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

Submission Type: Original Contribution
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 \( \alpha \)IIb\( \beta \)3 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 \( \beta \)1 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-\( \beta \), Calbiochem-Novabiochem Corp (La Jolla, CA). Neutralizing monoclonal antibody against \( \alpha \)IIb\( \beta \)3 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 1. 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.
Figure 1: Bar graph showing the percentage of BrdU labeled cells for different treatments. The x-axis represents different treatments: control, platelet, PDGF, TGF-β, and EGF. The y-axis represents the percentage of BrdU labeled cells. The platelet treatment shows a significantly higher percentage of labeled cells compared to the control and other treatments (* indicates statistical significance).
TSP-1 fluorescent intensity