Antibody Blockade of Thrombospondin Accelerates Reendothelialization and Reduces Neointima Formation in Balloon-Injured Rat Carotid Artery

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Background—Remodeling of the extracellular matrix plays an important role during the pathogenesis of atherosclerosis and restenosis. The matrix glycoprotein thrombospondin-1 (TSP1) inhibits endothelial cell proliferation and migration in vitro. In contrast, TSP1 facilitates the growth and migration of cultured vascular smooth muscle cells. Accordingly, we investigated the hypothesis that administration of anti-TSP1 antibody could facilitate reendothelialization and inhibit neointimal thickening in balloon-injured rat carotid artery.

Methods and Results—Sprague-Dawley rats were subjected to left common carotid artery denudation, after which arteries were treated with C6.7 anti-TSP1 or control antibody. Evans blue dye staining 2 weeks after injury disclosed significantly increased reendothelialization in arteries treated with C6.7 antibody compared with the control group, and this effect was associated with increased number of proliferating cell nuclear antigen–positive endothelial cells. In contrast, treatment with C6.7 antibody decreased the number of proliferating cell nuclear antigen–positive vascular smooth muscle cells in the injured arterial wall. Neointimal thickening was correspondingly attenuated to a statistically significant degree in arteries receiving C6.7 antibody versus the control group at both the 2-week and 4-week time points.

Conclusions—Intra-arterial delivery of antibody against TSP1 facilitated reendothelialization and reduced neointimal lesion formation after balloon denudation. (Circulation. 1999;100:849-854.)

Key Words: antibodies ▪ angioplasty ▪ endothelium ▪ carotid arteries ▪ muscle, smooth

Endothelial dysfunction triggers a cascade of events that contribute to the pathogenesis of atherosclerosis and restenosis, including platelet activation and aggregation, vascular smooth muscle cell (VSMC) proliferation and migration, and deposition of extracellular matrix (ECM) components into the vessel wall. It has become increasingly clear that multiple cytokines, in conjunction with the ECM and integrins, orchestrate vascular remodeling in response to arterial injury.

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The vascular ECM is a complex of different macromolecules organized into a highly ordered architectural framework that provides the structural supporting element for the vascular cells and surrounding tissues. ECM components also participate in the regulation of other highly specialized cellular functions triggered by growth factors and cytokines, including cell adhesion, migration, proliferation, and differentiation. The matrix protein thrombospondin-1 (TSP1) is synthesized and secreted by activated platelets and a variety of cell types including endothelial cells (ECs), macrophages, fibroblasts, and VSMCs. TSP1 is a 450-kDa homotrimeric glycoprotein that interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of functions. Cell culture experiments have demonstrated the ability of TSP1 to induce arrest of EC growth. The spontaneous development of angiogenic tube-like structures is also inhibited by TSP1 both in vitro and in vivo. In marked contrast, TSP1 promotes VSMC proliferation and migration and plays a stimulatory role in platelet activation and aggregation. These findings suggest that TSP1 may play an important role in the pathogenesis of atherosclerosis and restenosis. Consistent with this notion, TSP1 expression has been associated with atherosclerotic lesions, acute vascular injury, hypercholesterolemia, and hypertension. In the present study, monoclonal neutralizing anti-TSP1 antibody was locally delivered to the arterial wall after balloon angioplasty in the rat carotid artery.
artery to evaluate in vivo the role of TSP1 on vascular repair after acute injury. We show that local administration of anti-TSP1 antibody expedited reendothelialization and reduced neointimal thickening.

Methods

Balloon Angioplasty, Antibody Delivery, and Evaluation of Reendothelialization and Neointimal Thickening

Balloon angioplasty in the left common carotid artery of male Sprague-Dawley rats was performed essentially as described by Clowes et al.\(^1\) Immediately after angioplasty, protein A–purified mouse monoclonal anti-TSP1 antibody C6.7\(^2\) or control nonspecific IgG antibody MOPC-21 (Sigma Chemical) was delivered intra-arterially by use of the dwell technique (100 \(\mu\)g of IgG in 100 \(\mu\)L of sterile 0.1% BSA/PBS; 30 minutes). This was followed by 1 week of continuous periarterial delivery by osmotic pump (Alzet, model 2 ML1, Alza) as described previously.\(^3\),\(^4\) In brief, the common carotid artery was further isolated and dissected free of fat and connective tissue and a superficial longitudinal incision into the adventitia was made with a modified coronary artery surgical blade to ensure penetration of the antibody to the external muscular layer of the media. A sterile microcatheter was placed adjacent to the injured portion of the artery and secured by suturing it directly to the adjacent musculature. The proximal end of the microcatheter was heat-sealed, and longitudinal perforations were made on the catheter at the site adjacent to the injured arterial segment. The distal end of the microcatheter was passed through the lateral neck and connected to the osmotic pump. The pump was filled with C6.7 or MOPC-21 control antibody (400 \(\mu\)g IgG in a final volume of 2 mL of sterile PBS). The wounds were cleaned with saline and wiped dry with a sterile cotton swab. Fascia surrounding the artery was sutured closed. The pump was placed and sutured in a pocket made in the back of the rat. One week after infusion at 10 \(\mu\)L/h, the pump and the microcatheter were removed from the rats under anesthesia.

The study comprised 13 arteries treated with C6.7 antibody (n = 7, 2 weeks; n = 6, 4 weeks) and 18 arteries treated with control antibody (n = 9, 2 weeks; n = 9, 4 weeks). Two carotid arteries from the control group (1 at each time point) developed thrombosis after balloon injury and were therefore discarded. All vessels treated with C6.7 antibody were patent at the time the animals were killed. Animals received an intravenous injection of 1 mL 0.5% Evans blue 30 minutes before they were killed to identify nonendothelialized areas. The extent of reendothelialization and neointimal thickening were evaluated by planimetric analysis as previously described.\(^5\),\(^6\)

To assess antibody delivery into the arterial wall, animals were killed 3 days after angioplasty. Methanol-fixed arteries were sectioned longitudinally for immunohistochemistry with the use of rat-adsorbed, biotinylated horse anti-mouse antibody (Vector Laboratories) and a biotin/streptavidin-horseradish peroxidase detection system (Signet Laboratories). A polyclonal horse anti-goat antibody was used for negative control.

Antibody Bioassay

VSMC migration was assessed with a modified Boyden chamber (Neuroprobe) and platelet-derived growth factor (PDGF) BB (10 ng/mL) as the chemoattractant.\(^7\) Rat aortic VSMCs were isolated as previously described,\(^8\) seeded in the upper compartment (2.5 \(\times\) 10\(^4\) cells in 50 \(\mu\)L of 1% FBS/DMEM), and incubated for 30 minutes in the absence or in the presence of C6.7 antibody. After 5 hours of incubation, migration was quantified by counting the number of cells on the lower side of the filter from 3 randomly chosen high-power (>400) fields.

Expression Studies and Assessment of Proliferative Activity After Balloon Injury

Arteries were harvested and adventitia and connective tissues were removed as cleanly as possible. Preparation of arterial extracts and Western blot analysis were carried out as previously described.\(^9\) Blots were probed with rabbit polyclonal anti-TSP1 antibody (a gift from Dr Jack Lawler) and mouse monoclonal anti-tubulin antibody (Calbiochem). After incubation with horseradish-peroxidase– conjugated secondary antibodies, immunocomplexes were visualized with ECL reagent (Amersham). Proliferative activity in injured arteries was evaluated by immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) expression as previously described.\(^10\) ECs were identified by immunohistochemistry with the use of a mouse monoclonal anti-CD31 antibody (1:40 dilution in 1% BSA/PBS) (PharMingen).

Statistical Analysis

Results are expressed as mean±SEM. Differences in the number of migrating cells and PCNA-immunoreactive cells were evaluated by use of a 2-tailed, unpaired Student’s \(t\) test. Differences in the extent of reendothelialization and neointimal thickening were evaluated by use of ANOVA and Schefﬂe’s \(F\) post hoc test. Differences were considered significant at \(P<0.05\).

Results

Induction of TSP1 Expression After Balloon Angioplasty and Biological Activity of C6.7 Antibody

TSP1 protein expression in the rat carotid artery was greatly upregulated as early as 1 day after angioplasty, and its expression was maintained up to 2 weeks (Figure 1A). These findings are consistent with previous Northern blot and immunohistochemical analysis demonstrating a rapid induction of TSP1 in balloon-injured arteries.\(^11\)

We next evaluated the biological activity of neutralizing anti-TSP1 antibody C6.7. Treatment with C6.7 antibody inhibited in a dose-dependent manner PDGF BB–dependent migration of cultured VSMCs (Figure 1B). When C6.7 antibody solution containing 50 \(\mu\)g/mL IgG was incubated for 6 days at 37°C, its inhibitory effect on PDGF BB–mediated VSMC migration was comparable to that elicited by the same concentration of fresh antibody. Thus prolonged incubation of C6.7 antibody at body temperature did not appear to reduce its biological activity.

C6.7 Antibody Delivery Facilitates Reendothelialization After Balloon Angioplasty

To assess the role of TSP1 on the vascular response to injury, neutralizing C6.7 antibody was delivered intra-arterially by use of the dwell technique followed by continuous delivery with an osmotic pump. We first evaluated the presence of mouse monoclonal C6.7 antibody in the injured arterial wall 3 days after angioplasty. Longitudinal sections were incubated with horse anti-mouse IgG, which elicited immunoreactivity within the media and adventitia (Figure 1C, left), No signal was detected when horse anti-goat IgG was used as the secondary antibody (Figure 1C, right). These studies demonstrated the presence of mouse monoclonal C6.7 antibody in the injured arterial wall.

To evaluate the effect of C6.7 antibody on reendothelialization and neointimal thickening, rats were implanted with the osmotic pump delivery system for 1 week after balloon angioplasty. Animals received an intravenous injection of 1 mL 0.5% Evans blue dye before they were killed to evaluate the extent of reendothelialization (Figure 2A). Two weeks after angioplasty, C6.7-treated arteries disclosed a 60% in-
immunostaining was used to assess the effect of C6.7 antibody delivery on cellular proliferation in balloon-injured arteries. Two weeks after angioplasty, C6.7-treated arteries disclosed a higher number of PCNA-positive cells at the luminal surface (1.57\(\pm\)0.26 mm\(^{-1}\)) in the control group vs 3.09\(\pm\)0.49 mm\(^{-1}\) in the C6.7 group, P<0.05 (Figure 2C). As expected, the number of PCNA-positive cells at 4 weeks was reduced in the neointima of both control and C6.7-treated arteries (data not shown).

Collectively, the above results suggest that administration of anti-TSP1 antibody inhibited VSMC hyperplasia in vivo. The intima-to-media (I/M) ratio was correspondingly reduced by \(\approx 63\%\) in the C6.7 group at both the 2- and 4-week time points (1.33\(\pm\)0.04 in the control group vs 0.83\(\pm\)0.05 in the C6.7 group at 2 weeks, P<0.0001; 1.57\(\pm\)0.07 in the control group vs 0.94\(\pm\)0.04 in the C6.7 group at 4 weeks, P<0.0001) (Figure 2B). Of note, although control arteries showed a statistically significant higher I/M ratio at 4 weeks as compared with the 2-week time point (P=0.03), neointimal thickening in the C6.7-treated arteries remained nearly unchanged during the same time interval.

**Discussion**

The expression pattern of TSP1 is consistent for a role of this matrix protein on the pathogenesis of atherosclerosis and restenosis\(^{43,45–47}\) (this study). Previous in vitro studies also support this notion. For example, TSP1 inhibits migration and proliferation of cultured ECs,\(^{21,22}\) and several studies have suggested that this might translate into inhibition of angiogenesis in vivo.\(^{21,24,45–47}\) The extent to which these observations may be extrapolated to reendothelialization after balloon injury, however, has never been tested. Given the ability of TSP1 to promote VSMC proliferation and migration in vitro, TSP1 would potentially represent a unique matrix protein with dual effects on ECs and VSMCs that might both delay EC regeneration and facilitate neointimal lesion formation after balloon injury. The present study demonstrates that administration of the anti-TSP1 antibody C6.7 after balloon angioplasty in the rat carotid artery expedited reendothelialization and reduced neointimal thickening, and these effects were associated with increased EC proliferation and reduced VSMC growth.

Recent studies have demonstrated direct effects of TSP1 on ECs that are consistent with our observations. For example, addition of TSP1 to ECs that had already formed...
stable focal adhesions on a fibronectin substrate stimulated focal adhesion disassembly.48 Moreover, TSP1 inhibited angiogenesis both in vitro and in vivo,23–25 and some observations suggested that TSP1 might act as a physiological inhibitor of angiogenesis.24,45–47 The ability of C6.7 antibody to inhibit neointimal thickening after balloon angioplasty is also in agreement with previous in vitro studies demonstrating a direct role of TSP1 in promoting VSMC growth and migration.26,27,49–51

In addition to the direct effect of TSP1 on VSMC proliferation, it is noteworthy to point out that several studies in the rat carotid model of vascular injury have clearly established an inverse relation between endothelial integrity and VSMC proliferation.52–55 Moreover, application of various EC mitogens that accelerated reendothelialization attenuated neointimal lesion formation.38,56,57 Thus expeditious reendothelialization in C6.7-treated arteries probably contributed to reduced neointimal thickening.

In conclusion, this study demonstrates a favorable effect of anti-TSP1 antibody on both reendothelialization and neointimal hyperplasia after balloon angioplasty. Thus the results imply the potential utility of a novel treatment strategy in which inhibition of a matrix protein simultaneously promotes EC growth and reendothelialization and inhibits VSMC proliferation. This could be a powerful therapeutic strategy to inhibit neointimal thickening after balloon denudation.

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**References**


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