Aortic Smooth Muscle Cells Interact with Tenascin-C through Its Fibrinogen-like Domain*

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The extracellular matrix protein tenascin-C is a multidomain protein that regulates cell adhesion. We used two different smooth muscle cell subtypes derived from adult and newborn rat aorta to investigate the interaction of tenascin-C or its various domains with these cells using an adhesion assay. Newborn cells were three times more adherent to tenascin-C than adult cells. Tenascin C-adhering cells remained round, whereas they spread rapidly on a fibronectin substrate. Adhesion assays showed the interaction between tenascin-C and newborn cells to be predominantly RGD-independent. Mg²⁺ increased newborn cell adhesion to tenascin-C in a concentration-dependent manner, whereas Ca²⁺ had no effect. To analyze the structure-function relationships of different domains of tenascin-C, we used recombinant full-length fibronectin-like and fibrinogen-like domains and various subdomains corresponding to the alternatively spliced regions of tenascin-C. The cells adhered to the fibrinogen-like domain but not to the fibronectin-like domain or its subdomains. As with the intact tenascin-C molecule, adherent cells remained round, and the Mg²⁺, but not Ca²⁺, promoted this interaction. The interaction of cells with the fibrinogen-like region was further mapped to a 30-amino acid peptide located near the carboxyl-terminal part of the tenascin-C molecule. The same 30-amino acid peptide was active in promoting cell migration. Our results provide a basis for understanding the mechanism of interaction of tenascin-C with smooth muscle cells and a framework for isolating membrane binding sites that mediate the cellular responses to this molecule.

Tenascin-C is an oligomeric glycoprotein composed of multiple domains that has been implicated in cell migration (1–7). Human, mouse, and chicken tenascin-C contain a cysteine-rich segment at their amino termini through which the six tenascin-C monomers link into a hexamer. This segment is followed by epidermal growth factor-like repeats, fibronectin-type III repeats (FN-L), and a globular carboxyl terminus homologous to fibrinogen (Fbg-L) (8). These domains mediate the interaction between the tenascin-C molecule and cells. For example, endothelial cells interact with tenascin-C through its fibrinogen-like domain (9), whereas the FN-L domain of tenascin-C mediates interaction with fibroblasts (10). The specific domain of tenascin-C that mediates its interaction with SMCs is unknown.

We have previously demonstrated that chemotactic factors involved in the remodeling of vascular tissues including platelet-derived growth factor BB and angiotensin II markedly induce tenascin-C gene expression in SMCs (11, 12). To gain insight into the molecular mechanism of tenascin-C-SMC interactions, we mapped the active site of tenascin-C. Using recombinant proteins corresponding to the Fbg-L and FN-L domains of tenascin-C, we demonstrated that the Fbg-L domain, but not FN-L domain, is the active domain. We further mapped the active site of the Fbg-L domain and demonstrated that the activity of the Fbg-L domain can be duplicated by a 30-amino acid peptide.

EXPERIMENTAL PROCEDURES

Materials—Tenascin C was purified from conditioned media of baby hamster kidney cells overexpressing tenascin-C, as described (10). The recombinant proteins corresponding to the full-length Fbg-L and FN-L domains were expressed and purified from the bacteria BL-21 Escherichia coli provided by Dr. Harold Erickson (Duke University). Dr. John Peters (Cedar-Sinai Medical Center) kindly provided the recombinant fibronectin type III unit 10. Prestained protein standards were from Bio-Rad. Other chemicals were of reagent grade quality and obtained from Sigma.

Cell Culture—Adult aortic SMCs were cultured as described (12). Newborn (9 days old) aortic SMCs were obtained from Dr. Stephen M. Schwartz (University of Washington) and cultured (13). Briefly, rat aortic SMCs were isolated by enzymatic digestion of rat (Sprague-Dawley, 2 months old, 270 g) aorta. Cells were grown in DMEM/F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). After reaching confluence, cells (between the third and sixth passages) were used for adhesion studies.

Adhesion Assay—Microtiter plates (Falcon; Becton Dickinson, Oxford, CA) were coated with the respective substrate for 1 h at 37 °C. Nonspecific sites were blocked with 1 mg/ml BSA in phosphate-buffered saline. Subconfluent cells exhibited higher adhesion activity than confluent cultured cells; therefore, cells were split and plated at half confluence the day before the assay. SMC subtypes were detached by trypsin/EDTA, washed once in DMEM/F-12 medium, 2.5 mg/ml BSA, and 1 mg/ml trypsin inhibitor (adhesion buffer) and plated at 4 x 10³ cells/well. After incubation for 60 min at 37 °C, nonadherent cells were removed with gentle washing with phosphate-buffered saline. The number of attached cells were quantified by staining cells with 0.2% crystal violet in 20% methanol, lysing with 1% SDS, and measuring the absorbance at 550 nm (14).

To determine the concentration for the adhesion assays, microtiter plates were coated with increasing concentrations (1–100 µg/ml) of substrates, and the number of adherent cells was quantified. Adhesion of cells to tenascin-C reached saturation levels at coating concentrations of 10 µg/ml (not shown); therefore, all the subsequent adhesion assays were performed by coating the wells with 10 µg/ml substrate.

Expression of Recombinant FN-L Subdomains—The PET expression...
system was used to express the recombinant FN-L subdomains (10). The primers were targeted to the exact boundaries of the alternatively spliced fibronectin type III repeats corresponding to each isoform of tenasin-C. A BamHI site together with the NdeI site provided for unidirectional ligation downstream from the T7 promoter in the expression vector pET11a (Novagen, Madison, WI). The cloned subunits were resequenced to ensure that no errors had been introduced during the cloning process. The resultant construct was transformed into the E. coli expression host BL21 (DE3) (Novagen). Clonal cultures were grown in LB medium containing 50 \( \mu \text{g/ml} \) carbenicillin and induced with isopropyl-\( \beta \)-D-galactopyranoside for 3 h. Polyclonal antibodies to tenasin-C were used to identify the recombinant proteins. To further assess the integrity of the recombinant proteins, their amino acid composition and partial amino acid sequences were determined (UCLA amino acid sequencing core facility). Both corresponded exactly to the predicted values (not shown).

**Peptide Synthesis**—Peptides corresponding to the full-length Fbg-L domain were synthesized in the UCLA peptide synthesis core facility. The peptides were synthesized using Fmoc (9-fluorenyl)methoxycarbonyl (Fmoc) strategies on an Advanced Chem Tech multiple synthesizer model 396, cleaved at room temperature (cleavage mixture: 90% trifluoroacetic acid, 5% thioanisole, 5% ethanedithiol, 2% anisole), purified by reverse phase high performance liquid chromatography, and characterized by mass spectral analysis (at the UCLA mass spectroscopy facility) and high performance capillary electrophoresis (Beckman 2200 HPCE). The core facility was unable to synthesize peptides II and VII, presumably due to the formation of a strong secondary structure that prevented elongation of the peptide chain.

**Migration Assay**—Migration was measured by a modification of the Boyden’s chamber method using microchemotaxis chambers (Neuro Probe Inc). Polycarbonate filters were coated with 10 \( \mu \text{g/ml} \) concentrations of substrates overnight at 4 °C. Newborn rat SMCs were suspended at a concentration of 10\(^5 \) cells/ml in serum-free DMEM supplemented with 1 mg/ml BSA. A volume of 50 \( \mu \text{l} \) of cell suspension was placed in the upper chamber, and 30 \( \mu \text{l} \) of 10% bovine calf serum in DMEM was placed in the lower chamber. In some experiments, cells were suspended in DMEM supplemented with 0.1 \( \mu \text{M} \) Fbg-L or 1 \( \mu \text{M} \) peptide VIII before addition to the upper chamber. The chamber was incubated at 37 °C under 5% CO\(_2\) in air for 4 h. The filter was removed, and the cells on the upper side of the filter were scraped off. The SMCs that had migrated to the lower side of the filter were fixed in methanol, stained with Diff-Quick staining solution (Baxter), and counted under a microscope.

**RESULTS**

**Effect of Tenasin C on Cell Adhesion in Different SMC Subtypes**—Interaction between aortic SMCs and tenasin-C was assessed by adhesion assay. Adult rat aortic SMCs avidly adhered to wells coated with fibronectin and vitronectin (Fig. 1). By contrast, adhesion to tenasin-C was 3–4 times lower than attachment to the adhesive proteins. Tenasin-C-adherent cells remained round, whereas spreading and flattening followed attachment to fibronectin and vitronectin (not shown).

The reduced adhesion of adult SMCs with tenasin-C indicates that either complete binding equilibrium did not occur at 60 min or that adult SMCs have diminished the ability to interact with tenasin-C. We found that the interaction reached equilibrium levels at 60 min (not shown); therefore, the reduced SMC-tenasin-C interaction most likely reflects a lower adhesive capacity of adult SMCs for tenasin-C.

Since tenasin-C is prominently expressed during embryogenesis (8, 15), we postulated that SMCs derived from newborn arteries might express a higher number of tenasin-C receptors. To explore this possibility, we examined the adhesion of cultured newborn SMCs to tenasin-C (Fig. 1). The levels of adhesion of newborn SMCs to tenasin-C were 3–4 times higher than adult cells and were comparable to the attachment levels observed with fibronectin (Fig. 1).

**The Role of RGD Motif in Cell Binding**—It has been shown that the interaction of some cells with tenasin-C is mediated by the RGD motif (9, 16). Therefore, we determined the role of RGD in the interaction between tenasin-C and SMCs by an adhesion assay. The specificity of the adhesion was determined by coating the wells with the recombinant 10th-type III repeat of fibronectin (17). As shown in Fig. 2, GRGDS peptide inhibited the attachment of newborn SMCs to the recombinant fibronectin fragment, and complete inhibition was observed at 0.1 mg/ml. Attachment of SMCs to tenasin-C was inhibited by approximately 30% in the presence of 0.1 mg/ml GRGDS peptide, and higher concentrations did not significantly alter adhesion levels (Fig. 2). To establish the sequence specificity of the RGD-mediated interaction, the effect of GRGDS peptide was compared with inactive GRFDS peptide. The GRFDS peptide had no effect (not shown), demonstrating that the RGD-mediated interaction of tenasin-C is specific.

**Effect of Cations on the Interaction between SMCs and Tenasin C**—To determine the role of cations in the interaction between SMCs and tenasin-C, the adhesion assay was performed in the presence of increasing concentrations of EDTA (Fig. 3). The intact fibronectin molecule and its recombinant subunit were used as a positive control. Newborn cells adhered well to tenasin-C, fibronectin, or the recombinant fragment in the absence of EDTA. The addition of 1 mM EDTA reduced adhesion of newborn cells to the intact fibronectin molecule or
Contrast, 

Tenascin-C by 50%, and nearly complete inhibition of cell adhesion to all substrates was observed with 10 mM EDTA (Fig. 3). These data suggest that cations are essential for adhesion of newborn SMCs to tenascin-C.

The type of cation markedly affects the association rate constant of the ligand for integrin (18–21). To determine which cation influences adhesion of SMCs to tenascin-C, we examined the effect of both Ca$^{2+}$ and Mg$^{2+}$ cations on cell adhesion. The level of newborn SMC adhesion to tenascin-C or to the recombinant fibronectin fragment increased as the Mg$^{2+}$ concentration increased (Fig. 4). Maximal cell adhesion to both substrates was observed in the range of 5–10 mM Mg$^{2+}$. In contrast, Ca$^{2+}$ was ineffective. Thus, we conclude that there is the potential for a 3-fold increase in the interaction between newborn SMCs and tenascin-C in the presence of Mg$^{2+}$.

Since Ca$^{2+}$ ion can reverse the Mg$^{2+}$-dependent adhesion of some integrins, particularly $\alpha$5$\beta$1 (19), we examined its effect on the adhesion of newborn SMCs to tenascin-C in the presence of Mg$^{2+}$ cation. We found no indication that the presence of Ca$^{2+}$ up to 10 mM, a concentration that completely reversed the Mg$^{2+}$-dependent adhesion of human fibroblasts (22), could inhibit the Mg$^{2+}$-dependent adhesion of newborn cells (not shown).

**Mapping of the Active Domain of Tenascin C**—We next mapped the active domain of tenascin-C using recombinant proteins corresponding to full-length Fbg-L and FN-L domains as well as FN-L subdomains 6–8, A–D, A1A2, and D. The ability of these domains or subdomains to interact with newborn SMCs was determined by an adhesion assay. The level of adhesion to the intact tenascin-C molecule was high compared with the fibronectin (Fig. 5). Newborn cells adhered to the Fbg-L domain, and although the level of adhesion was slightly lower, it was comparable to the intact tenascin-C (Fig. 5). In contrast, SMCs did not adhere either to the entire FN-L domain or its recombinant subdomains (Fig. 5). As with the intact tenascin-C, the Fbg-L-adherent cells remained round and did not spread (not shown). Similar results were obtained with adult SMCs, although the level of adhesion to the Fbg-L domain was markedly lower (not shown).

The inability of FN-L domain or subdomains to promote cell adhesion could be explained by reduced capacity of the FN-L repeats to coat tissue culture dishes, by the loss of function as a result of binding to tissue culture dishes, or by the lack of activity of the FN-L domains. To distinguish between these possibilities, the recombinant FN-L proteins were labeled with 125I, and their ability to coat the tissue culture dishes was compared with the Fbg-L domain. We found no difference in the coating efficiency of these recombinant proteins (not shown). To determine whether binding to tissue culture dishes influenced the activity of the recombinant proteins, we examined the ability of soluble Fbg-L or FN-L domains and subdomains to inhibit the interaction between intact tenascin-C and SMCs. The soluble FN-L domain or subdomains were inactive, whereas 200 µg/ml soluble Fbg-L reduced adhesion of SMCs to tenascin-C by 70% (Fig. 6). The soluble recombinant Fbg-L had no effect on the adhesion of SMCs to either intact fibronectin or its recombinant fragment (Fig. 6). Further, adhesion of SMCs to the Fbg-L domain was controlled by the same factors that regulated adhesion of cells to tenascin-C, i.e., adhesion was completely inhibited by EDTA and promoted by Mg$^{2+}$ cation but not Ca$^{2+}$ (not shown). Taken together, these data led us to conclude that the Fbg-L domain, but not FN-L domain, mediates adhesion of SMCs to tenascin-C.

**Mapping of the Active Site of Fbg-L Domain**—To map the active site of the Fbg-L domain, we designed nine synthetic peptides, each containing a sequence of 30 amino acids, which together constituted the entire isolated Fbg-L domain. Some degree of overlap was included to avoid the possibility of splitting the active site and thereby losing the activity (Fig. 7).
The ability of the synthetic peptides to directly interact with newborn cells were determined by an adhesion assay. Tissue culture plates were coated with increasing concentrations of peptides from 0.1 to 10 μg/ml, and adhesion of newborn cells was measured. Cell adhesion was promoted as peptide concentration was increased from 0.1 to 1 μg/ml (not shown). Adhesion reached saturation levels at 1 μg/ml, and no significant change was observed beyond 10 μg/ml; therefore, tissue culture plates were coated with a solution of 10 μg/ml of peptides in all subsequent experiments. As shown in Fig. 8A, peptide VIII was the only peptide capable of strongly promoting newborn cell adhesion when coated on the tissue culture dishes, and it accounted for most (80%) of the adhesion activity of the Fbg-L domain. Peptide IX exhibited 30% activity, and other peptides were inactive. As with the interaction with intact tenascin-C or the isolated Fbg-L domain, peptide VIII-adherent cells remained round. Further, as with the whole Fbg-L domain, adhesion to peptide VIII was blocked by EDTA and promoted by Mg²⁺ (not shown).

The inability of other peptides to promote SMC adhesion suggests that peptide VIII was the only peptide that matched the active site of the Fbg-L domain. However, we cannot rule out the possibility that either other peptides did not bind to tissue culture dishes or that they lost their function as a result of binding to tissue culture dishes. We found no difference in the ability of peptides to coat tissue culture dishes (not shown). To determine whether peptides lose their function as a result of binding to tissue culture dishes, an adhesion assay was performed in the presence of soluble peptides. As shown in Fig. 8B, in addition to peptide VIII, soluble peptides III and VI completely blocked adhesion of newborn cells to either intact tenascin-C or the recombinant fibrinogen-like domain. Other peptides were either partially active (IX) or were completely inactive (I, IV, V). This suggests that peptide III or VI may lose their function as a result of conformational changes induced after binding to tissue culture dishes. If true, these peptides may represent a sensitive active site. It is thus unclear whether these sites remain active when soluble tenascin-C is incorporated into the insoluble extracellular matrix substrate.

We next mapped the active domain of tenascin-C that is involved in cell migration by determining the ability of the
In addition, the type of cations increases SMCs adhesion, and maximal adhesion of SMCs on tenascin-C substrate, and most likely this occurs through the peptide VIII. The cation-dependent adhesion of SMCs to tenascin-C may mediate the interaction of tenascin-C with endothelial cells (9, 32). Thus, the presence of Ca$^{2+}$ cations in the adhesion buffer may negatively impact adhesion of some cell types to tenascin-C. Therefore, the concentration of cations and their type may at least partly explain the long controversy about cell-tenascin-C interaction.

Mg$^{2+}$-mediated modulation may be relevant to the remodeling of the injured arteries after balloon angioplasty. Under normal physiological conditions, the extracellular environment has a higher concentration of Ca$^{2+}$ than Mg$^{2+}$ (33). In contrast, the intracellular Mg$^{2+}$ concentration in a typical mammalian cell is between 15 and 30 mM, whereas intracellular Ca$^{2+}$ is only about 1–2 μM (34–36). After balloon angioplasty, it is possible that a local increase in extracellular Mg$^{2+}$ levels might occur as the damaged tissue releases its cellular content. It is conceivable that such an increase in the extracellular Mg$^{2+}$ gradient, set up locally from the site of injury, along with growth factors released by the platelets at the injured site, could stimulate tenasin-C-SMC interaction possibly through an integrin receptor. This would then provide the stimulus and directional signaling necessary to mobilize SMCs.

We have identified the factors that control adhesion of SMCs to tenasin-C and mapped the active domain as well as the active site of the tenasin-C molecule. Based on these data, we propose that the adhesion of SMCs to tenasin-C is most likely mediated by integrin, which is different from other cell types. It is different from fibroblasts because heparin sulfate proteoglycans mediate adhesion of fibroblasts to tenasin-C (10). It is also different from endothelial cells, because adhesion of endothelial cells to tenasin-C is completely blocked by RGD peptide (9). In contrast, the adhesion of SMCs to tenasin-C is only partially (30%) blocked by RGD peptide. This suggests that at least two receptors, one of them RGD-dependent and the other RGD-independent, mediate the adhesion of SMCs to tenasin-C. Several lines of evidence suggest that these two receptors are likely integrins, as the adhesion was 1) completely blocked by EDTA, 2) promoted by Mg$^{2+}$ cation, but not Ca$^{2+}$, and 3) completely blocked by the soluble Fbg-L domain or peptide VIII.

The cation-dependent adhesion of SMCs to tenasin-C may address some of the controversial issues related to the adhesion of cells to tenasin-C. Previous studies reported no cell adhesion to tenasin-C-coated culture dishes (23–26), whereas other reports showed a weak adhesion of cells (16, 27–31). In these reports, adhesion assays were performed with a buffer containing both Ca$^{2+}$ and Mg$^{2+}$. We observed that increasing Mg$^{2+}$ concentrations increased SMC adhesion, and maximal adhesion was achieved at 5–10 mM. In addition, the type of cations profoundly affects cell-integrin interaction, and in some cases, it is a deciding factor whether there is any adhesion at all. For example, the binding activity of αβ1 integrins is promoted by Mg$^{2+}$ cations, and Ca$^{2+}$ reverses the effect of Mg$^{2+}$ (19). This is particularly relevant to adhesion to tenasin-C, as it has been suggested that αβ1 mediates the interaction of tenasin-C with endothelial cells (9, 32). Thus, the presence of Ca$^{2+}$ in the adhesion buffer may negatively impact adhesion of some cell types to tenasin-C. Therefore, the concentration of cations and their type may at least partly explain the long controversy about cell-tenasin-C interaction.
39). We found, however, that neither the FN-L domain or its subdomains, including the full-length alternatively spliced region, had neither adhesive- nor migration-promoting activities for detached SMCs and that the Fbg-L domain can account for nearly all of the activities. However, we cannot exclude the possibility that adherent SMCs may need the alternatively spliced region for cell detachment, because our migration assay was performed with detached SMCs. It is thus conceivable that the alternatively spliced region is needed to down-regulate focal adhesion points of adherent SMCs and to promote cell detachment. Once detached, however, the Fbg-L domain alone may be sufficient to maintain cell movement.

Tenascin C is largely expressed during embryonic development, but it is down-regulated in adult tissue (8). We found differences in the ability of newborn and adult SMCs to adhere to tenascin-C, which was consistent throughout multiple isolates and passages of adult and newborn cells. These data suggest that the newborn cells have a greater number of stable cell surface receptors for tenascin-C. In many circumstances, the basic cellular mechanisms that originally were used during embryonic development may be reactivated under pathological conditions. We have reported that formation of neointima during wound healing in the balloon-injured adult rat carotid artery is dependent on reexpression of developmentally regulated gene(s), and the reactivation of these genes may be responsible for the formation of neointima (40–44). The differential ability of the newborn and adult SMCs to adhere to tenascin-C suggests that the reexpression of a developmentally regulated gene like tenascin-C provides a suitable substratum for the subpopulation of aortic SMCs to migrate and form neointima.

In summary, we have characterized the parameters that determine the interaction between SMCs and tenascin-C. We have shown that the Fbg-L domain, but not the FN-L domain, is involved in SMC adhesion and migration. We further mapped the active site of the Fbg-L domain to a 30-amino acid peptide, peptide VIII, which is located near the carboxyl-terminal part of the domain. Based on these data, we hypothesize that the interaction between SMCs and the Fbg-L domain of tenascin-C is essential for cell adhesion and migration, and blocking this interaction may blunt SMC migration from media and ultimately affect neointimal formation.

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