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Vascular smooth muscle cell migration induced by domains of thrombospondin-1 is differentially regulated

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Abstract

BACKGROUND: Thrombospondin-1 (TSP-1) stimulates vascular smooth muscle cell (VSMC) migration via defined intracellular signaling pathways. The aim of this study was to examine the signaling pathways whereby TSP-1 folded domains (amino-terminal [NH_2], procollagen homology [PCH], all 3 type 1 repeats [3TSR], and a single recombinant protein containing the 3rd type 2 repeat, the type 3 repeats, and the carboxyl-terminal [E3T3C1]) induce VSMC migration.

METHODS: Quiescent VSMCs were pretreated with serum-free media or inhibitors: PP2 (c-Src), LY294002 (phosphatidylinositol 3-kinase), FPT (Ras), Y27632 (Rho kinase), SB202190 (p38 kinase), and PD98059 (extracellular signal-regulated kinase). Migration induced by serum-free media, TSP-1, NH_2, PCH, 3TSR, and E3T3C1 was assessed using a modified Boyden chamber.

RESULTS: TSP-1, NH_2, 3TSR, and E3T3C1 induced VSMC chemotaxis ($P < .05$), but PCH did not ($P > .05$). PP2, FPT, SB202190, and PD98059 attenuated chemotaxis stimulated by TSP-1, NH_2, 3TSR, and E3T3C1 ($P < .05$). LY294002 inhibited TSP-1-induced and E3T3C1-induced ($P < .05$) but not NH_2-induced or 3TSR-induced ($P > .05$) chemotaxis. Y27632 inhibited NH_2-induced, 3TSR-induced, and E3T3C1-induced ($P < .05$) but not TSP-1-induced ($P > .05$) induced chemotaxis.

CONCLUSIONS: TSP-1 folded domains are differentially dependent on intracellular signaling pathways to induce migration.

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Intimal hyperplasia is an exaggerated response to injury after arterial angioplasty. Arterial injury causes vascular smooth muscle cells (VSMC) to transform from a contractile to synthetic state, migrate to the intima, proliferate, and produce extracellular matrix proteins, narrowing the artery lumen.

Thrombospondin-1 (TSP-1), a "matricellular" glycoprotein, is upregulated after arterial injury. "Matricellular" refers to TSP-1's ability to bind cell-surface receptors, mediate cell-cell interactions, and bind proteins in the extracellular matrix. TSP-1 induces migration by way of the following intracellular signaling kinases: c-Src, phosphatidylinositol 3-kinase (PI3K), Ras, p38 kinase (p38), and extracellular signal-regulated kinase (ERK); interestingly, TSP-1-induced VSMC migration was not Rho dependent.[1–4]

Intact TSP-1 has 6 domains (amino-terminal [NH_2], procollagen homology [PCH], type 1 repeat, type 2 repeat, type

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3 repeat, and carboxyl-terminal [COOH]). It consists of 3 identical polypeptide chains attached by disulfide bonds that are between the NH_2 and PCH domain. TSP-1's domains have multiple receptor sites imparting different functions, including platelet aggregation and VSMC adhesion and migration. Our previous work using fusion proteins to specific TSP-1 domains demonstrated that NH_2, type 3 repeat, and COOH stimulated VSMC migration.[5]

In this study, we examined the ability of TSP-1 recombinant fragments (with domains to NH_2, type 1 repeat, type 2 repeat, type 3 repeat, and COOH) to induce VSMC migration. Additionally, we examined whether these folded domains used the same signaling pathways to induce VSMC migration as intact TSP-1. We hypothesized that the folded domains preferentially depend on these signaling pathways to induce VSMC migration.

Methods

Materials

Purified TSP-1, derived from human platelets, was obtained from Athens Research (Athens, GA). Recombinant folded TSP-1 domains (NH_2, PCH [domain not associated with VSMC chemotaxis], all 3 type 1 repeats [3TSR, domain not associated with VSMC chemotaxis], and a single recombinant protein containing the 3rd type 2 repeat, the type 3 repeats, and the COOH [E3T3C1]) were synthesized in *Drosophilia* S2 cells and purified as described previously.[6] Cell signaling inhibitors (obtained from EMD Chemicals, Gibbstown, NJ) used were PP2 (c-Src), LY294002 (PI3K), FPT (Ras), LY294002 (PI3K), SB202190 (p38), and PD98059 (ERK).

Cell culture

VSMCs from plaque-free human thoracic aortas were maintained at 37°C in human smooth muscle cell growth media (Cell Applications, Inc, San Diego, CA) with 1% penicillin/streptomycin/amphotericin (Lonza, Walkersville, MD). VSMCs at 80% confluence were made quiescent at passages 4 to 5. Specifically, VSMCs were cultured in serum-free media (SFM; 1× Dulbecco's modified Eagle's medium, 25 mmol/L glucose [Innovative Research, Novi, MI]) for 48 hours before experimentation. These VSMCs were then treated with the aforementioned inhibitors 20 hours (FPT) or 20 minutes (PP2, LY294002, Y27632, SB202190, and PD98059) before performing the chemotaxis assay.

Cell viability assay

Cell viability was tested with a trypan blue exclusion assay (Countess Cell Counter, Invitrogen, Carlsbad, CA).

Chemotaxis assay

VSMC migration was assessed using a modified Boyden microchemotaxis chamber, as previously described.[5] In the 1st set of experiments, chemotaxis of quiescent VSMCs were placed in the top wells of the chamber and chemoattractants (SFM or TSP-1, NH_2, PCH, 3TSR, and E3T3C1 [5, 10, 20, 40, or 80 μg/mL]) were placed in the lower wells. In the 2nd set of experiments, quiescent VSMCs were pretreated (20 minutes) with SFM, PP2 (10 μmol/L), LY294002 (25 μmol/L), Y27632 (2 μmol/L), SB202190 (10 μmol/L), or PD98059 (10 μmol/L) and migrated to the following chemoattractants: TSP-1, NH_2, 3TSR, and E3T3C1 (20 μg/mL) or SFM. FPT (10 μmol/L) cell pretreatments were for 20 hours before chemotaxis. VSMCs were counted under high power (400×) for 5 fields per well. Each experiment was performed in triplicate and repeated 3 times.

Statistical analysis

The number of migrated VSMCs was calculated for each experimental group and is reported as the mean ± SEM. Comparisons between 2 treatment groups were analyzed using unpaired Student's t tests, while those among several treatment groups were analyzed using analysis of variance. P values $< .05$ were considered significant.

Results

Cell viability

Viability of SFM and acutely treated VSMCs with the inhibitors was 95 ± 2%. Viable cells averaged 22 ± 3 μm in size. Cells pretreated with FPT were 95 ± 2% viable and averaged 22 ± 2 μm in size.

Intact TSP-1-induced and domain-induced chemotaxis

Intact TSP-1, NH_2, 3TSR, and E3T3C1 induced VSMC chemotaxis in a concentration-dependent manner compared to negative control (SFM). PCH did not induce VSMC chemotaxis at any concentration (Table 1).

Inhibitor effects on intact TSP-1-induced and folded domain-induced chemotaxis

Results are expressed as percentage migration compared to the positive control (respective folded domain). For TSP-1, PP2 (62 ± 1%), LY294002 (51 ± 4%), FPT (55 ± 5%), SB202190 (21 ± 3%), and PD98059 (24 ± 2%) inhibited TSP-1-induced VSMC chemotaxis ($P < .05$ for all inhibitors); however, Y27632 (95 ± 3%) did not affect chemotaxis ($P = .19$).