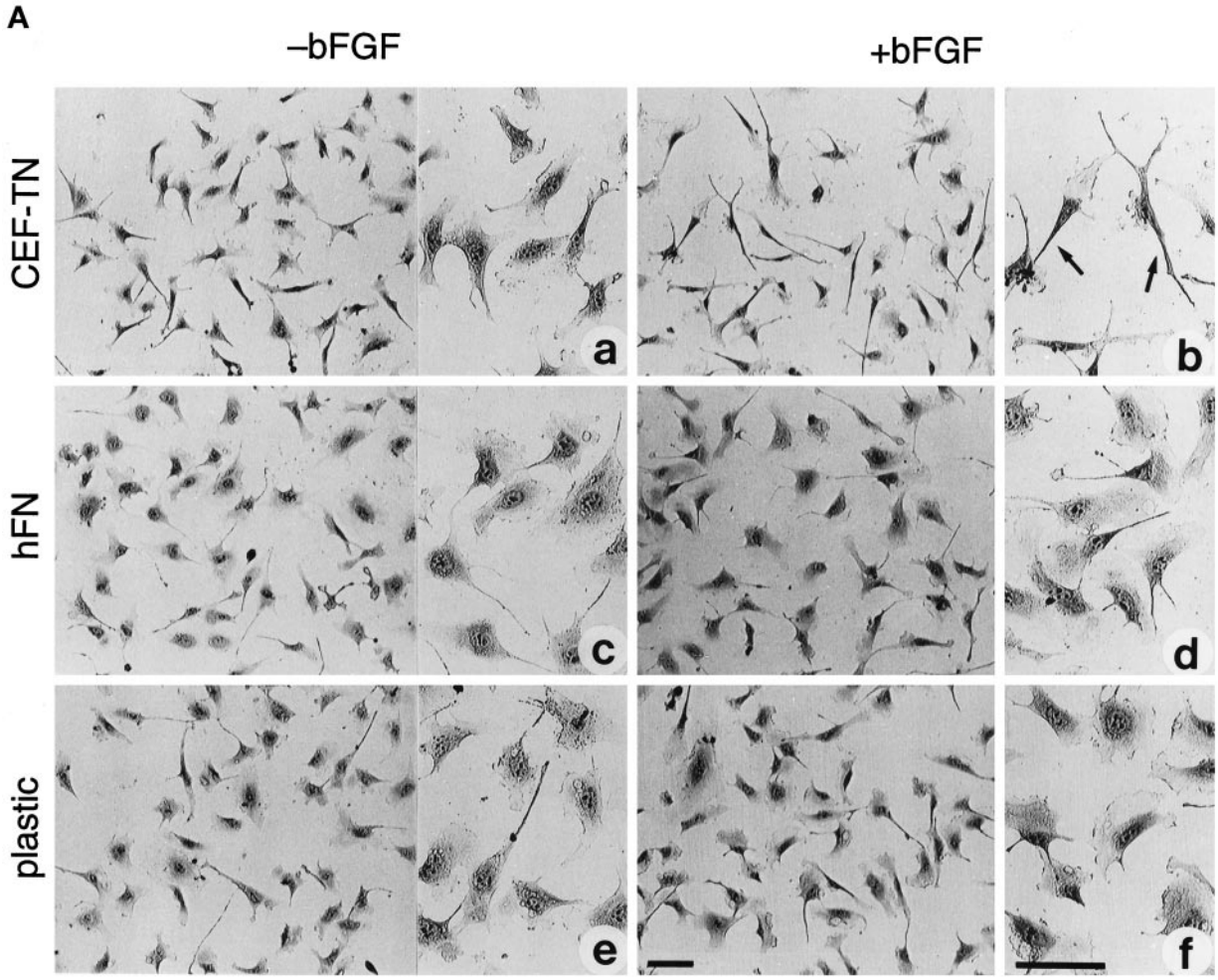
The Fibrinogen Globe of TN-C Promotes Endothelial Elongation in Response to bFGF

CEF-TN is a mixture of the three major splice variants produced by chick embryo fibroblasts in culture. These variants arise from alternative mRNA splicing within the FN type III repeats and are known as TN-230, TN-220, and TN-190 on the basis of the molecular weight of the respective subunits (Figure 1). To assess whether different TN-C splice variants are responsible for the elongated phenotype, nonsprouting BAECs were plated on tissue-culture plates coated with recombinant full-length TN-C splice variants (TN-230, TN-190). After 48 h of incubation, cells were stained and photographed, and elongated cells were counted.

TN-230 (the largest TN-C splice variant isolated from chick embryo fibroblasts) and TN-190 (the smallest TN-C splice variant lacking all alternatively spliced FN-III repeats) promoted endothelial cell elongation in response to bFGF to the same extent as CEF-TN (Figure 5). This indicated that the alternatively spliced region does not play any role in promoting cellular elongation in response to bFGF. We therefore concluded that other constant domains such as the EGF-like repeats, the constant FN-III repeats, or the fibrinogen globe must be responsible for the observed effects. To distinguish the effects of specific domains we used recombinant deletion mutants lacking certain types of domains. Neither deletion of the constant FN-III repeats (TN-FN$^-$) nor deletion of the EGF-like repeats (TN-EGF$^-$) reduced elongation of nonsprouting BAECs in response to bFGF; however, deletion of the fibrinogen globe (TN-FB$^-$) resulted in the abolishment of endothelial elongation. Thus, the fibrinogen globe is required for cellular elongation on TN-C substrates in response to bFGF.

The Presence of the Fibrinogen Globe Reduces Cell Adhesion to TN-C

Elongation of endothelial cells requires their detachment from the substratum. We therefore hypothesized that the combined effects of TN-C and bFGF on endothelial elongation would require decreased cell adhesion to the substratum. Thus, functional in vitro studies have revealed that in most cell types intact TN-C is a poor adhesion substrate compared with FN. In short-term adhesion assays we tested whether decreased cell adhesion correlates with the observed phenotypic modulation, i.e., endothelial elongation, induced by bFGF on TN-C substrates. Nonsprouting BAECs were plated in serum-free medium on various TN-C sub-



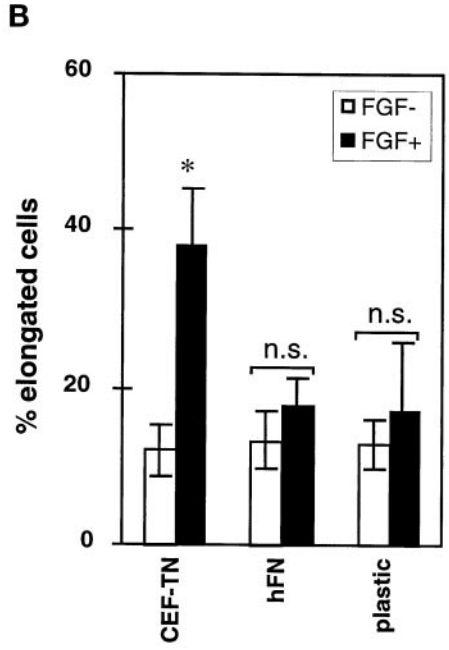


Figure 3. (a) Only the combination of TN-C (CEF-TN) and bFGF promote an elongated phenotype in nonsprouting BAECs. Tissue culture dishes were coated with CEF-TN (a, b) or hFN (c, d) or left uncoated (e, f). Nonsprouting BAECs were plated in the presence or absence of bFGF. In the presence of bFGF (b, d, f), a strong elongation of the cells was found on CEF-TN (b, arrows) but not on hFN (d) or plastic (f). No phenotypic change was found in the absence of bFGF on either substrate (a, c, e). Photos were taken after 48 h of incubation. Bars, 75 μm. (b) Quantification of elongated phenotypes in nonsprouting BAECs plated on CEF-TN, hFN, or plastic in the presence or absence of bFGF. Data represent the percentage of elongated cells from the total cell population. Mean $\pm$ SD from one representative experiment is shown (n = 3). Statistical analysis: significant (*), not significant (n.s.); $P < 0.05$.

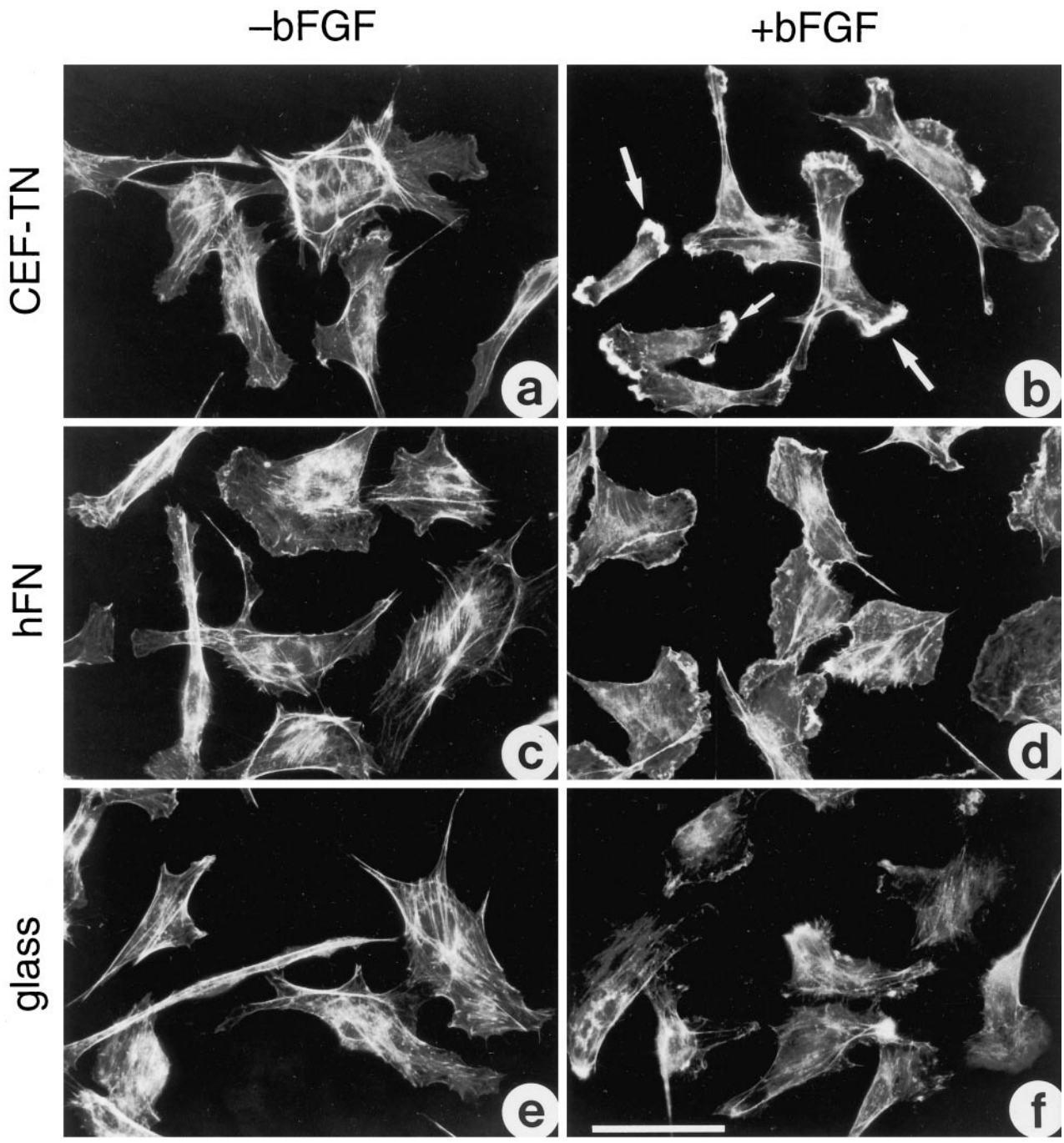


Figure 4. TN-C in conjunction with bFGF alters cytoskeletal organization. Nonsprouting BAECs were grown on CEF-TN (a, b), hFN (c, d), or glass (e, f) in the presence or absence of bFGF. After 24 h the cells were fixed, and the actin cytoskeleton was stained with TRITC-phalloidin. In the absence of bFGF (a, c, e), endothelial cells showed prominent stress fibers on either substrate. In the presence of bFGF (b, d, f), cells adhering to CEF-TN show an intense staining within areas of membrane ruffles (b, indicated by arrows), whereas cells on hFN (d) or glass (f) showed only a slight reduction in the number of stress fibers. Bar, 50 μm.

strates, hFN, plastic, or BSA. After 45 min, cells were fixed and stained, and adherent cells were counted.

As expected, cells adhered well to hFN (540 ± 60 cells per field) or plastic (574 ± 79 cells per field), and most cells spread completely on hFN within 45 min (our unpublished results). Moderate adhesion with mostly rounded cells was found on intact TN-C (CEF-TN, TN-230, TN-190) as well as on the deletion mutants TN-FN$^-$ and TN-EGF$^-$ (for quan-

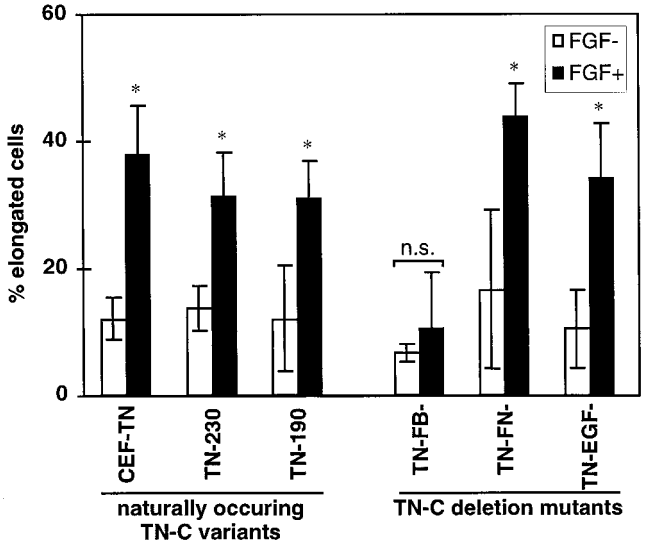


Figure 5. The fibrinogen globe of the TN-C molecule is necessary for the bFGF-induced elongated phenotype. Nonsprouting BAECs were plated on various TN-C substrates and incubated in the presence or absence of bFGF. After 48 h, cells were fixed, stained, and counted. Data are expressed as the percentage of elongated cells from total cell number. The TN-C deletion mutant lacking the fibrinogen globe (TN-FB$^-$) caused significant reduction of elongated cells in the presence of bFGF compared with the other variants. Data are given as mean ± SD from one representative experiment (n = 3). Statistical analysis: significanct (*), not significant (n.s); $P < 0.05$.

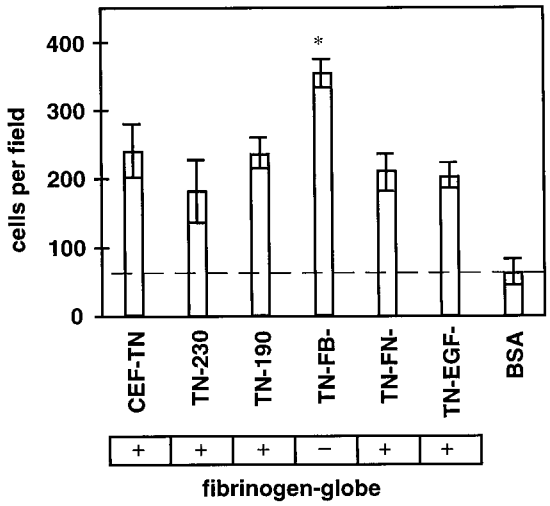


Figure 6. The fibrinogen globe renders TN-C less adhesive. Nonsprouting BAECs were plated in serum-free medium on tissue-culture dishes coated with TN-C variants, hFN, plastic, or BSA. After incubation of 45 min, cells were fixed and stained, and adhering cells were counted. Cells rapidly adhered to hFN or plastic (for numbers see Results), but only moderate adhesion was found on TN-C substrates. Deletion of the fibrinogen globe (TN-FB$^-$) increased the number of adhering cells significantly (*, $P < 0.05$), whereas deletion of the FN type III repeats (TN-FN$^-$) or EGF-like repeats (TN-EGF$^-$) did not change cell adhesion compared with full-length TN-C (CEF-TN, TN230, TN190). Data are given as mean ± SD (n = 3).

titative analysis see Figure 6). In contrast, cells were more adherent with the deletion mutant lacking the fibrinogen globe (TN-FB$^-$), and many cells were remarkably well spread.

This indicates that the fibrinogen globe is responsible for reduced adhesion of endothelial cells to intact TN-C. This agrees well with the finding that elongated endothelial cell morphology occurs only in the presence of bFGF together with intact TN-C or on deletion mutants containing the fibrinogen globe.

We conclude that the fibrinogen globe of TN-C contributes to endothelial elongation in response to bFGF by reducing the adhesive strength.

DISCUSSION

During angiogenesis, endothelial cells change their morphology from tubular in the parent vessel to flat and elongated in growing sprouts and back to tubular as new blood vessels are established. Sprouting is initiated by various angiogenic stimuli such as TGF-β, tumor necrosis factor-α, PDGF-BB, vascular endothelial growth factor, aFGF, and bFGF. Endothelial cells respond to these factors by increased proliferation, by expression of proteolytic enzymes, and by synthesis of specific ECM molecules (Folkman and Shing, 1992; Montesano, 1992; Battegay, 1995). Initiation of angiogenesis requires the detachment of endothelial cells from there original substratum. This is followed by endothelial elongation in association with elements of the ECM.

In this study we aimed to better define the specific role of TN-C and its constituent domains in endothelial elongation in vitro. We could show that TN-C is specifically expressed by sprouting and cord-forming endothelial cells but not by nonsprouting endothelial cells, whereas FN is expressed by both types of cells. Furthermore, only the combination of bFGF and TN-C but not FN induced cytoskeletal reorganization and an elongated (sprouting) phenotype in nonsprouting endothelial cells. In contrast to FN, TN-C was found to be a moderately adhesive substrate for endothelial cells. Only the fibrinogen globe of TN-C, together with bFGF, was found to induce an elongated phenotype of endothelial cells. In addition, the fibrinogen globe was found to mediate the anti-adhesive properties of TN-C. Therefore, we suggest that TN-C, more specifically the fibrinogen globe of this molecule, may play an important role in early angiogenesis by modulating the action of bFGF on endothelial cells. Specifically, in conjunction with bFGF, the fibrinogen globe of TN-C may ease detachment of endothelial cells and induce cytoskeletal reorganization and endothelial sprouting.

bFGF is an angiogenic stimulus in vivo, and it induces proliferation as well as migration of endothelial cells in vitro (Rifkin and Moscatelli, 1986); however, different parameters such as the cell density or the local microenvironment can modulate the cellular responses to a given growth factor such as bFGF (Schubert, 1992). For example, the effects of soluble TN-C and bFGF on cell proliferation were only found in confluent but not in subconfluent endothelial cells (Chung *et al.*, 1996). Cell spreading and growth was pro-

moted at high coating concentrations of FN or collage type IV in the presence of bFGF. In contrast, on moderate coating concentrations bFGF promoted cell extensions and formation of branching tubular networks. This suggests that bFGF-stimulated endothelial cells may be switched between growth and differentiation by altering the adhesivity of their ECM (Ingber and Folkman, 1989).

TN-C is a poor adhesion substrate for many cells; however, the ability to recognize TN-C varies between different cell types, and endothelial cells adhere better to TN-C than, for example, fibroblasts (Joshi *et al.*, 1993). In our experiments only half as many endothelial cells adhered to TN-C than to FN; however, clearly more cells adhered to TN-C than to BSA. Thus, TN-C exerts moderate adhesive strength on endothelial cells and thereby may promote cellular elongation in response to bFGF.

Reports discussing adhesion properties of TN-C found that cells bind to the fibrinogen globe of the molecule (Spring *et al.*, 1989; Prieto *et al.*, 1992; Joshi *et al.*, 1993). For example, the fibrinogen globe, but not the fibronectin type III repeats, allowed lymphocyte rolling on TN-C substrate (Clark *et al.*, 1997). This effect was independent of integrin interaction and was mediated by a yet unknown receptor that recognizes the fibrinogen globe (Clark *et al.*, 1997). We speculate that the set of receptors expressed on a specific cell type determines and modulates its adhesive interactions with different TN-C subdomains. Hence, in contrast to lymphocytes, in endothelial cells interaction with the fibrinogen globe is mediated via the $\alpha_2\beta_1$ integrin (Sririmarao *et al.*, 1993). In addition, two more TN-C receptors have been described in endothelial cells. Annexin II recognizes the alternative spliced region of TN-C (Chung and Erickson, 1994), whereas TN-C binding to $\alpha_v\beta_3$ has been mapped to the third FN type III repeat. This repeat contains an RGD-sequence in human and chicken but not in mouse TN-C (Jones *et al.*, 1988; Nies *et al.*, 1991; Weller *et al.*, 1991). Species variation as well as the observation that this RGD may be a cryptic site, covered up by the adjacent second FN type III repeat (Joshi *et al.*, 1993), raised questions about the functional significance of an RGD-dependent cell binding to the third FN III repeat.

Our experiments have revealed specific responses of endothelial cells to the fibrinogen globe of TN-C. Using TN-C deletion mutants lacking either the FN type III repeats, the EGF-like repeats, or the fibrinogen globe, we have shown that the anti-adhesive effect of TN-C was mediated by the fibrinogen globe. This is in agreement with earlier experiments in which the adhesive behavior of 12 different cell lines on TN-C substrates was investigated. Most cell lines confirmed the general anti-adhesive nature of TN-C, and this was shown to be linked to the presence of the fibrinogen globe (Chiquet and Fischer, unpublished results). In addition to the effects on cell adhesion, we found that TN-C in combination with bFGF induced an elongated phenotype in nonsprouting BAECs. This effect was dependent on the fibrinogen globe. Here we show for the first time that the fibrinogen globe of TN-C contributes to phenotypic changes occurring in early angiogenesis, i.e., sprouting.

bFGF-induced elongation of BAECs on TN-C is due to a profound alteration of the actin cytoskeleton. In the absence of bFGF, cells assembled stress fibers that were lost in the presence of bFGF. In contrast, cells plated on TN-C with bFGF showed broad actin staining in areas of membrane ruffles. This effect was specific to TN-C; cells plated on FN or glass did not show this pattern of actin staining in the presence of bFGF. The actin cytoskeleton mediates various essential biological functions. In addition to providing a structural framework that defines cell shape and polarity, its dynamic properties provide the driving forces for cells to divide or to move. Formation of lamellipodia is a prerequisite for cell migration (Huttenlocher *et al.*, 1995). Before migration, lamellipodia that pull on the cell are extended in all directions. As the cell starts to stretch, the formation of additional lamellipodia is suppressed, resulting in a bipolar and finally unipolar cell shape with a lamellipodium pointing in the direction of migration. Successful migration requires the complex integration of motility-promoting and motility-inhibiting signals. These include growth factors, cytokines, proteases, and ECM components. One class of motility-promoting molecules are anti-adhesive ECM components such as SPARC, thrombospondin, and TN-C (Murphy-Ullrich, 1995). The formation of lamellipodia in endothelial cells plated on TN-C in the presence of bFGF supports the hypothesis that TN-C might promote migration; however, it is important to note that in the presence of bFGF lamellipodia formation on TN-C is not unidirectional. Very often lamellipodia protrude into two or more directions, indicating that TN-C alone is not sufficient to allow migration.

TN-C and the adhesion-promoting ECM molecule FN share a wide distribution in organs of developing animals (Crossin *et al.*, 1986); however, TN-C exhibits a much more restricted tissue distribution, and it was hypothesized that in vivo one of the major functions of TN-C is to modulate action of FN (Mackie *et al.*, 1987; Probstmeier *et al.*, 1990). This idea is supported by experimental data in vitro, where soluble TN-C inhibited adhesion and spreading of fibroblasts on FN (Chiquet-Ehrismann *et al.*, 1988). Furthermore, mixed substrates of TN-C and FN (but not FN alone) up-regulate gene expression of matrix metalloproteinases (Tremble *et al.*, 1994), which suggests that alterating the composition of ECM by adding proteins such as TN-C may be crucial to processes such as migration. Recently TN-C was found to enhance FN-mediated migration of glioma cells in vitro (Deryugina and Bourdon, 1996). Interestingly, the effect of TN-C was completely blocked by antibodies to $\alpha_2\beta_1$ integrin, which interacts with the fibrinogen globe of the TN-C molecule. This again implies a possible function of the fibrinogen globe of TN-C in mediating cell migration by reducing cell adhesion to FN.

It is not known how TN-C expression is regulated in endothelial cells. Several angiogenic growth factors such as aFGF, bFGF, TGF-β, tumor necrosis factor-α, or PDGF-BB have been shown to up-regulate TN-C expression in various nonendothelial cells in vitro (Chiquet-Ehrismann *et al.*, 1995). Interestingly, TN-C induction by bFGF in Swiss 3T3 cells (Tucker *et al.*, 1993) as well as in astrocytes (Meiners *et al.*, 1993) is paralleled by elongation of these cells, similar to the phenotypic changes induced by the combination of TN-C and bFGF in nonsprouting BAECs. So far we could not detect any induction of TN-C in response to bFGF in our endothelial cell system (our unpublished results); however, spontaneously sprouting cells within our cord-forming BAECs are exposed to various growth factors present in

FCS. Therefore, it is possible that the combination of two or more growth factors and/or cytokines is needed to upregulate TN-C expression. Moreover, TN-C expression can also be induced by mechanical forces generated and experienced by cells in culture (Chiquet-Ehrismann *et al.*, 1994). Sprouting endothelial cells apply high tractional forces to their surrounding ECM, and it is believed that the resulting alignment of ECM forms pathways for cellular migration (Vernon and Sage, 1995).

The results described in this article suggest that TN-C could play a significant role in early angiogenesis. By expressing TN-C, endothelial cells modify the ECM composition in a way that results in reduced adhesive strength, which may facilitate bFGF-induced endothelial sprouting and migration. The finding that bFGF-induced effects of TN-C on endothelial cells are dependent on the fibrinogen globe of the TN-C molecule implies a role of $\alpha_2\beta_1$ integrin. We suggest that TN-C might activate intracellular signals by binding to $\alpha_2\beta_1$, which cross-talk with the signaling pathways activated by bFGF.

In conclusion, we suggest that TN-C, more specifically its fibrinogen globe, may play an important role in initiating angiogenesis by modulating the action of bFGF on endothelial cells. Specifically, in conjunction with bFGF, the fibrinogen globe of TN-C may ease detachment of endothelial cells and induce cytoskeletal reorganization and endothelial sprouting.

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