Thrombospondin-1 Mediates Smooth Muscle Cell Proliferation Induced by Interaction With Human Platelets

Takuya Ichii, Hidenori Koyama, Shinji Tanaka, Atsushi Shioi, Yasuhisa Okuno, Shuzo Otani, Yoshiki Nishizawa

Objectives—Platelet adherence and activation are associated with smooth muscle cell (SMC) proliferation and arterial restenosis. This study examined platelet-SMC interaction on fibrillar type I collagen and analyzed the role of thrombospondin (TSP)-1 in platelet-induced SMC proliferation.

Methods and Results—When SMCs cultured on fibrillar collagen were treated with human platelets (5 preparations), 7.45 ± 2.94% of the cells passed through S phase within 24 hours, as determined by bromodeoxyuridine nuclear labeling. The addition of platelets markedly induced SMC TSP-1 mRNA expression and cell surface protein accumulation, which colocalized with adhered platelets, as determined by αIIb integrin immunostaining. Direct interaction of platelets with SMCs was necessary for its effect on proliferation and TSP-1 accumulation, as determined in the transwell culture system. The anti–TSP-1 blocking antibody strongly inhibited platelet-induced SMC proliferation by ≈60%. Analysis of the receptors for TSP-1 accumulation on the SMC surface revealed that β1 integrins are mainly involved. The anti–β1 integrin blocking antibody, which potently suppressed TSP-1 accumulation on SMCs, also markedly inhibited platelet-stimulated SMC proliferation.

Conclusions—TSP-1 and β1 integrin interaction is involved in platelet-stimulated SMC proliferation. This in vitro coculture system could prove useful for examining the molecular mechanism underlying platelet-induced vascular remodeling and for studying the mechanism of a tested drug for restenosis. (Arterioscler Thromb Vasc Biol. 2002;22:1286-1292.)

Key Words: atherosclerosis • platelet-derived factors • integrins • collagen (type I) • vascular injury

The vascular endothelium plays a fundamental role in the local regulation of vascular tone and homeostasis.1 Injury of a vascular segment elicits a complex sequence of events characterized by platelet adhesion and aggregation, leukocyte infiltration, and smooth muscle cell (SMC) migration and proliferation.2,3 In experimental models, platelet aggregation and adhesion to the wall of injured vessels have been demonstrated in the early phase after balloon injury.4 Even though platelets disappeared from the arterial surface within a few days after injury, initial platelet adhesions appear to play important roles in intimal lesion formation.5 Platelet adherence and activation are associated with the local release of potent vasoactive factors, such as thrombin, platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, insulin-like growth factor-I, and thromboxane A2.6 These factors are known to stimulate the migration, proliferation, and synthesis of extracellular matrix molecules by SMCs that may lead to the development of an intimal lesion. However, the molecular mechanism behind the role of platelets in lesion formation is still not entirely clear.
promote neointimal formation after vascular injury. However, the role of TSP-1 in platelet-stimulated SMC proliferation has not been directly investigated.

We demonstrated that fibrillar type I collagen, in contrast to monomeric collagen, potently suppresses SMC proliferation stimulated by PDGF in vitro. Moreover, fibrillar collagen also strongly suppresses the expression of extracellular matrix molecules, including TSP-1, and induces many of the quiescent characteristics of arterial SMCs in normal media. By use of this system of culturing cells on fibrillar collagen, the present study was designed to model in vitro the local interaction of vascular SMCs with platelets and to explore the role of TSP-1 in this process. We show that SMC proliferation and TSP-1 accumulation on the SMC surface are potently stimulated by interaction with human platelets. Immunofluorescent microscopy and flow cytometry suggest direct interaction of SMCs with platelets, where TSP-1 is predominantly accumulated. Moreover, an anti–TSP-1 blocking antibody markedly inhibited platelet-induced SMC proliferation, suggesting an involvement of TSP-1 in SMC proliferation induced by direct interaction with platelets.

Methods

Reagents and Preparation of Human Platelets

For reagents and preparation of human platelets, please refer to the expanded Methods section in the online supplement (which can be accessed at http://atvb.ahajournals.org).

Human Arterial SMC Culture and Coculture With Platelets

Human SMCs were obtained and cultured as previously described. The cells were isolated from umbilical arteries and express SMC markers, including smooth muscle α-actin, calponin, and SM22α. SMCs were cultured on polymerized collagen fibrils as described. They were serum-deprived for 48 hours, trypsinized, and cultured on fibrillar collagen. In this condition, SMCs are completely arrested in the G1 phase of the cell cycle and are less responsive to PDGF and growth factors than are cells on monomeric collagen.

Moreover, this culture system mimics many of the characteristics of quiescent SMCs in media in vivo. After culture of SMCs on fibrillar collagen for 24 hours, platelets (100-fold the numbers of SMCs) were added, and SMC DNA synthesis was determined by BrdU nuclear labeling. As previously reported on fibrillar collagen, <3% of the cells went through S phase within 48 hours in the absence of platelets, whereas the addition of platelets dramatically induced SMC DNA synthesis under these culture conditions (Figure IA and 1B). This effect of platelets on SMC proliferation was observed as early as 12 hours, and ~10% of the cells passed through S phase within 24 hours (Figure 1B). The effects on SMC proliferation (24 hours) of independently prepared platelets from healthy subjects (n=5) were 7.45±2.94% (mean±SD, range 5.16% to 11.63%). To examine whether direct interaction of platelets with SMCs is necessary for this mitogenic effect, we used a transwell culture system in which direct cellular interaction is eliminated. When platelets were added to the upper chamber of the transwell coated with thin fibrillar collagen and SMCs were cultured in the bottom chamber on fibrillar collagen, platelet-stimulated proliferation of SMCs was barely observed (Figure 1C). Thus, direct interaction with SMCs may be required for mitogenic effects of platelets. As previously described, growth-regulatory factors that are released from platelets, including PDGF, TGF-β, and EGF, did not show potent mitogenic effects on fibrillar collagen (see Figure I, which can be accessed online at http://atvb.ahajournals.org).

TSP-1 and Platelets Predominantly Accumulate on the Surface of SMCs Cultured on Fibrillar Collagen

TSP-1 is secreted by platelets and by SMCs and is known as a mitogen and chemoattractant for SMCs. In the in vivo balloon-injury model in rat carotid arteries, a neutralizing antibody against TSP-1 effectively suppressed the neointimal formation. Thus, TSP-1 is a candidate for a molecule that links platelet-SMC interaction and mediates SMC phenotypic changes in injured arteries. We have recently shown that TSP-1 mRNA and protein expression are potently suppressed in SMCs cultured on fibrillar collagen compared with monomeric collagen and that PDGF is unable to induce TSP-1 mRNA on fibrillar collagen.

Twenty-four hours after the addition of platelets to the quiescent SMCs cultured on fibrillar collagen, much TSP-1 expression on the SMC surface. Some strong staining of TSP-1 was colocalized with αv integrin staining, implying that some, but not all, of the platelets colocalized with TSP-1. No staining of αv integrin was detected on SMCs, and TSP-1 staining was faint when platelets were not added (Figure 2A). Confocal microscopy revealed αv integrin staining on the surface of SMCs (data not shown). Thus, coculture with platelets increases TSP-1 expression on the SMC surface.

To quantify the changes in levels of TSP-1 on the surface of SMCs, flow cytometric analyses were performed. For this experiment, SMCs on fibrillar collagen were incubated with or without platelets for 24 hours, suspended by collagenase digestion, and extensively washed with PBS. The TSP-1

Flow Cytometry, Immunocytochemistry, Confocal Microscopy, BrdU Nuclear Labeling, and Northern Blotting

For flow cytometry, immunocytochemistry, confocal microscopy, bromodeoxyuridine (BrdU) nuclear labeling, and Northern blotting, please refer to the online supplement (which can be accessed at http://atvb.ahajournals.org).

Results

Platelets Markedly Induce SMC Proliferation Cultured on Fibrillar Collagen

It is well known that in the injured artery, platelets rapidly accumulate on the injured surface, SMC proliferation is induced, and neointimal lesions develop. To model possible platelet-SMC interactions observed in arterial lesions, we have used a coculture system in which platelets were added to SMCs cultured on fibrillar collagen. We have shown that SMCs cultured on fibrillar collagen are arrested in the G1 phase of the cell cycle and are less responsive to PDGF and growth factors than are cells on monomeric collagen.

To quantify the changes in levels of TSP-1 on the surface of SMCs, flow cytometric analyses were performed. For this experiment, SMCs on fibrillar collagen were incubated with or without platelets for 24 hours, suspended by collagenase digestion, and extensively washed with PBS. The TSP-1
expressed on the cell surface was determined by flow cytometry. As shown in Figure 2B, the addition of platelets significantly increased the abundance of TSP-1 on SMCs. In this condition, >10% of platelet-treated SMCs were positive for α_{IIb} integrin, indicating the adhesion of platelets to SMCs (Figure 2C). Thus, direct platelet-SMC interaction was observed in parallel with the accumulation of TSP-1 on the SMC surface. When direct platelet interaction with SMCs was prevented in the transwell culture system, no significant increase in TSP-1 accumulation was observed on the SMC surface (Figure 2D). Thus, direct interaction between platelets and SMCs appears to be necessary for TSP-1 accumulation on SMCs. Furthermore, PDGF (10 ng/mL) and EGF (20 ng/mL) were not as effective as platelets in inducing TSP-1 accumulation in SMCs on fibrillar collagen, although TGF-β (10 ng/mL) increased SMC TSP-1 expression on the cell surface in this experimental system (see Figure II, which can be accessed online at http://atvb.ahajournals.org).

Not only did TSP-1 protein on SMCs increase on interaction with platelets, but TSP-1 mRNA expression in SMCs increased markedly (Figure 3). The addition of platelets rapidly increased TSP-1 mRNA abundance as early as 1 hour (512±76%, mean±SD), with its level maximal at 3 hours (624±86%), and the level gradually decreased up until 24 hours (220±52%). Thus, synthesis and secretion of TSP-1 from SMCs may at least partly contribute to the accumulation of TSP-1 on SMCs.

**TSP-1 Mediates SMC Proliferation Induced by Platelets**

To understand which type of receptor is involved in the accumulation of TSP-1 on the SMC surface after the addition of platelets, we examined the effect of blocking reagents on TSP-1 levels at the SMC surface as determined by flow cytometry (Figure 4). SMCs cultured on fibrillar collagen for 24 hours were treated with platelets in the presence of blocking reagents. SMCs were suspended by collagenase digestion, incubated with phycoerythrin-labeled anti-TSP-1 antibody, and analyzed by flow cytometry. As shown in Figure 4, cell surface accumulation of TSP-1 was signifi-
cantly suppressed by anti–α2, anti–α5, and anti–α6β1 integrins, as well as by CD47 antibodies, and was potently inhibited by anti–β1 integrin blocking antibody. Thus, β1 integrins complexed with α2 and α5 integrins appear to be major receptors for platelet-induced accumulation of TSP-1 on SMCs.

Finally, to investigate the involvement of TSP-1 in SMC proliferation induced by platelets, we next examined the effect of anti–TSP-1 blocking antibody. Anti–TSP-1 antibody (50 μg/mL), C6.7 monoclonal antibody, is known to suppress the C-terminal of TSP-112 and has been successfully used to inhibit SMC migration in vitro and balloon catheter–induced carotid neointimal formation in vivo.21 In our experimental system, anti–TSP-1 blocking antibody (50 μg/mL) effectively inhibited the accumulation of TSP-1 on SMCs after the addition of platelets (Figure 5A), even though it is less effective than anti–β1 integrin antibody. Compared with control mouse IgG, anti–TSP-1 blocking antibody suppressed platelet-stimulated SMC proliferation by ≈60% (Figure 5B). Disruption of the β1 integrin, one of the major receptors for platelet-induced TSP-1 accumulation, also potently inhibited platelet-stimulated SMC proliferation (Figure 5B). The β1 integrin expressed on the SMC surface mediates the cell proliferation induced by direct interaction with platelets. Anti–α5β1 integrin that was added only to platelets, in contrast to that added to SMCs alone, failed to inhibit SMC proliferation (Figure 5C). An anti–αIIbβ3 integrin antibody, a potent inhibitor for platelet aggregation, barely had any inhibitory effect against SMC proliferation (Figure 5B).

Discussion

The present study describes a unique in vitro coculture system with which to examine the interaction between platelets and SMCs, which could be used as a model for an injured artery in vivo. Our results show that TSP-1 accumulation at the SMC surface mediates the cell proliferation induced by direct interaction with platelets.

When SMCs are cultured on fibrillar type I collagen compared with cells on monomeric collagen, growth factor–stimulated proliferation is potently inhibited.22 SMC culture
on fibrillar collagen mimics many of the characteristics of arterial SMCs in vivo. Thus, by using a coculture system on fibrillar collagen rather than a regular 2D culture system, the interaction between platelets and SMCs could be examined in more physiological conditions.

After treatment with platelets, proliferation and TSP-1 mRNA induction were observed in SMCs cultured on fibrillar collagen. In our recent report, PDGF failed to induce TSP-1 expression in SMCs cultured on fibrillar collagen. In the present study, we showed that TSP-1 accumulation and SMC proliferation on fibrillar collagen was not markedly induced compared with untreated SMCs. TSP-1 mRNA abundance was determined by Northern blot analysis. A representative result of triplicate experiments is shown.

Figure 3. Platelets stimulate TSP-1 mRNA induction in SMCs. SMCs, which had been cultured on fibrillar collagen for 24 hours, were treated with human platelets on fibrillar collagen for the periods indicated. Total RNA was isolated, and TSP-1 mRNA abundance was determined by Northern blot analysis. A representative result of triplicate experiments is shown.

Our data strongly imply that TSP-1 mediates the effects of platelets on SMC proliferation. Strong TSP-1 immunostaining on the SMC surface colocalized with some platelet staining, and the TSP-1 blocking antibody potently suppressed SMC proliferation stimulated with platelets. Because anti–TSP-1 blocking antibody also significantly inhibits TSP-1 accumulation on the SMC surface treated with platelets, cell surface accumulated TSP-1 may mediate the SMC proliferation stimulated by human platelets. The expression kinetics of TSP-1 in carotid arteries after balloon injury is very rapid and is consistent with a role for this matrix molecule in the progression of atherosclerosis. We have also shown that TSP-1 mRNA induction is detected as early as 6 hours after balloon injury. The data of Raugi et al showed that prominent medial SMCs and large foci of TSP-1 immunostaining on the luminal surface of the vessel were present just 1 hour after deendothelialization. The rapid induction and accumulation of TSP-1 after injury are consistent with the notion that platelet adhesion is an important trigger for TSP-1 accumulation. In rat carotid arteries, anti–TSP-1 blocking antibody efficiently suppressed neointimal formation after balloon injury. In our coculture system, TSP-1 accumulation appears to mediate SMC proliferation after the adhesion of platelets. Given its ability to promote SMC proliferation and migration, TSP-1 may be a key mediator for SMC proliferation after arterial injury.

Figure 4. Effects of blocking reagents on platelet-induced accumulation of TSP-1 on the SMC surface. SMCs and platelets were cultured on fibrillar collagen for 24 hours in the presence of 20 μg/mL of various blocking antibodies. SMCs were suspended by collagenase digestion, extensively washed, stained with PE-conjugated anti–TSP-1 blocking antibody, and analyzed by flow cytometry. Each column shows the mean ± SD (n = 3) of percent inhibition by blocking reagents. The increase in TSP-1 staining stimulated with platelets in the presence of control IgG is expressed as 100%. *P < 0.05 vs control IgG, by ANOVA with multiple comparison (Scheffé type).
TSP-1 accumulation. In SMCs, β3 integrins appear to play roles in the interaction with TSP-1.\textsuperscript{14–20} The αβ3 integrin is also a platelet collagen receptor.\textsuperscript{32} In our present study, treatment of platelets alone with anti–β1 integrin antibody failed to inhibit platelet-stimulated SMC proliferation. Because pretreatment of the platelets with anti–β3 integrin antibody does not completely inhibit platelet adhesion to collagen and platelet activation, as determined by P-selectin expression (data not shown), the suppressive effect of the antibody on SMC proliferation appears mainly mediated through inhibiting β1 integrin on SMCs. Likewise, inhibiting platelet αmβ3 integrins (the fibrinogen receptors involved in platelet aggregation)\textsuperscript{35} barely had suppressive effects on platelet adhesion, platelet activation, and platelet-stimulated SMC proliferation.

Previous studies have found that TSP-1–stimulated SMCs or β3 integrin–expressing HEK cell proliferation is significantly inhibited by αβ3 integrin blocking reagents.\textsuperscript{34,35} Because the suppression of TSP-1–induced proliferation by the reagents was partial, receptors other than αβ3 integrin may also be involved in TSP-1–stimulated signals, leading to proliferation. Our data suggest that together with β3 integrin, β1 integrin also appears to be involved in TSP-1–stimulated cell proliferation. In the present study, anti–β1 integrin antibody was even more potent than anti–TSP-1 blocking antibody. Thus, our data do not eliminate the possibility that the disruption of β1 integrin may also block signals distinct from TSP-1, which is necessary for SMC proliferation.

Restenosis is currently the major limitation of percutaneous transluminal coronary angioplasty. There is only limited effective therapy available for restenosis. The role of platelets in the development of thrombosis and abrupt closure after percutaneous transluminal coronary angioplasty is well recognized. However, the effects of platelets in angioplasty extend well beyond the early phase.\textsuperscript{56} Although anti-platelet agents, such as αmβ3 integrin antagonists, have been reported to reduce target-vessel revascularization, major controversies exist.\textsuperscript{37} The present in vitro culture system could be useful in unveiling the molecular mechanism behind the role of platelets in restenosis and in studying the mechanism of a tested drug for restenosis.

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Materials and Methods

Reagents

We purchased reagents from the following companies: type I collagen (Vitrogen 100), Collagen Corporation (Fremont, CA); FITC- or PE-conjugated anti-human αIIbβ3 integrin, FITC-conjugated p-selectin, and PE-conjugated anti-human TSP-1, Immunotech (Marseille, France); FITC-conjugated anti-BrdU antibody, Becton Dickinson (San Jose, CA); neutralizing monoclonal antibody against human TSP-1 (C6.7), Lab Vision Corporation (Fremont, CA); neutralizing monoclonal antibody against human β1 integrin (P4C10), GIBCO BRL, Life Technologies Inc. (Rockville, MD), recombinant human PDGF-BB and human recombinant epidermal growth factor (EGF), Genzyme (Cambridge, MA); human TGF-β, Calbiochem-Novabiochem Corp (La Jolla, CA). Neutralizing monoclonal antibody against αIIbβ3 integrin (YM337) was kindly provided by Yamanouchi Pharmaceutical Company (Tokyo, Japan).

Preparation of human platelets

Aliquots (prepared for matching for transfusion) of pooled human platelets collected from healthy subjects were obtained from the blood transfusion center of Osaka City University Medical School, or freshly isolated from healthy volunteers as previously described. Generally, about 35% of pooled platelet aliquots and less than 15% of freshly isolated platelets were positive for p-selectin as determined by flow cytometric analyses. For either preparation, 100% of cells expressed p-selectin following culture on fibrillar collagen (data not shown) and the effects on SMC proliferation and TSP-1 expression were identical. Thus, platelet aliquots were used for
most of the experiments.

**Flow cytometry, immunocytochemistry and confocal microscopy**

Flow cytometric analysis, immunocytochemistry, and confocal microscopic analysis were performed as described previously \(^2\).

**BrdU nuclear labeling**

Cell proliferation was determined from the incorporation of BrdU into the nucleus. In brief, BrdU was added to SMCs simultaneously with platelets and cultured up until 48 h. Cells were fixed in 70% ethanol for 30 min at room temperature (RT), immersed in 0.07 M NaOH for 2 min, and washed in PBS. BrdU incorporated into the nucleus was immunostained with FITC-labeled anti-BrdU antibody.

**Northern blot analysis**

Northern blot analyses were done as described previously \(^2\). A cDNA fragment for human TSP-1 was cloned as a gene in SMCs suppressed on fibrillar collagen \(^2\).


Figure I: Effects of growth factors on SMC proliferation on fibrillar collagen. Quiescent human SMCs on fibrillar collagen were treated with human platelets (100-fold the numbers of SMCs), PDGF-BB (10 ng/ml), TGF-β (10 ng/ml), or EGF (20 ng/ml) together with 10 µM BrdU for 24 hours. Cell proliferation was determined as in Figure 1A. Each column represents mean SD of triplicate experiments. *; p<0.05 vs control, ANOVA with multiple comparison (Scheffe’s type).
Figure II: Effects of growth factors on TSP-1 expression in SMC on fibrillar collagen. Quiescent human SMCs on fibrillar collagen were treated with human platelets (100-fold the numbers of SMCs), PDGF-BB (10 ng/ml), TGF-β (10 ng/ml), or EGF (20 ng/ml) together with 10 µM BrdU for 24 hours. TSP-1 expression was determined by flow cytometry as described in Figure 2B. Control; black line, platelet; red line, PDGF; blue line, TGF-β; green line, EGF; yellow line. Upper range shows the range of negativity for TSP-1 staining.