Introduction

Neointimal formation can be a critical event in the development of various arterial diseases such as atherosclerosis and pulmonary hypertension. Neointimal formation also occurs after angioplasty, stent implantation and vascular surgery resulting in restenosis. Neointimal formation is associated with proliferation and migration of smooth muscle cells (SMCs) (Ross, 1993; Davies and Hagen, 1994). SMCs are localized only within media in normal artery, but after vascular injury, SMCs show migration from the media into the intima, accompanied by a phenotypic change from a quiescent-contractile type to a synthetic-proliferative one (Ross, 1993; Davies and Hagen, 1994; Raines, 2004). In vascular lesions, it is well known that increased expression of growth factors occurs, such as those of platelet-derived growth factor (PDGF) , basic fibroblast factor, heparin-binding epidermal growth factor (EGF)-like growth factor, insulin-like growth factor-1 and transforming growth factor β (Ross, 1993). Levels of PDGF-β chain gene transcripts are elevated in atherosclerotic carotid plaque as determined by RNA transfer blot and dot blot hybridization analysis (Barrett and Benditt, 1987). Immunohistochemical studies of neointima have shown expression of PDGF-B and PDGF receptor (PDGFR)-β in neointima by SMCs (Rubin et al., 1988; Tanizawa et al., 1996). PDGF-A and -B chain mRNA and PDGF receptor (PDGFR)-α and -β mRNA were also detected in carotid plaques by in situ hybridization (Wilcox et al., 1988). PDGF and PDGFR may be a major regulatory pathway inducing the proliferation and migration of SMCs (Ross, 1993; Raines, 2004).

After vascular injury, sequential changes of extracellular matrix (ECM) composition of the neointima have been observed (Davies and Hagen, 1994; Imanaka-Yoshida et al., 2001). In the early stage of neointimal formation after percutaneous transluminal coronary angioplasty of human coronary artery, tenascin-C (TNC), a large hexameric ECM glycoprotein, is upregulated in the developing neointima (Imanaka-Yoshida et al., 2001). TNC

deposition is found in the deep layer of neointima of human carotid artery after stent implantation (Toma et al., 2003). TNC deposition is also increased in the neointima after balloon injury in rat models (Hedin et al., 1991; Wallner et al., 2002), while the deposition is limited in the media of normal rat carotid artery. In TNC-null mice, neointimal formation after surgical injuries of arterial walls and arterial anastomoses is attenuated (Yamamoto et al., 2005; Sawada et al., 2007). We have also reported that implantation of TNC-coated platinum coils accelerates organization of arterial lumens in rat aneurismal model (Toma et al., 2005). These findings suggest promotive effects of TNC on cell proliferation and migration in SMCs. A major cellular source of TNC in the neointima is SMCs (Hedin et al., 1991; Yamamoto et al., 2005; Sawada et al., 2007). PDGF is a strong inducer of larger variants of TNC in cultured rat aortic SMCs (Mackie et al., 1992; LaFleur et al., 1994). Therefore, it can be hypothesized that TNC modulates PDGF signaling in the SMCs, while PDGF upregulates TNC expression.

In this study, we examined whether TNC promotes proliferation and migration of SMCs by PDGF stimulation in vitro, using A10 cell line, which is derived from the thoracic aorta of embryonic rat and resembles a synthetic-proliferative phenotype of vascular SMC (Rao et al., 1997). Furthermore, we explored the molecular mechanism of the modulatory effect of TNC on PDGF signaling.

Materials and methods

Antibodies

Anti-PDGFR-α, PDGFR-β, SRC, FAK, phosphorylated (p) SRC (at Y418) and pFAK (at Y925 or Y397) antibodies were purchased from Cell Signaling Technology. Monoclonal anti-integrin αv (clone21) antibody was purchased from BD Bioscience (San Jose, CA). Polyclonal anti-pPDGFR (α:Y572/574, β:Y579/581) antibody was purchased from Biosource (Camarillo, CA). Monoclonal anti-integrin αvβ3 (clone LM609) antibody was purchased from

Millipore (Billerica, MA). Monoclonal anti-α-tubulin and GAPDH antibodies were purchased from Cederlane (Ontario, Canada) and Ambion (Austin, TX), respectively. Production of mouse monoclonal anti-TNC antibody (4F10TT, IBL, Takasaki, Japan) was as previously described (Imanaka-Yoshida et al., 2002).

Cell culture

A10 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely cultured in IMDM with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in 5% CO_2. For every experiment, sub-confluent cells were used.

BrdU incorporation assay

TNC was purified from conditioned medium of human glioma cell line U251MG as previously described (Yoshida et al., 1999). Cover glasses (18 mm diameter) were coated with TNC (4 μg/glass) or collagen type I (4 μg/glass) and air-dried. Control cover glasses were not coated with any substrates. The A10 cells were suspended at 5×10^4 cells/ml in IMDM with 0.4% FBS. One ml of the suspension was poured into a well (12-well plate, BD Falcon, Franklin Lakes, NJ), and serum-starved at 37°C for 48 h. The quiescent cells were stimulated with PDGF-BB (0 to 3 ng/ml) for 16 h. Then, the cells were labeled with 5-bromo-2'-deoxyuridine (BrdU, 10 μg/ml, Sigma-Aldrich, St. Louis, MO) for 2 h, fixed with 100% ethanol at -20°C for 30 min and treated with 1N HCl solution at 37°C for 30 min. Labeled nuclei were detected with anti-BrdU antibody (Dako Japan, Kyoto, Japan) and peroxidase-labeled anti-mouse IgG (MBL, Nagoya, Japan). The BrdU-positive nuclei and total number of nuclei (more than 500) were counted. The proportion of labeled nuclei was determined.

Migration assay

To examine the effect of TNC on PDGF-BB-induced chemotactic migration of A10 cells, transwell assays were performed using cell culture inserts (8-μm pore size, BD Bioscience). The upper surfaces of the inserts were coated with TNC (1 μg/insert) or collagen type I and air-dried. The control inserts were not coated with any substrates. The A10 cells were suspended at 5×10^4 cells/ml in IMDM with 0.4% FBS, and 0.3 ml of the suspensions were placed on the upper surfaces of the inserts. The medium (0.7 ml) containing PDGF-BB (10 ng/ml, R&D system) as a chemoattractant was poured in the lower well (Falcon 24-well plate, BD Falcon). The cells were allowed to migrate to the lower surface of the membrane for 4 h at 37°C. The unmigrated cells on the upper surfaces were wiped off and the inserts were fixed with 100% ethanol and stained with 0.1% crystal violet/50% ethanol for 20 min. The stained cells under four fields were counted under a 20× objective lens. The migratory activity was denoted as the average number of migrated cells in an area of 1 mm^2.

Immunofluorescent staining of integrin αv and phosphorylated SRC and FAK

The A10 cells were plated on cover glass, which was coated with or without TNC (4 μg/well), and serum-starved as described above. The cells were stimulated with PDGF-BB (3 ng/ml) for 0 to 10 min. For detection of integrin αv subunit, the cells were fixed with acetone at -20°C for 3 min. For detection of SRC pY418, FAK pY397 and pY925, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized with PBS with 0.5% Triton X-100 for 2 min. The fixed cells were blocked in PBS with 5% BSA for 15 min, and incubated with primary antibodies (100-fold diluted) at room temperature for 2 h. Then, the cells were incubated with fluorescein-labeled anti-mouse IgG Fab' or rabbit IgG Fab' (MBL) for 1 h. The cells were observed under an epifluorescence microscope with 60× objective lens (Olympus, Tokyo, Japan) and photographs were taken

using cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

Immunoblotting analysis and immunoprecipitation

The A10 cells were plated on cover glass, which was coated with TNC (4 μg/well), followed by serum starvation as described above. The cells were stimulated with PDGF-BB (3 ng/ml). The cells were lysed with 1% SDS, 50 mM Tris-HCl, pH 6.8, and 1 mM sodium orthovanadate. The protein amounts in the lysates were determined using BCA assay (Pierce, Rockford, IL). The lysates were mixed with an equal volume of 2× Laemmli's sample buffer with or without 2-mercaptoethanol, and boiled for 10 min. The proteins were subjected to SDS-PAGE with 5-20% polyacrylamide gradient gels (Atto, Tokyo, Japan) and transferred onto Immobilon membrane (Millipore, Bedford, MA). The membranes were blocked with 5% BSA at room temperature for 2 h. The blots were incubated with primary antibodies, and then incubated with peroxidase-labeled anti-mouse IgG (GE Healthcare Bioscience, Waukesha, WI) or anti-rabbit IgG (Sigma-Aldrich). Immunoreactivity was detected with ECL or ECL plus system (GE Healthcare Bioscience).

To immunoprecipitate integrin αvβ3, the cells were lysed in ice-cold lysis buffer, 1% Nonidet P-40, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium orthovanadate and protease inhibitor tablet (complete mini, EDTA-free, Roche Diagnostics, Mannheim, Germany). To immunoprecipitate integrin αv subunit, the cells were lysed in another lysis buffer, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1 mM sodium orthovanadate and protease inhibitor tablet. The lysates were centrifuged at 15,000×g for 20 min. The supernatants were precleared with protein G beads (Sigma-Aldrich) for 2 h, and then incubated with specific antibody or mouse IgG, as a negative control, at 4°C for 4 h. The complexes were precipitated with protein G beads at 4°C overnight. The beads were washed 3 times with each lysis buffer and boiled with sample buffer with or without 2-mercaptoethanol

for 10 min. The samples were subjected to immunoblotting as described above.

Intensities of bands were quantified using Image J software. The values of protein amounts were normalized by intensities of α-tubulin bands. The values of PDGFR-β or FAK phosphorylation were normalized by the intensities of the total PDGFR-β or FAK bands. The amount of PDGFR co-immunoprecipitated with integrin αvβ3 or SRC with integrin αv subunit was normalized by the intensity of the total αv band. All quantitative analyses were performed at least in triplicate.

Quantitative polymerase chain reaction (qPCR)

To analyze the expression of α-tubulin mRNA, total RNA was isolated by using ISOGEN Reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to cDNA by Transcriptor First Strand cDNA Synthesis Kit using oligo-dT18 primer (Roche Diagnostics, Mannheim, Germany). qPCR was performed on a LightCycler instrument (Roche) using LightCycler FastStart DNA Master SYBR Green I (Roche). The sequences of primers for α-tubulin isoforms (TUBA1A and B; accession number V01227for TUBA1A) were 5'-TACCCTCGCATCCACTTCCCT-3' (sense) and 5'-CGCTTGGTCTTGATGGTGGCA-3' (antisense) (Roy et al., 2004). Primers for GAPDH were purchased from Qiagen (Rn_Gapd_1_SG QuantiTect Primer Assay). The amplification procedure was consisted of denaturation at 95°C for 10 min and 40 cycles of amplification reactions at 95°C for 10 sec, 55°C for 10 sec and 72°C for 20 sec, followed by the melting curve analysis initiated at 65°C with elevation of the temperature up to 95°C.

Statistical analysis

All values are provided as mean±standard deviation. Statistical comparisons were made using Student's t test. Significance was defined at the $P<0.05$ level.

Results

TNC enhances PDGF-BB-induced proliferation and chemotactic migration of A10 cells

We examined the effects of TNC on PDGF-BB-induced proliferation of the A10 cells by BrdU incorporation assay. BrdU-labeling indices in the cells with PDGF-BB (3 ng/ml) treatment on TNC-coated substrate were significantly higher than those in the cells on a collagen type I-coated or non-coated one, while the indices on collagen type I were also higher than those on non-coated substrate (Fig. 1A,B). However, without PDGF-BB stimulation, there was no significant difference in the indices between TNC and control groups. The enhanced effect of TNC on cell proliferation by PDGF-BB stimulation was shown for various concentrations of PDGF-BB from 0.1 to 3 ng/ml (Fig. 1C).

We also examined the effect of TNC on chemotactic migration to PDGF-BB in transwell migration assay. TNC coating significantly promoted the migration compared with those with type I collagen and control, whereas type I collagen substrate showed an inhibitory effect (Fig. 1D,E). The enhanced migration by TNC was exhibited in a dose-dependent manner (Fig. 1D,F).

TNC promotes PDGFR-β phosphorylation at Y579/581

Next, we examined PDGF signaling in A10 cells. In immunoblotting of A10 cell lysates, intensity of α-tubulin band in TNC-coated group was always weaker than that in control group, in spite of loading equal amounts of proteins (Fig. 2, see also Fig. 5). When band intensities of GAPDH as another intrinsic standard in control and TNC-coated groups were compared, the weaker bands were also observed in TNC samples. The intensity ratios of TNC-coated group against the control were 0.54±0.05 in GAPDH and 0.60±0.05 in α-tubulin. In qPCR, mRNA levels of and GAPDH were not different between TNC-coated and control

groups (1.17±0.16 and 1.00±0.07 for α-tubulin, and 1.19±0.14 and 1.00±0.06 for GAPDH, respectively). Whereas the mRNA expression was not decreased, these protein contents were concomitantly decreased in the lysates of cells on TNC substrate. TNC-treated samples may contain the exogenous TNC and, possibly, the protein-rich matrices formed by TNC on the substrates other than the cellular proteins. Therefore, we normalized protein amounts of PDGFRs by intensities of α-tubulin bands. Immunoblotting of A10 cell lysate could detect PDGFR-β, but not PDGFR-α. The amounts of PDGFR-β were not different between the cells on TNC substrate and control (Fig. 2A: 0.96±0.11 and 1.00±0.02, respectively, P=0.61) after normalization by α-tubulin, while the band intensity in TNC-coated group was weaker than that in control group, at a ratio of 0.68± 0.05 as low as 0.71±0.12 of α-tubulin. We considered that PDGFR-β is dominant in A10 cells and TNC does not affect the expression of PDGFR-β. We also examined the effect of TNC on the PDGFR-β phosphorylation at Y579/581 in immunoblotting analysis. In serum-starved A10 cells, a minimum extent of the PDGFR-β phosphorylation was detected. PDGF-BB stimulation induced autophosphorylation of PDGFR-β at Y579/581. TNC significantly enhanced the phosphorylation 10 min after PDGF-BB stimulation in comparison with the control (Fig. 2B). The phosphorylation after 30 min of the treatment was also augmented in TNC-treated group, although the difference was not significant.

TNC induces upregulation of integrin αv subunit and the recruitment to focal adhesion

Integrins α2/α7/α8/α9β1 and αvβ1/β3/β6 are known as TNC receptors (Orend and Chiquet-Ehrismann, 2006; Midwood and Orend, 2009). Integrin αvβ3 is expressed in activated SMCs (Jones et al., 1997; Perlstein et al., 2003). Therefore, we examined expression of integrin αv subunit using immunofluorescence and immunoblots. The immunofluorescent staining demonstrated that TNC promoted the clustering of integrin αv in focal adhesions of

the cell periphery (Fig. 3A). In immunoblotting, intensity of α-tubulin band in control group was also higher than that in TNC-treated group (Fig. 3B). TNC coated on the glass surface was extracted with SDS-containing lysis buffer and was detected by immunoblots using TNC antibody, whereas TNC was barely detectable in the extract of the cells on non-coating glass, indicating little production of TNC by serum-starved A10 cells (Fig. 3B). Band intensities of αv subunit after normalization by α-tubulin amounts showed significant upregulation of the subunit in TNC-coating group (Fig. 3B,C).

PDGFR-β is associated with αvβ3 integrin

Previous reports suggest that synergistic collaboration between αvβ3 integrin and PDGFR enhances cell proliferation and migration in microvascular endothelial cells and fibroblasts on vitronectin substrate (Schneller et al., 1997; Woodard et al., 1998; Eliceiri, 2001). We also examined the effect of TNC on the association of αvβ3 integrin with PDGFR-β in SMCs by immunoprecipitation followed by immunoblotting. The analysis demonstrated that, in the absence of PDGF-BB, PDGFR-β was co-precipitated with integrin αvβ3 and the amount was not different between TNC treatment and control (Fig. 4A: 1.08±0.14 and 1.00±0.11, respectively, after normalization by αv subunit. P=0.48). After 30 min of PDGF-BB stimulation, the amount of co-precipitated PDGFR-β in TNC-treated cells was significantly larger than that in control cells (Fig. 4B), while the phosphorylated fraction of PDGFR-β was not changed between TNC-treated cells and control cells (Fig. 4C).

TNC enhances FAK phosphorylation at Y397 and Y925

FAK is recruited to integrins and autophosphorylated at Y397 (Giancotti and Ruoslahti, 1999; McLean et al., 2005; Mitra and Schlaepfer, 2006). To the phosphorylated site, SRC is bound and activated, which is followed by FAK phosphorylation at Y407, 576, 577, 861 and

925 (Giancotti and Ruoslahti, 1999; McLean et al., 2005; Mitra and Schlaepfer, 2006). The Y925 is an exclusive phosphorylated site for SRC (McLean et al., 2005). We examined FAK phosphorylation at Y397 and 925 by immunofluorescent staining and immunoblots.

Immunofluorescence using antibodies against FAK pY397 and pY925 showed bright fluorescence in focal adhesions of the cells after PDGF-BB treatment, and the staining was brighter and larger in the cells on TNC substrate (Fig. 5A). Immunoblotting demonstrated that TNC treatment significantly elevated the FAK phosphorylation at Y397 and Y925 after 10 min of PDGF-BB stimulation (Fig. 5B,C), while FAK expression level was not significantly changed by TNC treatment (0.91±0.06 and 1.00±0.08 under the condition with TNC and control, respectively, P=0.20) after normalization by α-tubulin. Before normalization, band intensity ratios of TNC-coated group against the control were 0.65±0.04 in total FAK and 0.72±0.07 in α-tubulin.

TNC enhances SRC recruitment to integrin αv complex

SRC also binds to autophosphorylation sites (Y579/581) of PDGFR-β (Mori et al., 1993; Heldin et al., 1998) as well as FAK pY397, followed by the activation and intermolecular autophosphorylation at Y418 (Heldin et al., 1998; Brunton and Frame, 2008). We also examined SRC activation and its association with αv integrin complex. In immunofluorescence, SRC pY418 was localized in focal adhesions, of which the labeling became clearer after PDGF treatment (Fig. 6A). In immunoblots after immunoprecipitation, only a fraction of SRC was detected in protein complex precipitated by anti-αv subunit in the absence of PDGF-BB stimulation. After PDGF-BB stimulation for 30 min, SRC co-precipitated with αv subunit was increased, and further TNC treatment significantly raised the SRC amount in the precipitation, up to 3 times as much as with non-coating (Fig. 6B,C). TNC could enhance recruitment of SRC to the signaling complex of integrin/PDGFR after

PDGF-BB treatment.

Discussion

TNC, a large hexameric ECM glycoprotein, is expressed during fetal development. Although it is absent or reduced in most adult tissues, it is re-expressed in tissue growth and remodeling such as healing wounds, tumor tissues and inflammation (Orend and Chiquet-Ehrismann, 2006; Midwood and Orend, 2009). It has also been reported that TNC is highly expressed in various vascular lesions (Hedin et al., 1991; Imanaka-Yoshida et al., 2001; Wallner et al., 2002; Toma et al., 2003; Yamamoto et al., 2005). In this study, we demonstrated promotive effects of TNC on migration and proliferation of SMCs induced by PDGF-BB, using A10 cell line derived from the thoracic aorta of embryonic rat. Whereas TNC did not enhance proliferation of A10 cells without PDGF-BB stimulation, the growth with the stimulation of various concentrations was significantly promoted on TNC substrate. In transwell assay, the migration attracted by PDGF-BB was also enhanced by TNC coating in a dose-dependent manner. In previous studies, TNC enhanced proliferation of adult rat pulmonary artery SMCs induced by fibroblast growth factor and epidermal growth factor in vitro (Jones and Rabinovitch, 1996; Jones et al., 1997). These results indicate that TNC could promote the proliferation and migration of SMCs, maybe synergistically with growth factors. It has also been reported that cell growth and/or migration of fibroblasts and cancer cells are enhanced by TNC treatment (Yoshida et al., 1999; McKean et al., 2003; Tsunoda et al., 2003; Tamaoki et al., 2005; Trebaul et al., 2007).

PDGF induces signaling for cell growth and migration through PDGFRs (Mori et al., 1993; Heldin et al., 1998; Hollenbeck et al., 2004; Raines, 2004). Although there are two distinct PDGFRs: PDGFR-α and -β, a lack of functional PDGFR-α in A10 cells has been reported (Rao et al., 1997). We also detected PDGFR-β, but not PDGFR-α, by

immunoblotting. While the level of PDGFR-β expression was not altered by TNC treatment, PDGFR-β autophosphorylation at Y579/581 10 min after PDGF-BB stimulation was enhanced by TNC substrate.

Integrin binding to ECM causes their clustering and forms focal adhesion complexes associated with cytoskeleton. This in turn promotes further integrin clustering and then the cell spreads (Giancotti and Ruoslahti, 1999; Eliceiri, 2001). In our study, immunofluorescent staining showed larger integrin αv-positive adhesion plaques at cell periphery on TNC substrate. Immunoblot analysis demonstrated that the expression of integrin αv subunits is upregulated in TNC-treated cells. It has been reported that expression of integrin β3 is upregulated by SMC during neointimal formation after balloon injury to rat carotid artery (Bendeck et al., 2000) and that integrin αvβ3 of SMCs interacts with TNC in vitro (Jones et al., 1997; Perlstein et al., 2003). In addition, vitronectin and PDGF collaboratively potentiate proliferation and migration of fibroblasts through crosstalk signaling between integrin αvβ3 and PDGFR-β (Schneller et al., 1997). Therefore, we examined the association of PDGFR-β with integrin αvβ3 by immunoprecipitation using antibody specific to αvβ3. TNC treatment increased the amount of PDGFR-β co-precipitated with αvβ3, but not the phosphorylated fraction of PDGFR-β. These findings indicate that, in the cells on TNC substrate, integrin αv subunits, possibly αvβ3, are clustered at focal adhesions, being associated with the upregulated expression, and that PDGFR-β is recruited to focal adhesions by binding to αvβ3. However, phosphorylated fraction of PDGFR-β associated with the integrin is not increased, suggesting that TNC treatment may not directly augment PDGFR signaling by PDGF binding. The similar findings have been observed in NIH 3T3 cells on vitronectin (Schneller et al., 1997). Tyrosine phosphorylation of PDGFR itself after PDGF stimulation is not different between the cells on vitronectin and type I collagen, whereas vitronectin, but not collagen, enhances ERK1/2 phosphorylation. These data and ours indicate that binding of integrin αvβ3

to the ligands, vitronectin and TNC, can influence signal transduction downstream of the PDGFR by enhancing PDGFR recruitment to the integrin.

Integrins bound to ECM also activate FAK signaling (Giancotti and Ruoslahti, 1999; Mitra and Schlaepfer, 2006; Huveneers and Danen, 2009; Luo and Guan, 2010). FAK is primarily recruited to sites of integrin clustering, and increasingly autophosphorylates at Y397, which creates a high-affinity binding site for SH2 domain of SRC. The binding activates SRC itself, phosphorylating FAK at Y406, 576/577, 861 and 925 (McLean et al., 2005; Mitra and Schlaepfer, 2006; Brunton and Frame, 2008; Huveneers and Danen, 2009; Luo and Guan, 2010). We demonstrated that TNC significantly upregulates FAK phosphorylation at Y397 and Y925 after PDGF-BB stimulation, and that the phosphorylated FAK is localized in focal adhesions in immunofluorescence. It has been revealed that TNC treatment increases phosphorylation of FAK in microvascular endothelial cells (Zagzag et al., 2002).

The FAK/SRC complex integrates signals from integrins and growth factor receptors (Mitra and Schlaepfer, 2006; Brunton and Frame, 2008). SRC is bound to autophosphorylated sites at Y579/581 of PDGFR as well as FAK pY397. Tyrosine kinase of PDGFR phosphorylates SRC at Y215, upregulating the kinase activity (Heldin et al., 1998). The activation by the interaction with PDGFR and FAK causes the phosphorylation at Y418 in an intermolecular manner, followed by full activation. We demonstrated bright fluorescence of pY418 at focal adhesions after PDGF-BB stimulation. Furthermore, TNC augments FAK phosphorylation of Y925, a substrate site specific to SRC kinase, indicating SRC activation at focal adhesions. We also examined recruitment of SRC to integrin complex. While a small amount of SRC was present in integrin complex before PDGF-BB stimulation, SRC was recruited to the complex after the treatment and the association with the complex was increased up to 3-fold in the cells on TNC compared with that with non-coating. As a similar finding, the association of SRC with integrin $\alpha 2\beta 1$ has been observed in SMCs on collagen

type I, exhibiting a synergistic effect on cell proliferation with PDGF-BB, although the association level does not change when the cells are stimulated by PDGF-BB and/or collagen type I (Hollenbeck et al., 2004). The SH3 domain of SRC can constitutively and directly interact with integrin β3 cytoplasmic tail, followed by SRC activation, but not with β1 (Arias-Salgado et al., 2003). The primed SRC bound to integrin β3 may facilitate the downstream signaling of the integrin complex. A recent study has shown direct phosphorylation of FAK Y397 (autophosphorylation site) by SRC in vitro (Wu et al., 2008). Other tyrosine residues of FAK phosphorylated by SRC also serve as binding sites for SH2 domain-containing molecules, including SRC, although pY397 appears to be the main docking site (Cox et al., 2006). Thus, TNC substrate enhances formation of adhesion plaques clustering integrin αvβ3, to which PDGFR-β, FAK and SRC are joined. After PDGF stimulation, more SRC molecules are recruited to the signaling complex. Finally, the intracellular signaling induced by PDGF may be remarkably amplified in this complex.

A study using NIH3T3 cells transfected with wild-type FAK and mutants demonstrated that FAK/SRC complex formation promotes ERK activation, cyclin D1 upregulation and decreased p21 expression (Zhao et al., 1998). FAK autophosphorylation at Y397 also enhances cell proliferation by facilitating G1-S progression through regulation involving cyclin D1 and $p27^{kip1}$ in glioblastoma cells in vitro and in vivo (Ding et al., 2005). FAK Y397 also serves as a binding site for PI3K and Grb7 to promote cell motility. Active FAK/SRC promotes p130Cas binding and its subsequent phosphorylation, and Crk is bound to the phosphorylated site, facilitating Rac activation, lamellipodia formation and cell migration (Guarino, 2010). FAK also directly modulates signaling pathways related to cell migration and cytoskeleton remodeling, including paxillin, N-WASP and regulatory molecules of Rho subfamily of small GTPases (McLean et al., 2005; Mitra and Schlaepfer, 2006; Huveneers and Danen, 2009; Luo and Guan, 2010).

From this study, we can conclude that TNC promotes proliferation and migration of A10 cells induced by PDGF-BB, and that PDGF stimulation is augmented through enhanced crosstalk signaling between PDGFR-β and αvβ3 integrin by increased SRC recruitment and FAK activation on TNC substrate. During neointimal formation, TNC induced by various growth factors including PDGF may act on SMCs, in an autocrine fashion, as a critical regulator of cell behavior by modulating the signaling pathway. TNC could provide a target for future therapeutic and diagnostic approaches in vascular lesions.

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Fig. 1. Effects of TNC on PDGF-BB-induced proliferation and migration of A10 cells. A-C: Cells were plated on cover glasses, which were coated with/without TNC (4 μg/glass) or collagen type I (COLI), and serum-starved for 48 h. The quiescent cells were stimulated with 3 ng/ml PDGF-BB for 16 h, and treated with BrdU (10 μg/ml) for 2 h. A: The nuclei that incorporated BrdU were visualized by immunocytochemistry. Light counterstaining was carried out with hematoxylin. Bar, 100 μm. B: BrdU-labeling index in TNC-treated group was significantly greater than those in COLI-coated and control (non-coated) groups. C: Enhanced effect of TNC on cell proliferation was observed in the range of 0.1-3 ng/ml PDGF-BB, but not in a condition without PDGF-BB. #: $P<0.05$ compared with control.*: $P<0.01$ compared with control. D-F: Cells were plated on the upper surfaces of inserts (8-μm pore size), which were coated with TNC or COLI, treated with PDGF-BB (10 ng/ml) in the wells and allowed to migrate for 4 h. D: Cells migrating on the lower surfaces were fixed and stained with 0.1% crystal violet. Photographs show cells migrating onto the surfaces of the inserts in each condition. Bar, 100 μm. E: TNC significantly enhanced the migration against PDGF-BB. F: The effect of TNC was increased in a dose-dependent manner. #: $P<0.05$ compared with control.*: $P<0.01$ compared with control.

Fig. 2. TNC promotes PDGFR-β phosphorylation at Y579/581 in A10 cells. Cells were plated on cover glasses, which were coated with TNC, serum-starved for 48 h and stimulated with PDGF-BB (3 ng/ml) for 10 or 30 min. The control glasses were not coated with any substrates. Equal amounts of proteins in each sample were subjected to immunoblots. A: PDGFR-β bands were visible but PDGFR-α was hardly detectable. The expression of PDGFR-β was not different between TNC and control treatments. n.d.; not determined. B: The extents of PDGFR-β phosphorylation after 10 min and 30 min of PDGF-BB stimulation were also determined. The graph shows the quantified relative value of PDGFR-β phosphorylation at

Y579/581 against total PDGFR-β. TNC significantly enhanced the PDGFR-β phosphorylation at Y579/581 after 10 min of PDGF-BB stimulation. The phosphorylation after 30 min showed a similar tendency, although it was not significant.

Fig. 3. TNC induces upregulation of integrin αv and formation of focal adhesions. A: Cells were plated on the cover glasses coated with TNC, and serum-starved for 48 h. The control glasses were not coated with any substrates. Representative immunofluorescence photographs for integrin αv in non-coated and TNC-coated groups. Larger adhesion plaques positive for αv subunit were observed at the periphery of cells on TNC substrate. Bar, 20 μm. B: The cells were lysed and analyzed by immunoblotting. A10 cells barely produced TNC, but TNC coated on the surface of cover glass was retained. Integrin αv bands of cells on TNC substrate were apparently denser and broader than those of control. Amounts of α-tubulin were usually smaller in TNC-treated group than in control one, possibly due to TNC-rich extracellular matrix. C: The graph shows the relative values of αv integrin expression in each group. TNC induced significant upregulation of integrin αv subunit.

Fig. 4. PDGFR-β is associated with αvβ3 integrin. Cells were lysed with a lysis buffer, immunoprecipitated using αvβ3 antibody (LM609) and subjected to immunoblotting. A: PDGFR-β was co-precipitated with integrin αvβ3 even in the absence of PDGF-BB. As a control, an isotype-matched mouse IgG was used. B: The graph shows the quantified relative values of co-precipitated PDGFR-β to αv subunit after PDGF-BB stimulation. TNC treatment significantly increased amounts of PDGFR-β co-precipitated with integrin αvβ3. C: The ratio of phosphorylation did not change.

Fig. 5. TNC enhances FAK phosphorylation at Y397 and 925. A: Representative