Volume 335, number 2, 161–166 FEBS 13316 December 1993

The growth-supportive effect of thrombospondin (TSP1) and the expression of TSP1 by human MG-63 osteoblastic cells are both inhibited by dexamethasone

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Received 4 October 1993

Thrombospondin (TSP) is a 450-kDa extracellular matrix glycoprotein which supports the growth of human MG-63 osteoblastic cells [Abbadia et al., FEBS Lett., 329 (1993) 341–346]. In this study, we describe the effect of the glucocorticoid, dexamethasone, on cell proliferation and TSP expression by MG-63 cells. Using a serum-free mitogenesis assay, dexamethasone (25 to 500 nM) caused a dose-dependent decrease in [^{3}H]thymidine incorporation by MG-63 cels in culture, reaching 40% inhibition of cell proliferation at a concentration of 250 nM. Similarly, the stimulatory effect of TSP (500 ng/ml) on proliferation of MG-63 cells was totally abolished in the presence of dexamethasone (250 nM). In situ hybridization indicated that TSP mRNA level in dexamethasone-treated MG-63 cells decreased compared to quiescent cells. As judged by fluorescence-activated cell sorting analysis, dexamethasone treatment of MG-63 cells resulted in a 50 to 70% decrease in TSP cell surface expression compared to quiescent cells. Secretion of TSP in the culture fluid of dexamethasone-treated MG-63 cells also decreased by 40% while, under similar experimental conditions, a 180% increase in alkaline phosphatase activity was observed in dexamethasone-treated cells. Because glucocorticoids induce osteoporosis in vivo and reduce proliferation of osteoblasts in vitro, our results argue for an important role of TSP during bone formation.

Thrombospondin; Dexamethasone; 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, TSP promotes proliferation of fibroblasts and facilitates growth of smooth muscle cells in response to EGF [1]. In addition, exposure of smooth muscle cells to anti-TSP antibodies causes decreased proliferation [1]. TSP has been identified in the mineralized bone matrix of neonatal and young (growing) bone of many animal species [4], and we have reported that TSP, which is synthesized and secreted by human MG-63 osteoblastic cells in culture [5], promotes the growth of these osteoblastic cells through a prostaglandin-mediated mechanism [6]. Taken together, these findings [1,3–6] are consistent with an important role for TSP in cell proliferation.

Long-term treatment with glucocorticoids has been associated with osteoporosis, which is characterized by a decrease in bone mass [7]. Glucocorticoid-induced osteoporosis primarily involves decreased width of trabecular bone, suggesting reduction of trabecular osteoblast proliferation [7]. Osteoblastic cells have receptors for glucocorticoids [8], and cultured mouse MC3T3-E1 osteoblastic cells have been used to show that the glucocorticoid, dexamethasone, inhibits DNA synthesis and prostaglandin synthesis [9]. In addition, glucocorticoids also affect the expression of bone extracellular matrix proteins including type I collagen [10], osteocalcin [11,12], alkaline phosphatase [12,13], osteopontin [14], bone sialoprotein [15], and fibronectin [16]. All of these extracellular matrix proteins undoubtedly play an important role in bone cell morphology, and could be contributing factors to the reduced bone quality and reduced bone formation seen in glucocorticoid-induced osteoporosis. However, although alkaline phosphatase activity is inversely related to cell prolifer-

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Abbreviations: TSP, thrombospondin; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

ation [13], none of these extracellular matrix proteins have been shown to modulate the growth of osteoblastic cells directly. These findings, taken together with the fact that TSP stimulates the growth of human MG-63 osteoblastic cells [6], prompted us to examine whether the glucocorticoid, dexamethasone, could inhibit the growth of human osteoblastic cells through inhibition of TSP expression.

2. MATERIALS AND METHODS

*Eco*RI, *Bam*HI, [^{3}H]thymidine, and [α-^{35}S]UTP were purchased from Amersham. Proteinase K was from Boehringer. Bovine serum albumin (BSA) was purchased from Miles Laboratories Inc. (Elkhart). Human α-thrombin was from Fibrindex (Ortho Diagnostic Systems). Dexamethasone was from Sigma. Human platelet TSP1 was obtained from Stago (France). Mouse monoclonal antibody P10 and a rabbit polyclonal antibody directed against human blood platelet TSP1 have been described earlier [5,17]. Mouse monoclonal antibody MARK 1 directed against rat κ light chains was purchased from Immunotech (France). Goat anti-(mouse IgG) and goat anti-(rabbit IgG) conjugated with fluoroscein isothiocyanate were from Coulter Immunology (France).

The 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.

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% (mass/vol.) BSA for 24 h. Cells were then treated with α-thrombin (0.1 U/ml) or dexamethasone (250 nM) for 48 h. After washing, cells were fixed for 15 min in PBS containing 2% (m/v) paraformaldehyde, 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 [6].

Cell surface expression of TSP by MG-63 cells stimulated or not with dexamethasone (250 nM) was studied on a FACS flow cytometer (Becton Dickinson) using either anti-TSP antibodies or negative control antibody MARK 1 as the primary antibody, and fluoroscein isothiocyanate-conjugated IgGs as the secondary antibody. The experimental procedure was essentially as previously described [18].

Quantitation of TSP secreted by MG-63 osteoblastic cells was performed by enzyme-linked immunosorbent assay (ELISA). Cells resuspended in RPMI medium containing 10% (v/v) fetal calf serum were plated at a density of 13,000 cells/cm^2 in 6-well plates, and were grown to subconfluence in a 5% CO_2 incubator at 37°C. The monolayer was washed twice with serum-free RPMI medium to remove any residual serum components, and cells were cultured in RPMI medium containing 1% (mass/vol) BSA. After 24-h incubation at 37°C, cells were treated with increasing concentrations of dexamethasone (25 to 500 nM). Supernatants were removed at 48 h after the addition of serum-free medium supplemented with BSA. Cell numbers were determined using a hemocytometer. Supernatants were aliquoted and stored at −20°C prior to assay. TSP present in the supernatants was quantified using a double-antibody sandwich assay (Stago, France).

For cell proliferation, MG-63 cells resuspended in RPMI medium containing 10% (v/v) fetal calf serum were plated at a density of 5,000 cells/well 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 make cells quiescent. After 24-h incubation, quiescent cells were treated for 48 h with increasing concentrations of dexamethasone (25 to 500 nM) in the presence or absence of TSP (500 ng/ml). 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 dexamethasone were the mean ± S.D. of quadruplicate determinations.

Assays for alkaline phosphatase activity utilized a 0.016 M *p*-nitrophenyl phosphate substrate in 0.9 mM 2-amino-2-methyl-1 propanol buffer, pH 10.5, containing 1 mM $MgCl_2$ (alkaline phosphatase kit, Boehringer), and spectrophotometric measurement of reaction product at 405 nm. Untreated cells and cells treated with dexamethasone (250 nM) and/or TSP (500 ng/ml) were harvested after exposure to trypsin (0.05 mg/ml) and EDTA (0.5 mM). Trypsin/EDTA-treated cells were counted and solubilized in 0.05% Triton X-100 at 4°C for 30 min. After centrifugation to remove cellular debris, the activity of alkaline phosphatase was assayed in solubilized cells.

3. RESULTS

3.1. *Cell proliferation*

It has been shown that treatment of osteoblastic cells with dexamethasone reduced cell proliferation by 40 to 50%, depending on the cell lines used [9,13]. Experiments conducted with human MG-63 osteoblastic cells showed that dexamethasone induced a significant, dose-dependent decrease in [^{3}H]thymidine incorporation by these cells, reaching a maximum inhibitory effect (40%) at concentrations between 250 and 500 nM (Fig. 1). Because TSP induces proliferation of MG-63 osteoblastic cells at an optimal concentration of 500 ng/ml [6], cell proliferation assays were also performed with TSP (500 ng/ml) in the presence of increasing concentrations (25 to 500 nM) of dexamethasone (Fig. 2). TSP (500 ng/ml) alone induced a 760 ± 129% increase in [^{3}H]thymidine incorporation by MG-63 cells when compared to untreated cells (Fig. 2A vs. 2F). By contrast, dexa-

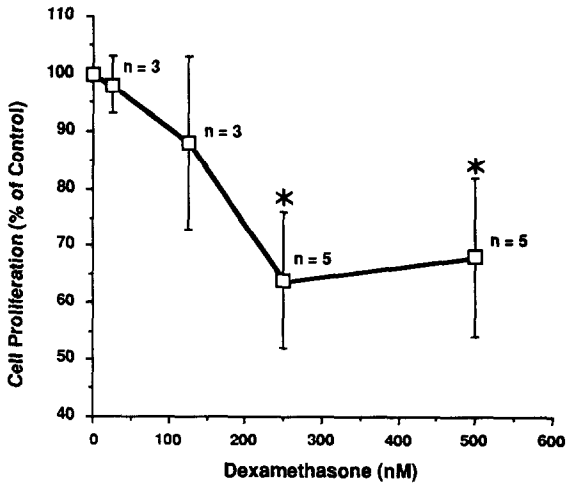

Fig. 1. Effect of increasing concentrations of dexamethasone on proliferation of human MG-63 osteoblastic cells. Data were obtained as cpm [^{3}H]thymidine incorporated by dexamethasone-treated cells and normalized as a percentage of the mean cpm incorporated by untreated cells. Results are the mean ± S.D. of *n* experiments. $*P \leq 0.01$ by Mann–Whitney test.

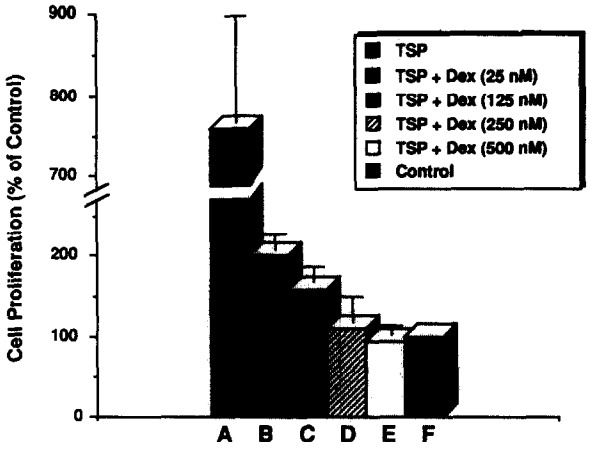

Fig. 2. Effect of increasing concentrations of dexamethasone (0 to 500 nM) on proliferation of human MG-63 osteoblastic cells induced by TSP (500 ng/ml). Results were expressed as described in the legend of Fig. 1, and are the mean ± S.D. of quadruplicate determinations. Two independent experiments were carried out with similar results.

methasone drastically reduced [^{3}H]thymidine incorporation by TSP-stimulated cells from 760 ± 120 to 98 ± 15% of the control value (Fig. 2B–E).

3.2. *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. 3A,B). As previously reported [6], 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. 3C) when compared to unstimulated cells (Fig. 3A). By contrast, treatment of MG-63 cells with dexamethasone (250 nM) induced a decrease in TSP1 mRNA expression (Fig. 3E) when compared to untreated cells (Fig. 3A). Using the sense probe, nonspecific hybridization for thrombin- and dexamethasone-treated MG-63 cells (Fig. 3D,F) was to a level similar to that observed for their unstimulated counterparts (Fig. 3B).

3.3. *Fluorescence-activated cell sorting analysis*

We have previously observed that TSP expression by thrombin-stimulated MG-63 cells is drastically increased at both mRNA and protein levels using in situ hybridization and immunofluorescence, respectively [6]. Because of the inhibition of TSP mRNA expression by dexamethasone-treated MG-63 cells (Fig. 3), cell surface expression of TSP was therefore studied by fluorescence-activated cell sorting analysis. Histograms of cell number vs. fluorescence intensity showed that the addition of an anti-TSP polyclonal antibody to untreated MG-63 cells increased the mean fluorescence intensity 14-fold when compared to an irrelevant antibody, producing a rightward shift in the histograms (Fig. 4, histograms c vs. a). Using similar experimental conditions, a 10-fold increase in the mean fluorescence intensity was observed with anti-TSP monoclonal antibody P10 (results not shown). By contrast, addition of an anti-TSP polyclonal antibody to dexamethasone-treated MG-63 cells produced a 70% decrease in fluorescence intensity compared to untreated cells (Fig. 4, histograms b vs. c). Using similar experimental conditions, a 50% decrease in fluorescence intensity was observed with anti-TSP monoclonal antibody P10 (results not shown).

3.4. *Enzyme-linked immunosorbent assay*

Secretion of TSP by MG-63 cells increased in a time-dependent manner (between 4 and 24 h), reaching a plateau at 48 h. Based on comparison with a standard curve, the amount of TSP in the culture fluid of the MG-63 cells at 48 h was found to be 40 ± 4.4 ng/10^6 cells (Fig. 5). Under similar experimental conditions, treatment of MG-63 cells with increasing concentrations of dexamethasone (25 to 500 nM) induced a significant dose-dependent inhibition on TSP secretion, reaching a maximum inhibitory effect at a concentration of 250 nM (24 ± 2 ng/10^6 cells; $P \leq 0.01$) (Fig. 5).

3.5. *Alkaline phosphatase activity*

Glucocorticoids stimulate alkaline phosphatase activity in a variety of osteoblastic cells [12,13]. Treatment of MG-63 osteoblastic cells with 250 nM dexamethasone led to a 180% increase in alkaline phosphatase activity when compared to untreated cells (10 ± 0.8 vs. 5.6 ± 0.7 U/L/10^6 cells; $P \leq 0.05$) (Fig. 6). For comparison, a 40% decrease in TSP secretion was observed with dexamethasone-treated MG-63 cells when compared to their untreated counterparts (24 ± 2 vs. 40 ± 4.4 ng/10^6 cells; $P \leq 0.01$) (Fig. 6).

4. DISCUSSION

Glucocorticoid-induced osteoporosis involves reduction of osteoblast proliferation [7–9,13], inhibition of prostaglandin synthesis [9] and inhibition of the expression of bone extracellular matrix proteins including type I collagen [10], osteocalcin [11,12], osteopontin [14], and fibronectin [16]. By contrast, glucocorticoids stimulate alkaline phosphatase activity [12,13] and bone sialoprotein expression by osteoblastic cells [15]. Using a super-physiological concentration of glucocorticoids (250 nM) known to occur in glucocorticoid-treated patients, the present study shows that dexamethasone concomitantly inhibits the expression of TSP and stimulates alkaline phosphatase activity in human MG-63 osteoblastic cells. The observation that dexamethasone inhibits TSP expression is based on a number of findings: (a) TSP mRNA expression is inhibited in dexamethasone-treated MG-63 cells, (b) cell surface expression of TSP is decreased by 50 to 70% in the presence of dexamethasone, and (c) secretion of TSP in the culture fluid

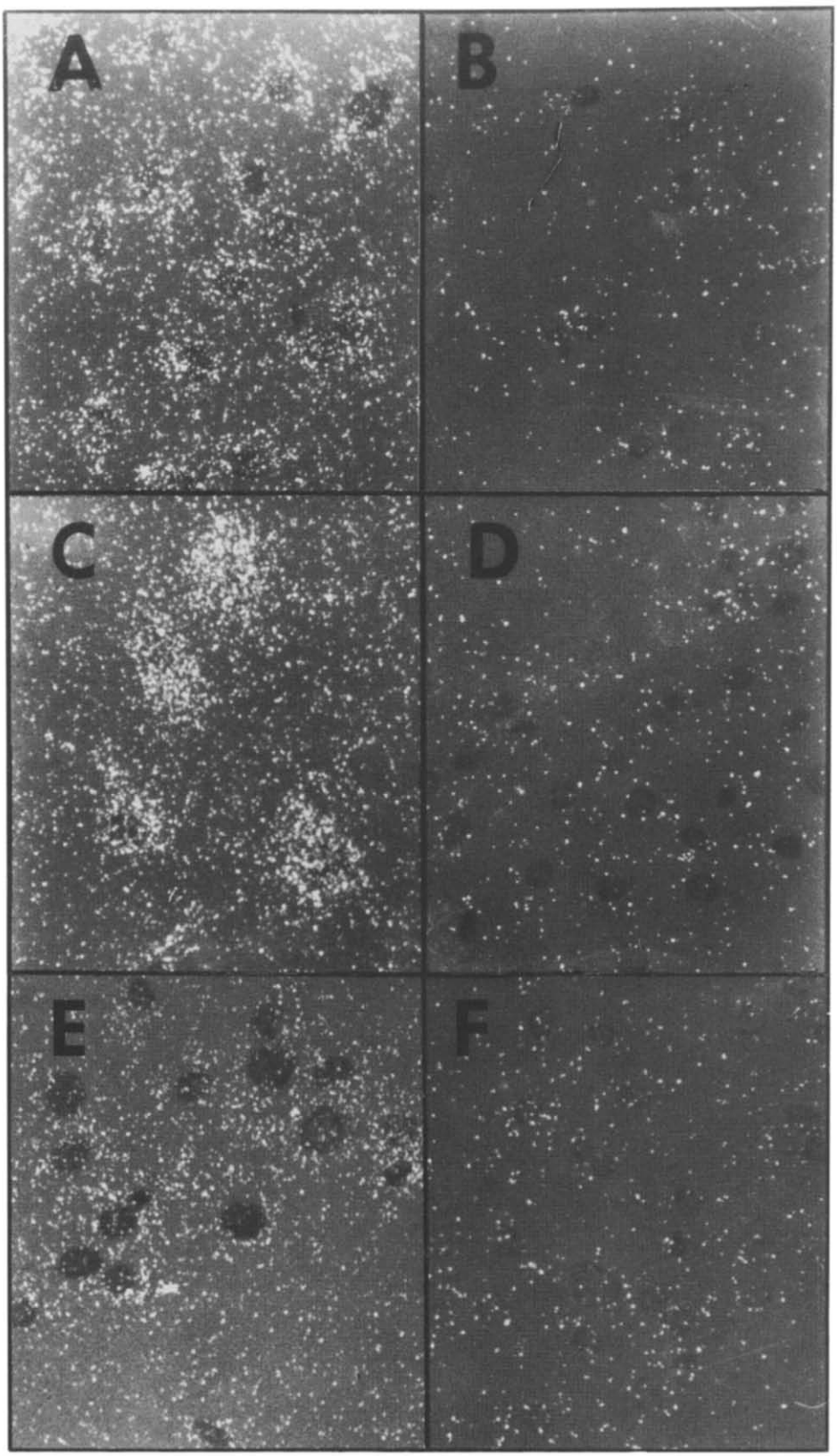

Fig. 3. Localisation of TSP1 mRNA in human MG-63 osteoblastic cells by in situ hybridization. (A) Darkfield photomicrograph of quiescent MG-63 cells hybridized with an antisense probe. Note the specific localization of silver grains over MG-63 cells when compared to the sense probe (B). (C and D) Darkfield photomicrographs of thrombin-treated MG-63 cells hybridized with an antisense probe and a sense probe, respectively. (E and F) Darkfield photomicrographs of dexamethasone-treated MG-63 cells hybridized with an antisense probe and a sense probe, respectively.

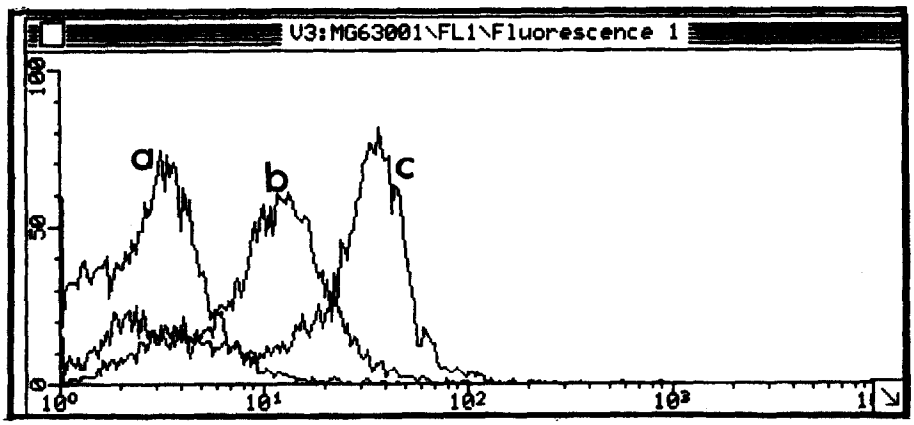

Fig. 4. Fluorescence-activated cell-sorting analysis of endogenous TSP expressed to the surface of MG-63 osteoblastic cells treated or not with dexamethasone. Osteoblastic cells were incubated with an anti-TSP polyclonal antibody or an irrelevant antibody, followed by fluorescein-conjugated goat anti-rabbit IgG. Cells were then analysed on a FACScan flow cytometer. Histogram a, binding of an irrelevant antibody to the surface of MG-63 cells treated or not with dexamethasone. Histogram b, binding of an anti-TSP polyclonal antibody to the surface of dexamethasone-treated MG-63 cells. Histogram c, binding of an anti-TSP polyclonal antibody to the surface of untreated MG-63 cells. *x*-axis, fluorescent intensity; *y*-axis, full scale counts. The experiment was carried two times with similar results.

of dexamethasone-treated MG-63 cells is decreased by 40%. The observation that dexamethasone stimulates alkaline phosphatase activity in human MG-63 osteoblastic cells confirms and extends previous findings obtained with human trabecular osteoblast-like cells [12] and rat ROS 17/2.8 osteoblastic cells [13]. As previously reported for human trabecular osteoblast-like cells [13,19], the increased activity of this enzyme is inversely related to proliferation of human MG-63 cells. Because TSP stimulates growth of MG-63 cells [6] and the growth-supportive effect of TSP is inhibited by dexamethasone (this study), it seems therefore that the decreased TSP expression observed in dexamethasone-treated MG-63 cells is directly related to the inhibition of cell proliferation. The mechanisms by which dexamethasone inhibits both TSP expression and cell growth are unknown. Cell surface heparan sulfate proteoglycans serve as a receptor for binding of TSP to many kinds of cells in culture [1], including MG-63 cells [20], and they also mediate degradation of TSP [21,22]. In this regard, dexamethasone could increase endocytosis of TSP-proteoglycan complexes, resulting in the decreased TSP cell surface expression seen in dexamethasone-treated MG-63 cells. A down-regulation of TSP receptors provides an alternative mechanism by which dexamethasone could also inhibit TSP expression. For example, TGF-β induces a marked decrease in adhesion of MG-63 cells to laminin which is related to a down-regulation of $\alpha_3\beta_1$-integrin [23]. By contrast, interleukin-1β up-regulates $\alpha_1\beta_1$-integrin expression in MG-63 cells [24], resulting in cell growth inhibition of interleukin-1β treated MG-63 cells [25]. Besides its effect on TSP expression, dexamethasone inhibits growth of human MG-63 osteoblastic cells (this study) as has been previously reported for cultured mouse osteoblast-like cells [8,9]. We have previously suggested that the growth-supportive effect of TSP could depend of a mechanism whereby cell surface-bound TSP stimulates secretion of prostaglandins which, in turn, allow cell proliferation to proceed [6]. Dexamethasone inhibits prostaglandin synthesis in mouse MC3T3-E1 osteoblastic cells and exogenous prostaglandin E_2 partially reverses growth inhibition induced by the glucocorticoid [9]. In this regard, it has been suggested that the inhibitory effect of dexamethasone may be partially mediated through a prostaglandin mechanism [9]. The proliferative action of prostaglandin E_2 in mouse MC3T3-E1 and rat C5.4E osteoblastic cells is mediated through

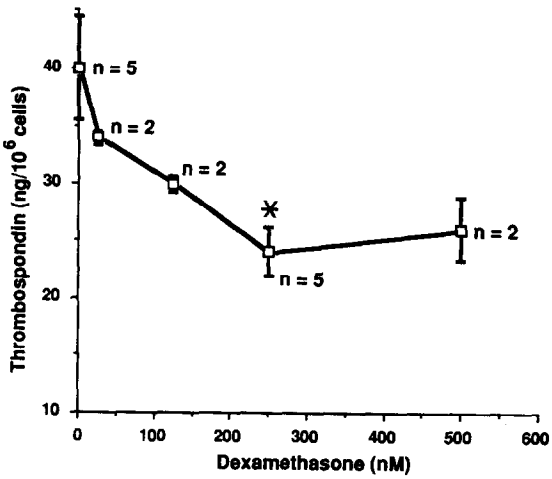

Fig. 5. Effect of dexamethasone on secretion of TSP by human MG-63 osteoblastic cells in culture. MG-63 cells were cultured in RPMI medium containing 1% (m/v) bovine serum albumin. After 24-h incubation at 37°C, cells were treated with increasing concentration of dexamethasone (0–500 nM) for 48 h at 37°C. TSP was measured in the culture supernatants using a double antibody sandwich assay. Results are the mean ± S.D. of *n* experiments. $^*P \leq 0.01$ by Mann–Whitney test.

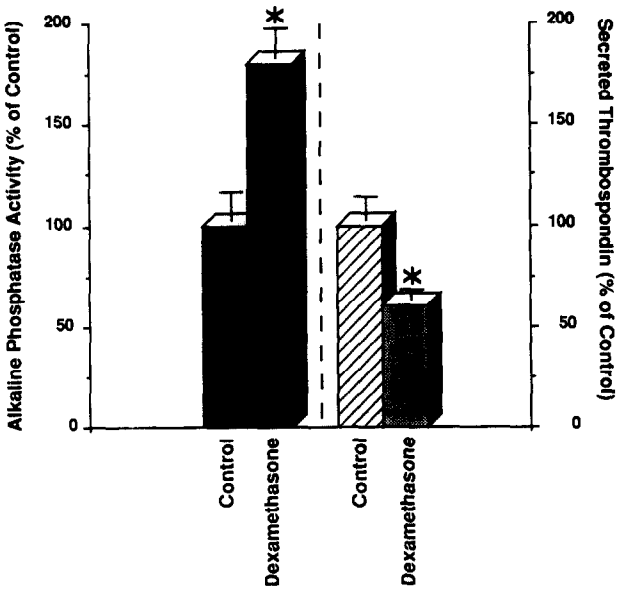

Fig. 6. Effect of dexamethasone on alkaline phosphatase activity and TSP secretion by human MG-63 osteoblastic cells. Alkaline phosphatase activity and TSP secretion were measured in the cell layer and the culture supernatant, respectively. Data values are expressed as percent of control from each of 5 experiments. The control value of alkaline phosphatase was 5.6 ± 0.7 U/l/10^6 cells. The control value of secreted TSP was 40 ± 4.4 ng/10^6 cells. Dexamethasone (250 nM) induced a 180% increase in alkaline phosphatase activity when compared to untreated cells (*$P \leq 0.05$ by Mann–Whitney test). For comparison, a 40% decrease in TSP secretion by MG-63 cells was observed in the presence of dexamethasone (*$P \leq 0.01$ by Mann–Whitney test).

activation of protein kinase C [26,27], and it is well established that protein kinase C is activated by diacylglycerol, a second messenger produced by agonists of phosphoinositide turnover [28]. Interestingly, submitogenic levels of TSP also stimulate phosphoinositide turnover in cultured smooth muscle cells [29]. Based on these findings [6,26–29], the growth-supportive effect of TSP may be mediated through protein kinase C activation and/or prostaglandin synthesis. It seems therefore that at least two different mechanisms could account for the reduced cell growth seen in glucocorticoid-treated osteoblastic cells: (a) the inhibition of TSP cell surface expression by dexamethasone induces a decrease in prostaglandin synthesis which results in reduced cell growth and (b) the stimulation of phosphoinositide turnover by TSP and/ or prostaglandin E_2 is inhibited by dexamethasone, leading to the inactivation of protein kinase C. In this regard, it will be very interesting to investigate whether or not the binding of TSP to cell surface heparan sulfate proteoglycans is able to transduce intracellular signals, or if other TSP receptors are involved in the signal transduction leading to cell proliferation.

Acknowledgements: The technical assistance of M. Mazet is gratefully acknowledged. This study was supported by grants from the Biologie Humaine, Université Claude Bernard, Lyon I [Z.A.] and the Association pour la Recherche sur les Tumeurs de la Prostate [P.C.].

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