Thrombospondin (TSP1) mediates in vitro proliferation of human MG-63 osteoblastic cells induced by α-thrombin

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Thrombospondin (TSP) is a 450-kDa glycoprotein synthesized and secreted by human MG-63 osteoblastic cells. In this study, we have first studied the effect of α-thrombin on TSP expression by human MG-63 cells. In situ hybridization indicated that TSP mRNA level in thrombin-treated MG-63 cells was increased when compared to unstimulated cells. As judged by immunofluorescence, thrombin-treatment of MG-63 cells resulted in increased cell surface expression of TSP when compared to quiescent cells. Because thrombin stimulates proliferation of osteoblastic cells, the involvement of TSP in proliferation of thrombin-stimulated osteoblastic cells was then investigated using a serum-free mitogenesis assay. Both α-thrombin (0.01 to 0.15 U/ml) and TSP (5 to 600 ng/ml) caused a dose-dependent increase in [^{3}H]thymidine incorporation by MG-63 cells. Proliferation of osteoblastic cells induced by α-thrombin or TSP was specifically and totally inhibited by anti-TSP monoclonal antibodies (3–10 μg/ml) or by indomethacin (1 μM), an inhibitor of prostaglandin synthesis. Anti-TSP antibodies which inhibited cell proliferation also inhibit TSP expression to the surface of these cells. Our experiments support the existence of a mechanism whereby TSP bound to the cell surface of thrombin-treated MG-63 cells stimulates secretion of prostaglandins which, in turn, allow cell proliferation to proceed.

Thrombospondin; Thrombin; Proliferation; Osteoblastic cell

1. INTRODUCTION

Thrombospondin (TSP) is a trimeric extracellular matrix glycoprotein of 450 kDa synthesized and secreted by a wide range of normal and transformed cells [1]. Recently, five distinct genes encoding for four structurally different TSPs (TSP1, TSP2, TSP3, and TSP4), and cartilage oligomeric matrix protein (COMP) have been described [2]. The functions of TSP2, TSP3, TSP4, and COMP are unknown. TSP1 has been described as a protein that modulates cellular proliferation. In situ, there is a precise regional and temporal appearance of TSP during organogenesis of mouse embryo, followed by a disappearance as differentiation proceeds [3]. In vitro, quiescent smooth muscle cells stimulated with platelet-derived growth factor (PDGF) rapidly secrete and elaborate a TSP-rich extracellular matrix which, in turn, facilitate growth of smooth muscle cells in response to EGF [4,5]. TSP also promotes proliferation of fibroblasts [6].

We have previously reported that human MG-63 and Saos-2 osteoblastic cells in culture synthesize and secrete TSP [7]. In addition, TSP has been identified in the mineralized bone matrix of neonatal and young (growing) bone of many animal species [8]. However, the significance of TSP in bone tissue is not known. The serine protease thrombin, best known for its central role in hemostasis, is emerging as a potent activator of both cell adhesion and proliferation [9], and has been shown to stimulate proliferation of human G292 and Saos-2 osteoblastic cells [10,11]. These findings, taken together with the fact that TSP stimulates the growth of fibroblasts and smooth muscle cells [4–6], prompted us to examine whether it is involved in the proliferation of human osteoblastic cells induced by thrombin.

2. MATERIALS AND METHODS

EcoRI, [^{3}H]thymidine and [α-^{35}S]UTP were purchased from Amersham Proteinase K was from Boehringer. Human α-thrombin was from Fibrindex (Ortho Diagnostic Systems). PPACK was purchased from Calbiochem. Human platelet TSP1 was obtained from Stago (France) or was purified as previously described [12]. Mouse monoclonal antibodies P10 and MA-II are directed at distinct epitopes of human blood platelet TSP1. The characterization and specificity of these antibodies have been described earlier [13,14]. Mouse monoclonal antibody MARK 1 directed against rat κ light chains was purchased from Immunotech (France).

Human osteosarcoma cell line designated MG 63 was obtained from the American Type Culture Collection (Rockville, MD). MG 63 cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum.

Human cDNA clone M9 specific for TSP1 was kindly provided by Dr. Jack Lawler (Boston, MA) [15]. The cDNA was inserted into the EcoRI site of pGEM-2 (Promega Biotec) and used to derived antisense and sense probes. RNA probes were generated using 100 μCi [α-^{35}S]UTP, and either SP6 or T7 polymerases as previously described

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Abbreviations: TSP, thrombospondin; BSA, bovine serum albumin; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethylketone; PBS, phosphate-buffered saline

[16]. For in situ hybridization, cells resuspended in RPMI medium containing 10% (v/v) fetal calf serum were plated at a density of 3,000 cells per cm^2 in glass chamber slides (Lab Tec) and allowed to attach overnight in a 5% CO_2 incubator at 37°C. After incubation, cells were washed and cultured in RPMI medium containing 1% (m/v) BSA for 24 h. Cells were then stimulated with α-thrombin (0 1 U/ml) for 48 h. After washing, cells were fixed in 2% (m/v) paraformaldehyde (in PBS) for 15 min, washed twice in PBS, dehydrated by immersion in ethyl alcohol, and air-dried. Cells were then pretreated for 5 min with PBS containing 5 mM $MgCl_2$, then incubated for 8 min with 5 μg/ml proteinase K (in PBS), rinsed twice in PBS and dehydrated in ethyl alcohol. Hybridization with the RNA probes, and the subsequent steps of the procedure have been detailed previously [16]

For cell proliferation, MG-63 cells resuspended in RPMI medium containing 10% (v/v) fetal calf serum were plated at a density of 13,000 cells/cm^2 in 96-well flat-bottom culture dishes. After 24 h incubation in a 5% CO_2 incubator at 37°C, cells were washed and cultured in RPMI medium containing 1% (m/v) BSA to made cells quiescent After 24 h incubation, quiescent cells were stimulated for 48 h with increasing concentrations of α-thrombin (0 01 to 0 15 U/ml) in the presence or absence of PPACK (1 μM). Alternatively, increasing concentrations of TSP (5 to 600 ng/ml) instead of α-thrombin were added to quiescent MG-63 cells. Inhibition studies were conducted in the presence of α-thrombin (0.1 U/ml) and increasing concentrations (3 to 10 μg/ml) of either anti-TSP mouse monoclonal antibody P10 or negative control mouse monoclonal antibody MARK 1. During the last 12 h of the 48-h incubation period, cells were pulsed with 1 μCi of [^{3}H]thymidine/well. At the end of the incubation, cells were washed three times with PBS and harvested after brief exposure to trypsin (0.5 mg/ml) and EDTA (0.5 mM). Trypsin/EDTA-treated cells were then resuspended and counted. Results obtained for each concentration of thrombin, TSP or antibody were the mean ± S.D. of quadriplicate determinations.

For immunofluorescence, cells resuspended in RPMI medium containing 10% (v/v) fetal calf serum were plated at a density of 3,000 cells per cm^2 in glass chamber slides, and allowed to attach and spread for 24 h in a 5% CO_2 incubator at 37°C. After incubation, cells were washed and cultured in RPMI medium containing 1% (m/v) BSA for 24 h. Quiescent cultured cells were left untreated or were exposed to α-thrombin (0.1 U/ml) in the presence of either anti-TSP antibody P10 or negative control antibody MARK-1 (5 μg/ml). After 48 h incubation at 37°C, cells were washed in PBS and fixed for 1 h at 4°C in 4% formaldehyde. After washing, cells were exposed to a rabbit polyclonal anti-TSP antibody (0.1 μg/ml) for overnight at 4°C. FITC-labeled secondary antibody (goat anti-rabbit antibody) was used at a 1/50 dilution and was incubated for 90 min at room temperature Cells were photographed on a Zeiss Axioplan microscope

3. RESULTS

3.1. In situ hybridization

With the specific antisense probe, there was high-intensity hybridization over quiescent MG-63 osteoblastic cells, while only minimal hybridization was observed with the sense probe (Fig. 1A,B). Stimulation of MG-63 cells with α-thrombin (0.1 U/ml) caused a drastic increase in TSP1 mRNA expression (as judged by grain densities) (Fig. 1C) when compared to unstimulated cells (Fig. 1A). Nonspecific hybridization for thrombin-stimulated cells (Fig. 1D) was to a level similar to that observed for their unstimulated counterparts (Fig. 1B).

3.2. Immunofluorescence

Untreated and α-thrombin-treated MG-63 cells incu-

bated with anti-TSP monoclonal antibody P10 or negative control monoclonal antibody MARK-1 were examined by immunofluorescence after formaldehyde fixation and staining with an anti-TSP polyclonal antibody. Under these conditions, cell membranes are not affected and the staining reflects only cell surface expression of TSP. As shown in Fig. 2, immunostaining of quiescent MG-63 cells revealed a uniform distribution of TSP over the cell surface. After α-thrombin-treatment in the presence of antibody MARK-1, cells became round and a strong cell surface staining was observed for TSP (Fig. 2). By contrast, the use of anti-TSP antibody P10 instead of antibody MARK-1, markedly decreased cell surface expression of TSP (Fig. 2). The use of α-thrombin in the presence of PPACK reduced cell surface expression of TSP to a level similar to that found with quiescent MG-63 cells. No immunofluorescence was observed when a non-immune antibody was used instead of the anti-TSP polyclonal antibody.

3.3. Cell proliferation

It has been recently shown that thrombin stimulates proliferation of human G292 and Saos-2 osteoblastic cells [10,11]. Experiments conducted with human MG-63 osteoblastic cells showed that α-thrombin caused a significant, dose-dependent increase in [^{3}H]thymidine incorporation by these cells, reaching a plateau at concentrations ranged from 0.1 to 0.15 U/ml (Fig. 3A). The effect of α-thrombin on MG-63 cells was abolished in the presence of PPACK (1 μM), a synthetic peptide which specifically inhibits thrombin activity. Increasing concentrations of TSP ranged from 5 to 600 ng/ml also caused a significant, dose-dependent increase in [^{3}H]thymidine incorporation by MG-63 cells when compared to untreated cells (Fig. 3B). The maximum effect was seen at a TSP concentration of 500 ng/ml. Addition of anti-TSP antibody P10 (5 μg/ml) to TSP (500 ng/ml) markedly reduced [^{3}H]thymidine incorporation from 760 ± 129% to 109 ± 21% of untreated cells.

Because α-thrombin stimulates TSP expression and proliferation of human MG-63 osteoblastic cells, MG-63 cells were therefore stimulated wit α-thrombin (0.1 U/ml) in the presence of increasing concentrations (3 and 10 μg/ml) of either anti-TSP antibody P10 or negative control antibody MARK-1 (Fig. 4). MARK 1 only exhibited slight inhibition of [^{3}H]thymidine incorporation by thrombin-stimulated cells (Fig. 4B,C). By contrast, P10 drastically reduced [^{3}H]thymidine incorporation by thrombin-stimulated cells from 777 ± 14% to 51 ± 1% of the control (Fig. 4D,E). Similar results were obtained with anti-TSP monoclonal antibody MA-II. Reduction in [^{3}H]thymidine incorporation by MG-63 cells observed with P10 and MA-II did not result of cell detachment by these antibodies, since direct cell counting failed to show any significant loss in cell number as a result of antibody treatment. The effect of indomethacin (a specific cyclooxygenase inhibitor) on prolifera-
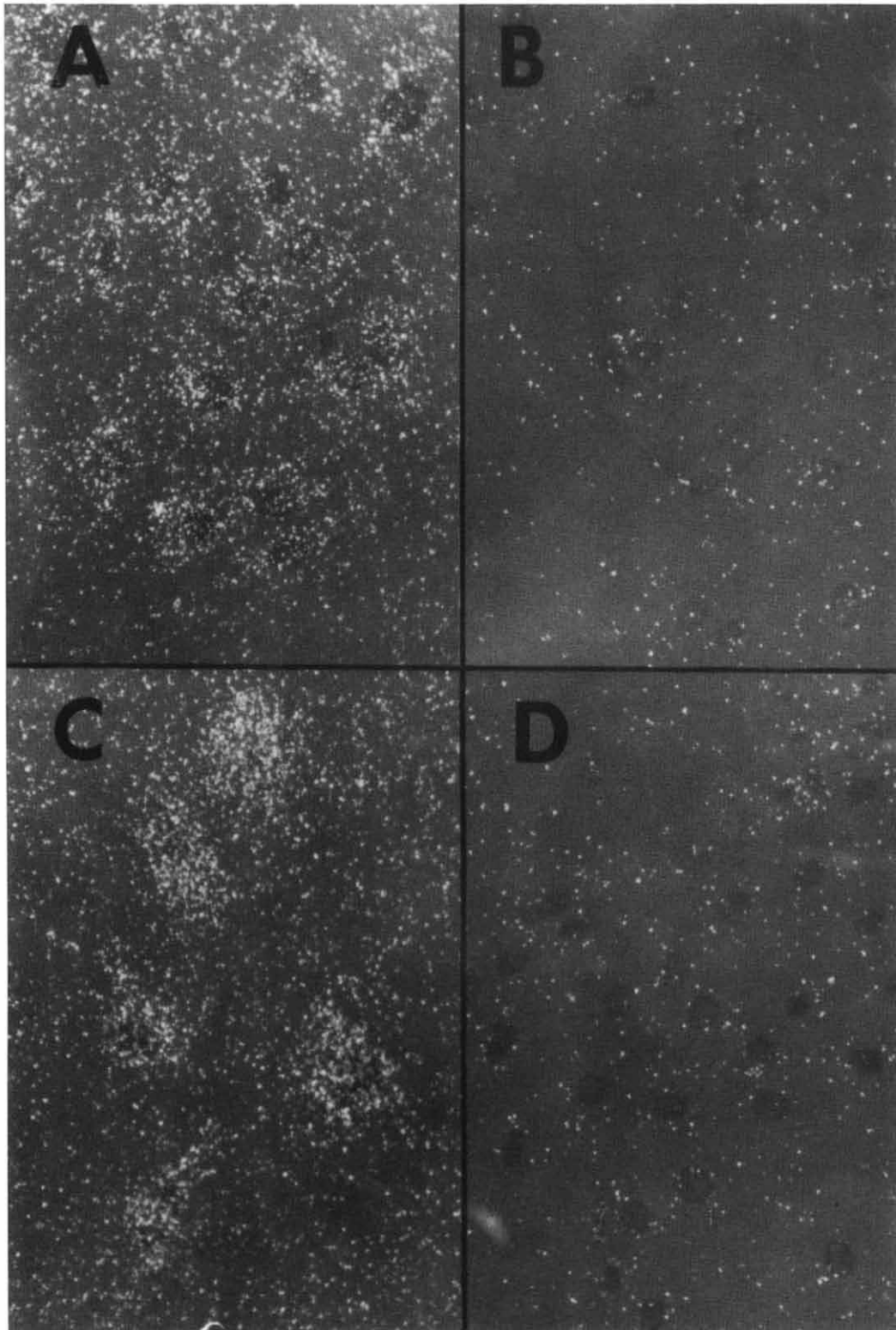
Fig. 1. Localization of TSP1 mRNA in human MG-63 osteoblastic by in situ hybridization. (A) Darkfield photomicrograph of quiescent MG-63 cells hybridized with an antisense probe. Note the specific localization of silver grains over MG-63 cells when compared to the sense probe (B). (C) Darkfield photomicrograph of thrombin-treated MG-63 cells with an antisense probe. A higher grain density is observed over thrombin-stimulated cells when compared to quiescent cells. (D) Nonspecific hybridization of thrombin-treated cells with a sense probe.
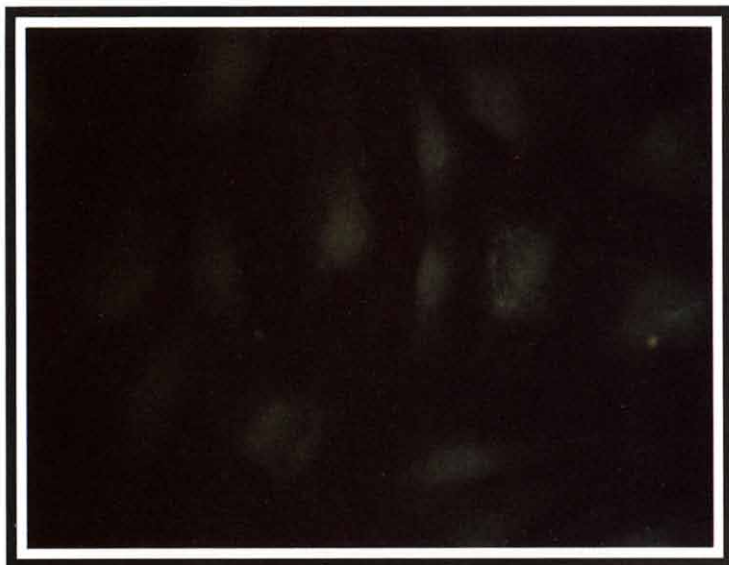
Control
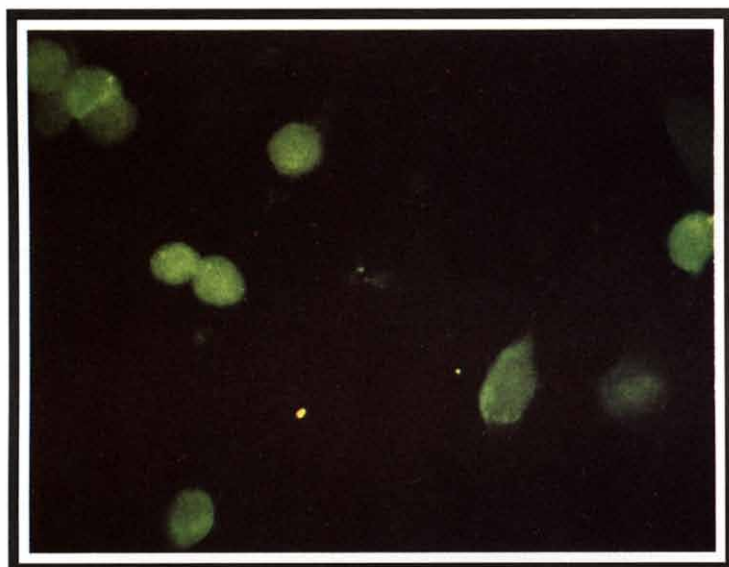
**Thrombin (0.1 U/ml)
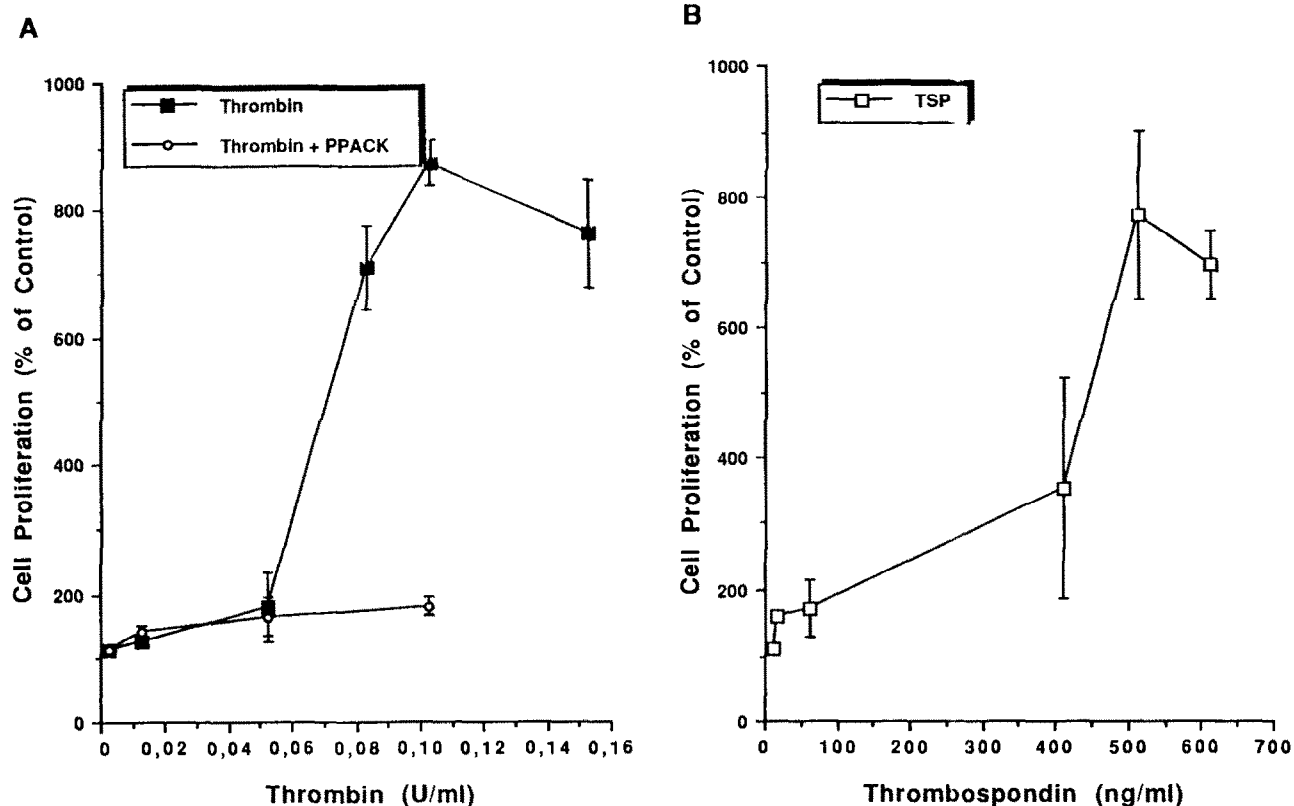+ MARK-1 (5 μg/ml)**
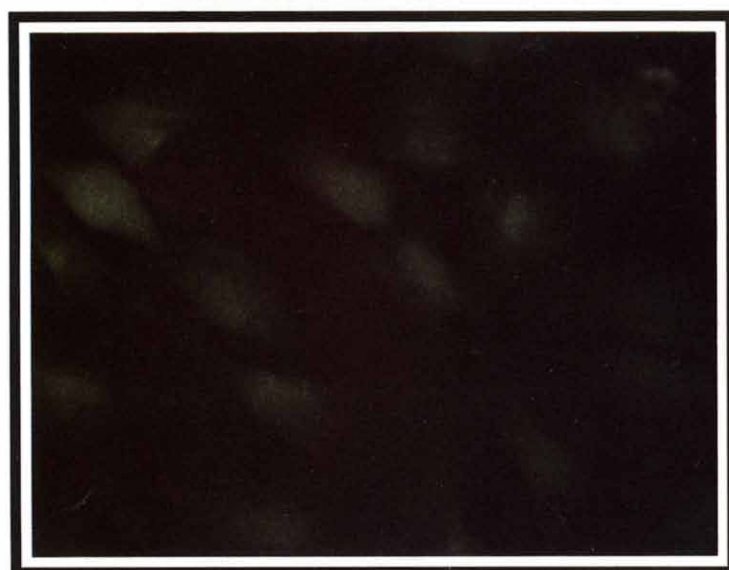
**Thrombin (0.1 U/ml)
+ P10 (5 μg/ml)**

Fig. 3. (A) Effect of increasing concentrations of α-thrombin on proliferation of human MG-63 osteoblastic cells. Data were obtained as cpm [³H]thymidine incorporated by thrombin-stimulated cells and normalized as a percentage of the mean cpm incorporated by untreated cells. Negative control experiments were conducted with thrombin in the presence of PPACK. Results are the mean ± S.D. of quadriplicate determinations. Five independent experiments were carried out with similar results. (B) Effect of increasing concentrations of TSP on proliferation of human MG-63 cells. Results were expressed as described above. Four independent experiments were carried out with similar results.

tion of human MG-63 cells was also studied (Fig. 5). In the presence of indomethacin (1 μM) and TSP (500 ng/ml), [³H]thymidine incorporation by MG-63 cells is only 242 ± 8% of the control value, while TSP alone induced a 760 ± 129% increase incorporation when compared to quiescent cells. Similarly, α-thrombin (0.1 U/ml) in conjunction with indomethacin (1 μM) reduced [³H]thymidine incorporation by MG-63 cells to 78.3 ± 18.2% of the control value, while α-thrombin alone induced a 862 ± 150% increase in [³H]thymidine incorporation by MG-63 cells when compared to untreated cells. [³H]Thymidine incorporation by MG-63 cells in the presence of indomethacin (1 μM) alone was 84.4 ± 6.7% of the control value.

4. DISCUSSION

We previously observed that human MG-63 osteo-

blastic cells synthesize, secrete and bind TSP in a receptor-like fashion [7,17]. The present study shows that, in vitro, TSP mediates proliferation of human MG-63 osteoblastic cells induced by α-thrombin. The observation that TSP mediates proliferation of MG-63 cells is based on a number of findings: (a) α-thrombin concomitantly stimulates TSP expression and proliferation of osteoblastic cells, and proliferation of thrombin-stimulated cells is specifically inhibited by anti-TSP monoclonal antibodies P10 and MA-II; (b) TSP itself induces proliferation of MG-63 cells; and (c) this proliferative effect of TSP is specifically inhibited by anti-TSP antibody P10. The growth supportive effects of α-thrombin and TSP are similar with respect to [³H]thymidine incorporation (862 ± 150% and 760 ± 129% increase of the control, respectively). The mechanisms by which α-thrombin and TSP stimulate cell growth are not known. Mouse osteoblasts and MC3T3-E1 osteoblastic cells re-

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Fig. 2. Immunofluorescence localization of TSP to the surface of human MG-63 osteoblastic cells. Untreated cells (control) and thrombin-treated cells incubated with anti-TSP monoclonal antibody P10 or negative control monoclonal antibody MARK-1 were examined after formaldehyde fixation and staining with an anti-TSP polyclonal antibody. The experiment was carried out two times with similar results.
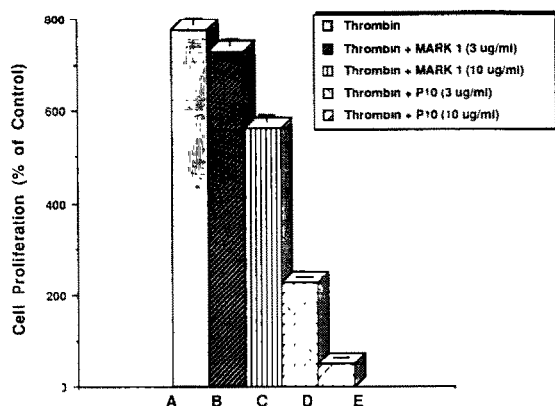
Fig. 4. Effect of anti-TSP monoclonal antibody P10 on proliferation of human MG-63 osteoblastic cells induced by α-thrombin (0.1 U/ml). Negative control monoclonal antibody MARK 1 is isotype-matched with P10. Results were expressed as described in the legend of Fig. 3. Three independent experiments were carried out with similar results
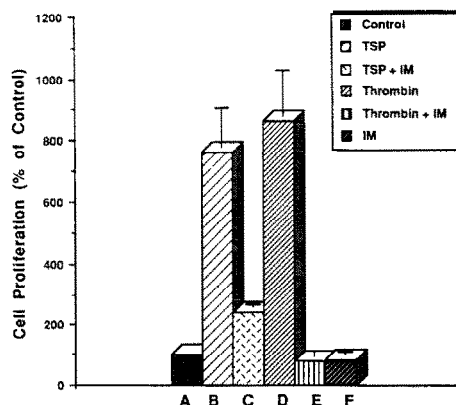

Fig. 5 Effect of indomethacin (1 μM) (i.m.) on proliferation of human MG-63 osteoblastic cells induced by TSP (500 ng/ml) or α-thrombin (0.1 U/ml). Results were expressed as described in the legend of Fig. 3. Three independent experiments were carried with similar results.

spond to thrombin with rapidly enhanced formation of prostaglandins such as PGE_2 [18], and PGE_2 stimulates proliferation of osteoblastic cells in vitro and bone formation in vivo [19]. We have shown in this study that indomethacin, which is known to inhibit prostaglandin synthesis [19], inhibits the effects of both α-thrombin and TSP on proliferation of MG-63 cells. In this respect, our data strongly suggest that the growth-supportive effects of α-thrombin and TSP on MG-63 cells are mediated by prostaglandins. Moreover, cell surface-associated TSP appears functionally essential for the proliferation of human MG-63 osteoblastic cells because anti-TSP monoclonal antibody P10 which inhibits proliferation of thrombin-stimulated osteoblastic cells also inhibits TSP expression on the surface of these cells. Similarly, anti-TSP antibodies drastically inhibit both proliferation of rat aortic smooth muscle cells and cell surface expression of TSP [5]. Taken together, our experiments support the existence of a mechanism whereby TSP bound to the cell surface of thrombin-stimulated MG-63 cells stimulates secretion of prostaglandins which, in turn, allow cell proliferation to proceed.

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