

Interleukin 10 Inhibits Transforming Growth Factor- β (TGF- β) Synthesis Required for Osteogenic Commitment of Mouse Bone Marrow Cells

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Abstract. Interleukin 10 (IL-10) suppressed TGF- β synthesis in mouse bone marrow cultures. Coincidentally, IL-10 down-regulated the production of bone proteins including alkaline phosphatase (ALP), collagen and osteocalcin, and the formation of mineralized extracellular matrix. The mAb 1D11.16 which neutralizes TGF- β 1 and TGF- β 2, induced suppressive effects comparable to IL-10 when administered before the increase of cell proliferation in the culture. It appears that mainly TGF- β 1 plays a role in this system since (a) TGF- β 2 levels were undetectable in supernatants from osteogenic cultures, (b) no effect was observed when the anti-TGF- β 2 neutralizing mAb 4C7.11 was added and (c) the suppressive effect of IL-10 could be reversed by adding exogenous TGF- β 1. It is unlikely that TGF- β 1 modulates osteogenic differentiation by changing the proliferative potential of marrow cells

since 1D11.16 did not affect [3 H]thymidine ([3 H]TdR) incorporation or the number of fibroblast colony forming cells (CFU-F) which harbor the osteoprogenitor cell population. Furthermore, 1D11.16 did not alter [3 H]TdR uptake by the cloned osteoprogenitor cell lines MN7 and MC3T3. Light and scanning electron microscopy showed that IL-10 and 1D11.16 induced comparable morphological changes in the marrow cultures. Control cultures contained flat adherent cells embedded in a mineralized matrix. In contrast, IL-10 and 1D11.16 treated cultures were characterized by round non-adherent cells and the absence of a mineralized matrix. In this study, the mechanism by which IL-10 suppresses the osteogenic differentiation of mouse bone marrow was identified as inhibition of TGF- β 1 production which is essential for osteogenic commitment of bone marrow cells.

BONE marrow stroma forms a network of fibroblasts, adipocytes, endothelial cells, and macrophages which supports and regulates hemopoiesis (Dexter et al., 1982; Allen et al., 1984) and harbors cells of the osteogenic lineage (Friedenstein et al., 1976; Owen et al., 1980). The latter is illustrated by the fact that marrow cells differentiate into bone when transplanted in ectopic sites (Ashton et al., 1980; Friedenstein, 1980; Budenz and Bernard, 1980) or when cultured in the presence of vitamin C and β -glycerophosphate (Howlett et al., 1986; McCulloch et al., 1991; Falla et al., 1993). In addition, a number of immortalized and transfected cell lines generated from bone marrow stroma elicited osteogenic characteristics in culture or when transplanted in vivo (Benayahu et al., 1989; Mathieu et al., 1992).

Although IL-10 was first described as "cytokine synthesis inhibiting factor" (CSIF)¹ (Fiorentino et al., 1989), subse-

quent studies revealed its pleiotropic activities. Apart from suppressing cytokine synthesis by murine Type 1 helper T cells (De Waal Malefijt et al., 1991a) and macrophage functions (De Waal Malefijt et al., 1991b), IL-10 stimulates the growth of mast cells (Thompson-Snipes et al., 1991), B cells (Go et al., 1990), and thymocytes (MacNeil et al., 1990). We recently demonstrated that IL-10 is a potent inhibitor of the osteogenic activity of murine bone marrow (Van Vlasselaer et al., 1993). This study revealed that by acting on early steps of osteogenic differentiation, IL-10 suppresses the synthesis of several bone proteins including alkaline phosphatase (ALP), collagen type I and osteocalcin, and the potential of cultured marrow to form a mineralized matrix. Hence IL-10 joins the list of cloned factors able to influence bone biology.

One aspect of the complex mechanisms governing bone formation is the network of osteotropic cytokines and growth factors (Gowen, 1992). Among them TGF- β was shown to elicit a broad range of biological activities (Sporn et al.,

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1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; CDP, col-

lagenase digestible protein; CFU-F, fibroblast colony-forming cells; CSIF, cytokine synthesis inhibiting factor.

1986; Massague, 1987) such as the control of proliferation and differentiation of several cell types specific to bone including mesenchymal precursor cells, chondrocytes, osteoblasts, and osteoclasts (Noda and Rodan, 1986; Centrella et al., 1987a; Gehron-Robey et al., 1987; Wrana et al., 1988; Shinar and Rodan, 1990; Joyce et al., 1990; Noda and Camilleri, 1989). TGF- β is synthesized by osteoblasts (Gehron-Robey et al., 1987), and is present in large amounts in the bone matrix where it may serve as a reservoir (Hauschka et al., 1986) and control the osteogenic differentiation of "naive" mesenchymal cells. In support of this view, TGF- β was reported to regulate chondrogenesis from mesenchymal cells (Gehron-Robey et al., 1987; Seyedin et al., 1987) and to modulate the expression of the osteoblast phenotype in vitro (Rosen et al., 1988). In addition, immunohistochemical staining and in situ hybridization revealed accumulation of TGF- β in areas of endochondral ossification (Carrington et al., 1988).

The goal of this study was to define the mechanism of action by which IL-10 suppresses bone protein synthesis and the formation of mineralized matrix in cultures of mouse bone marrow. This study showed that IL-10 activity resulted from its inhibitory effect on the synthesis of TGF- β 1 which appears to be a crucial factor for the initiation of osteogenic differentiation of mesenchymal cells from the mouse bone marrow.

Materials and Methods

Mice and Bone Marrow Cell Preparation

Eight to ten week old BALB/c mice were administered 5-Fluorouracil (5-FU) (Roche) at 150 mg/kg body weight by tail vein injection. 5 d later the marrow was flushed from the femora and dispersed into a single cell suspension. Red blood cells were removed by density centrifugation on a 70% Percoll (Pharmacia, Sweden) gradient.

Cell Culture

Bone marrow cells were cultivated in flat bottom 96 well plates at 5×10^4 cells per well in ISCOVE's medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin, ascorbic acid (100 μ g/ml), and β -glycerophosphate (0.6% wt/wt). Purified, recombinant murine IL-10 was added at a concentration of 2.5×10^3 U/ml. To determine cell proliferation, cultures were pulsed with [3 H]TdR (1 μ Ci/well) for 18 h, and were then harvested and read in a liquid scintillation counter. The anti-TGF- β mAbs, 1D11.16 and 3C7.14 (Dasch et al., 1979), were added at a final concentration of 10 μ g/ml at time points indicated in the text. 1D11.16 neutralizes the activity of TGF- β 1 and TGF- β 2 whereas 3C7.14 neutralizes the activity of only TGF- β 2.

Quantitation of Fibroblast Colony-forming Cells

The number of CFU-F was scored as described by Van Den Heuvel et al. (1987). Bone marrow cells were plated at 5×10^4 cells per 35-mm culture dishes. Replicate cultures were incubated at 37°C for 10 d and were then fixed with methanol and stained with May-Grunwald-Giemsa. Colonies of fibroblastoid cells were scored under a binocular (20 \times).

TGF- β Quantitation

TGF- β was determined by measuring the inhibition of growth of the mink lung cell line Mv1Lu as described by Danielpour et al. (1989). Mv1Lu cells were plated at 10^3 cells per well in flat bottom 96 well plates and allowed to adhere. Samples were added without treatment and after acidification. Acid activation of the supernatants was performed by the addition of HCl to reach pH 1.5 for 1 h. The medium was then reneutralized using NaOH.

After 72 h of cultivation, cell proliferation was determined using the MTT assay described by Mossman (1983). The suppressive effect of the culture supernatants on Mv1Lu cell growth was reversed with 1D11.16 (10 μ g/ml) indicating that the suppressive activity was due to TGF- β activity. TGF- β was quantitated in comparison with a dose response curve using a standard of biologically active TGF- β (British Biotechnology, UK). The amount of TGF- β 1 and TGF- β 2 in the samples was discriminated by adding the TGF- β neutralizing mAbs 1D11.16 and 3C7.14 separately. 1D11.16 blocks both TGF- β 1 and TGF- β 2 whereas 3C7.14 blocks specifically the activity of TGF- β 2 but not of TGF- β 1. The sensitivity of the assay was 1 pg/ml. The concentrations of TGF- β 1 and TGF- β 2 were determined in the FCS used in the medium and was subtracted from the experimental values. Consequently the results represent the amount of TGF- β secreted in the cultures. Both antibodies showed cross species specificity and were able to block bovine TGF- β 1 and TGF- β 2.

Measurement of ALP Activity

ALP activity was determined on day 15 of the culture as described by Lowry et al. (1954). The cultures were incubated with 0.1 M sodium acetate solution supplemented with 0.1% Triton X-100 and 5 mM *p*-nitrophenol phosphate (Sigma 104), pH 9.6 for 1 h at 37°C. Absorbance was determined at 405 nm and compared to a *p*-nitrophenol standard titration curve. ALP activity was expressed as nmol of *p*-nitrophenol formed per min.

Collagen Synthesis

Collagen synthesis was measured on day 18 of the culture by the incorporation of [3 H]proline (Amersham, UK) into collagenase digestible protein (CDP) (Peterkovsky and Diegelmann, 1985). Cell cultures were incubated with [3 H]proline (1 μ Ci/well) for 18 h at 37°C, and then were washed three times with PBS. Collagenase (0.1 mg/ml) was added for 1 h and the CDP was measured in a liquid scintillation counter. Collagenase was purchased from Worthington (UK) and was substantially free of nonspecific protease activity.

Osteocalcin Cell ELISA

The osteocalcin cell ELISA was performed as described by Van Vlasselaer (1992). The cultures were fixed with 4% cold formaldehyde for 30 min at 4°C, and then were washed with TRIS/HCl buffer (0.05 M pH 7.6). Endogenous peroxidase activity was blocked with 3% H₂O₂ for 5 min. The samples were then rinsed with TRIS/HCl buffer and blocked with normal goat serum (1:5 dilution, TAGO) for 1 h at 37°C. Rabbit-anti-mouse osteocalcin antiserum (kindly provided by Dr. R. Bouillon, K.U. Leuven, Belgium) was added (1:5,000 dilution) for 2 h at 4°C. The cultures were washed with TRIS/HCl buffer and incubated with HRP-conjugated goat-anti-rabbit Ig serum (1:2,000 dilution, TAGO) for 30 min at 4°C. After rinsing, the cultures were incubated at 37°C with substrate solution (ABTS, 1 mg/ml + H₂O₂ 0.1 ul/ml in citric acid/Na₂HPO₄, 10.5 gr/14.2 gr/500 ml H₂O) until coloration occurred. Absorbance was read at 450 nm with a reference of 650 nm. Osteocalcin was quantitated on day 24 of culture. Non-specific binding of the anti-mouse osteocalcin antiserum was determined using non-immune rabbit serum under the same conditions. The amount of osteocalcin incorporated in the culture was determined in comparison with a standard ELISA of purified mouse osteocalcin (kindly provided by Dr. R. Bouillon, K.U. Leuven, Belgium). The sensitivity of this assay was 0.3 ng/ml. No cross reactivity was observed with FCS.

Calcium Determination

Calcium was determined on day 27 of culture as described by Gitelman (1967). The cell cultures were washed with Ca²⁺ and Mg²⁺ free PBS and incubated overnight with 0.6 N HCl. The extracted calcium was complexed with *o*-cresol-phthalein-complexon (Test Combination Calcium, Boehringer Mannheim, Germany). The colorimetric reaction was read at 570 nm. The absolute calcium concentration was determined in comparison with a standard curve for calcium provided by the vendor.

RNA Preparation and Northern Blot

Total cellular RNA was prepared from 6-d old cultures by acid guanidine thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi (1987). RNA was fractionated by electrophoresis in 1.5% agarose containing 10 mM sodium phosphate after denaturation with glyoxal

and DMSO. RNA was transferred to Biodyne filters (Pall) and hybridized to a ^{32}P -labeled TGF- β cDNA probe (Derynck et al., 1986). Autoradiographic exposure was performed onto Kodak Ortho G X-ray films. Densitometry analysis was performed on a Imagestore 5000 device using SW 5000 software for analysis (Ultraviolet Products, Ltd., Cambridge, UK).

Results

Effect of IL-10 on TGF- β Synthesis

We previously showed that IL-10 suppresses in vitro bone protein synthesis and the formation of mineralized matrix in cultures of mouse bone marrow (Van Vlasselaer et al., 1993). Based on the CSIF activity of IL-10 and the role of TGF- β in bone biology, the question addresses whether IL-10 exerts this suppressive effect by down-regulating TGF- β production. To this end TGF- β concentrations were measured in marrow cultures performed in the absence or presence of IL-10 (2.5×10^3 U/ml). Supernatants were collected at 3-d intervals and their inhibitory activity was determined on the TGF- β sensitive Mv1Lu mink lung cell line. Biologically active and biologically inactive TGF- β was discriminated by adding the samples without previous treatment or after acidification, respectively. The levels of TGF- β 1 and TGF- β 2 were determined independently by neutralizing total TGF- β activity with the 1D11.16 and 3C7.14 mAbs separately. Fig. 1 shows that whereas no detectable levels of TGF- β 2 were observed, TGF- β 1 is synthesized throughout the culture period with maxima around days 6 and 27. In addition, the supernatants inhibited the growth of Mv1Lu cells without previous acidification indicating that at least part of the secreted TGF- β 1 is present in its biologically active form. During maximal synthesis, biologically active TGF- β 1 was present at concentrations of 0.015 and 0.021 ng/ml. This represents $\sim 23\%$ of the total amount of TGF- β 1 released in the medium. Hence the bulk of secreted TGF- β 1 is present in its biologically inactive form. TGF- β 1 concentrations were significantly reduced in IL-10-treated cultures. At days 6 and 27, the amount of latent TGF- β 1 was reduced up to 68 and 72%, and the amount of active TGF- β 1 up to 99 and 90%, respectively (Fig. 1). The suppressive activity of IL-10 on TGF- β 1 synthesis was furthermore illustrated at the

mRNA level. After 6 d of culture, the total amount of TGF- β 1 mRNA is reduced for 40% in the IL-10-treated cultures (Fig. 2). Whereas this correlates with the only partial reduction of biologically latent TGF- β 1 protein observed at the same time point of the culture, this appears to represent an almost complete reduction of the amount of biologically active protein (Fig. 1).

Effect of 1D11.16 and 3C7.14 on Bone Protein Synthesis and Mineralization

Thus far the data suggest that in vitro osteogenic differentiation of mouse bone marrow cells is regulated in particular by endogenous TGF- β 1 and not by TGF- β 2. To further address this idea, cultures were performed in the absence or presence of the TGF- β neutralizing mAbs 1D11.16 and 3C7.14 (both used at $10 \mu\text{g/ml}$) and the degree of ALP activity, collagen and osteocalcin synthesis, and the amount of matrix-bound calcium was determined at days 18, 21, 24, and 27, respectively. Fig. 3 shows that the synthesis of the different bone proteins and the degree of mineralization was significantly reduced in cultures treated with 1D11.16. Whereas ALP activity and collagen synthesis were suppressed by $\sim 35\%$, the effect of 1D11.16 was more pronounced on osteocalcin synthesis and mineralization which were reduced by more than 95%. In contrast, the addition of the TGF- β 2 neutralizing mAb 3C7.14 did not alter bone protein synthesis and mineralization. Taken together this illustrates once more that endogenous TGF- β 2 plays no role during in vitro osteogenic differentiation of mouse bone marrow cells (Fig. 3).

Reversal of IL-10 Activity by TGF- β 1

The possibility that IL-10 suppresses bone protein synthesis and mineralization by blocking TGF- β 1 production was further analyzed. Cultures were performed in the presence of IL-10 and supplemented from the start with exogenous TGF- β 1 (1 ng/ml). ALP, collagen and osteocalcin synthesis, and the degree of mineralization were determined at the time point of their maximal expression, e.g., at days 15, 18, 24, and 27 of culture, respectively (Falla et al., 1993). Whereas

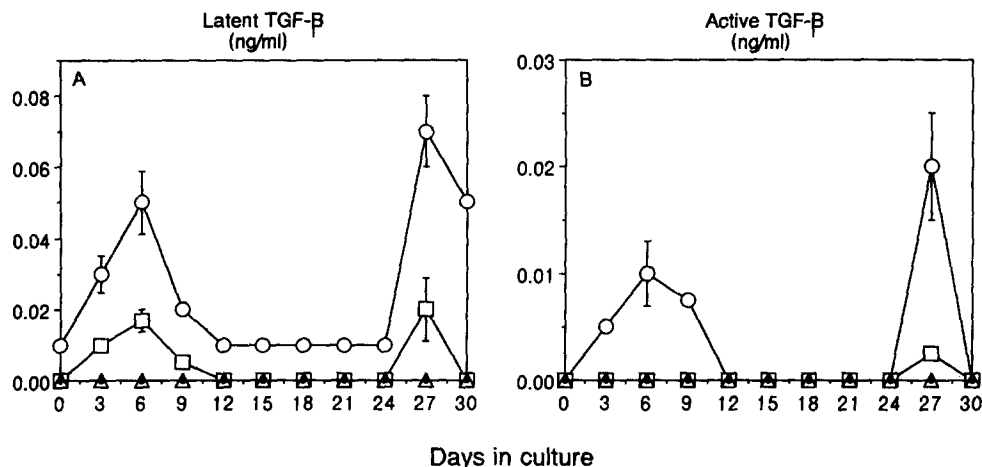


Figure 1. Effect of IL-10 on TGF- β 1 and TGF- β 2 synthesis. Cultures were set up under standard conditions and supernatants were collected at 3-d intervals. Biologically inactive and active TGF- β 1 and TGF- β 2 were determined using the Mv1Lu cell growth inhibition assay. \circ and \square represent TGF- β 1 levels in control and IL-10 (2.5×10^3 U/ml) treated cultures, respectively. \triangle and \bullet represent TGF- β 2 levels in control and IL-10 (2.5×10^3 U/ml) treated cultures, respectively. The results represent the mean \pm SD of triplicate cultures of one representative experiment.

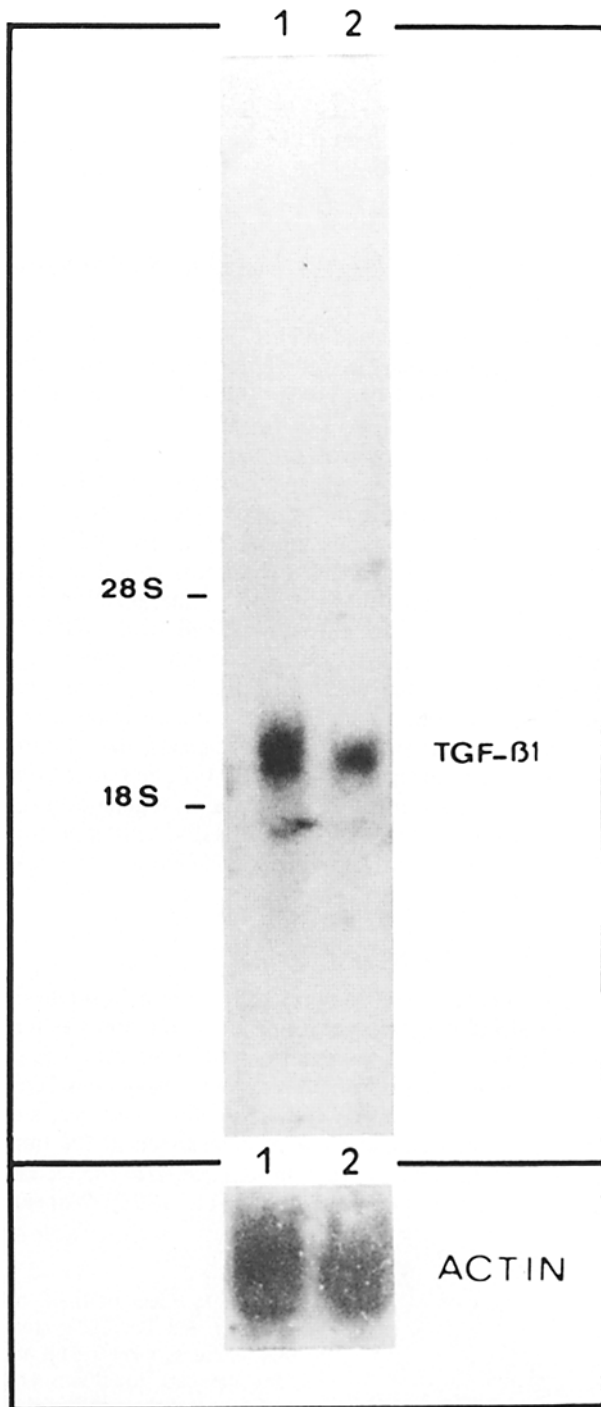


Figure 2. Effect of IL-10 on TGF- β 1 mRNA expression. Marrow cells were cultured for 6 d under standard conditions and the expression of TGF- β 1 mRNA was determined by Northern blot analysis. Lanes 1 and 2 represent mRNA from untreated and IL-10 treated cultures, respectively.

IL-10 reduced ALP activity and collagen synthesis for 54 and 40%, the suppressive effect was most obvious on osteocalcin synthesis and mineralization where IL-10 elicited suppressions up to 90%. The addition of exogenous TGF- β 1 abolished this IL-10 suppressive effect completely and resulted in normalized bone protein synthesis and mineralization

(Fig. 4). In addition it is of interest to note that bone protein synthesis and mineralization was slightly increased in control cultures supplemented with TGF- β 1.

Differential Effects of 1D11.16 on Early and Late Stages of Osteogenic Differentiation

It is conceivable that although TGF- β 1 is continuously present in marrow cultures, its role during osteogenic differentiation is restricted to a defined time period. Indeed, additional studies showed that the potential of 1D11.16 to elicit suppressive effects depended on the moment of its addition to the culture. When 1D11.16 was added before day 10, it suppressed ALP activity and collagen synthesis for 51% and 48% and blocked osteocalcin synthesis as well as mineralization for more than 90%. On the other hand 1D11.16 exerted no effect when it was added at later time points (Fig. 5).

Effect of 1D11.16 on Cell Proliferation and CFU-F Frequency

In vitro osteogenic differentiation is preceded by a sharp increase followed by a rapid decrease in cell proliferation (Owen et al., 1990; Falla et al., 1993). Consequently, one pathway via which TGF- β 1 may induce osteogenic differentiation is by modulating cell proliferation. To address this possibility, marrow cultures were performed in the absence and presence of 1D11.16 and the degree of [3 H]TdR incorporation was determined at 3-d intervals. Fig. 6 shows that cell proliferation in control cultures was maximal between days 9 and 12, e.g., beyond the time point at which TGF- β 1 exerts its osteoinductive activity. However, the presence of endogenous TGF- β 1 is not required for cell proliferation since the level of [3 H]TdR uptake was comparable between control and 1D11.16 treated cultures (Fig. 6). In addition, CFU-F frequency in the marrow (Fig. 7) and [3 H]TdR uptake by the cloned osteogenic cell lines MN7 (Mathieu et al., 1992) and MC3T3 (Sudo et al., 1983) were not affected by 1D11.16 (Fig. 8). Hence, it is unlikely that endogenous TGF- β 1 induces osteogenic differentiation by influencing the proliferation of osteoprogenitor cells.

Morphological Changes in IL-10 and 1D11.16 mAb Treated Marrow Cultures

Coinciding with their suppressive activity, IL-10 and 1D11.16 significantly changed the morphology of stroma cultures. Light microscopy revealed that 18-d old control cultures were composed of predominantly flat adherent cells. In contrast, 1D11.16 treated cultures contained large numbers of round non-adherent cells which formed cell aggregates in close contact with an adherent layer of fibroblastic and reticular cells (Fig. 9). In addition, scanning electron microscopy of 25-d old cultures revealed a prominent mineralized collagenous extracellular matrix in the control but not in the IL-10 and 1D11.16 treated cultures. The density of the mineralized matrix present in the control cultures at that time point made it impossible to distinguish individual cells. In contrast, IL-10 and 1D11.16 treated cultures were composed of large flat cells and small round cells with in between unoccupied areas. These observations illustrate again the suppressive effect of IL-10 and 1D11.16 on bone protein synthesis and mineralization.

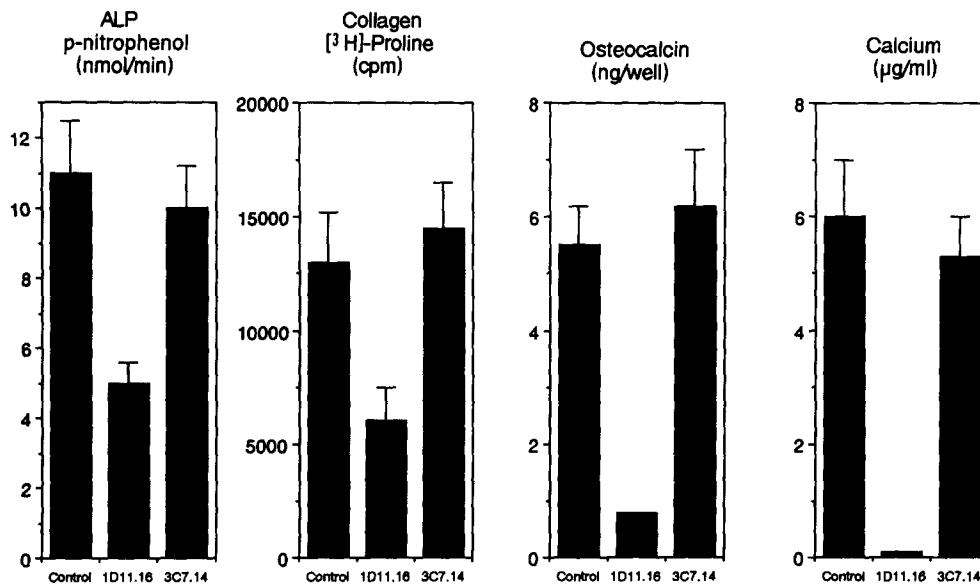


Figure 3. Effect of the neutralizing anti-TGF-β mAb, ID11.16, and 3C7.14 on bone protein synthesis and mineralization. Cultures were performed under standard conditions and ID11.16 and 3C7.14 mAb were added from the start at a final concentration of 10 μg/ml. ALP activity, collagen and osteocalcin synthesis, and mineralization were quantitated on days 18, 21, 24, and 27, respectively. Control cultures received no antibody. The results represent the mean + SD of quadruplicate cultures of one representative experiment.

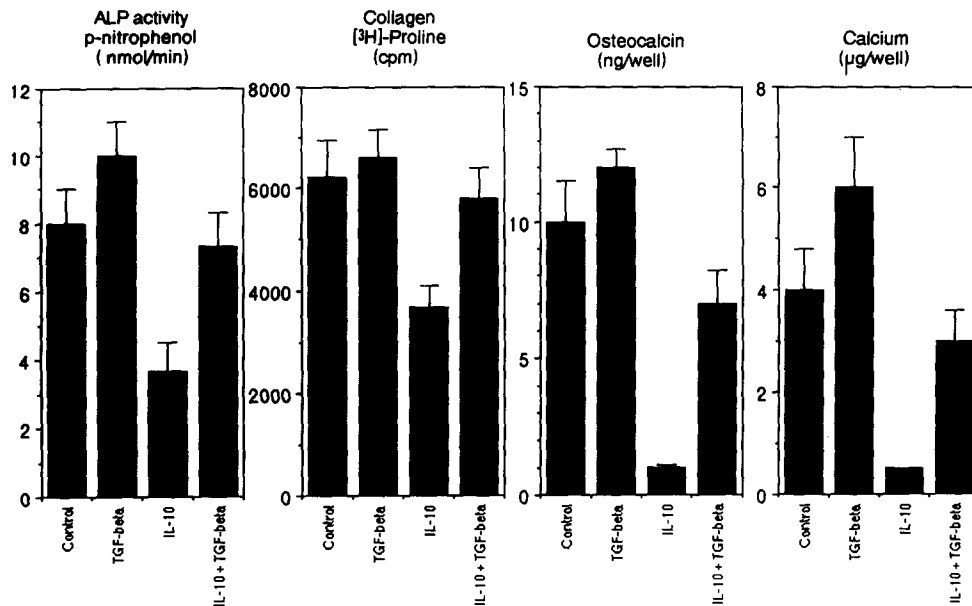


Figure 4. Effect of exogenous TGF-β1 on the suppressive activity of IL-10. Cultures were set up in the absence or presence of IL-10 (2.5×10^3 U/ml). TGF-β was added from the start of the culture at a concentration of 1 ng/ml. ALP activity, collagen and osteocalcin synthesis, and mineralization were quantitated on days 18, 21, 24, and 27, respectively. The results represent the mean + SD of quadruplicate cultures of one representative experiment.

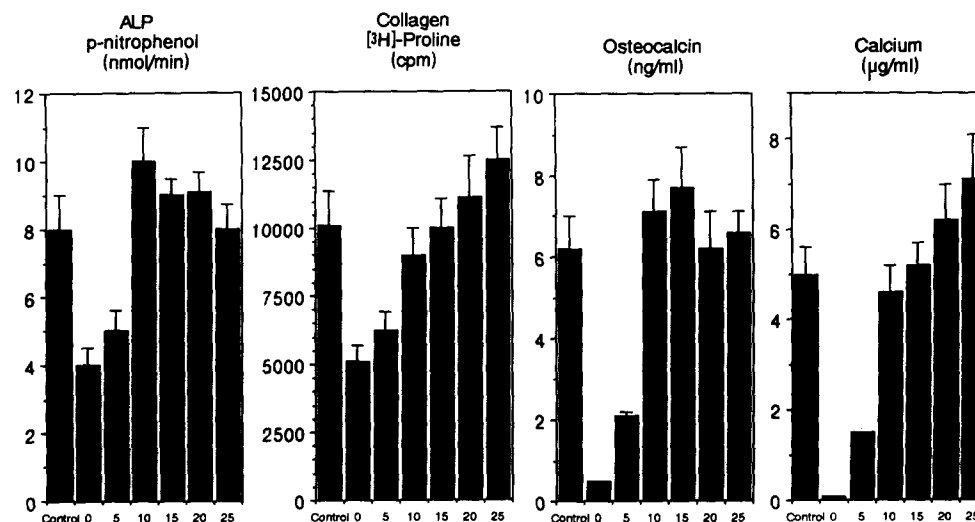


Figure 5. Effect of temporal addition of the neutralizing anti-TGF-β mAb, ID11.16 on ALP activity, collagen and osteocalcin synthesis, and mineralization. ID11.16 (10 μg/ml) was added at 5-d intervals starting at Day 0. Control cultures received no antibody. The results represent the mean + SD of quadruplicate cultures of one representative experiment.

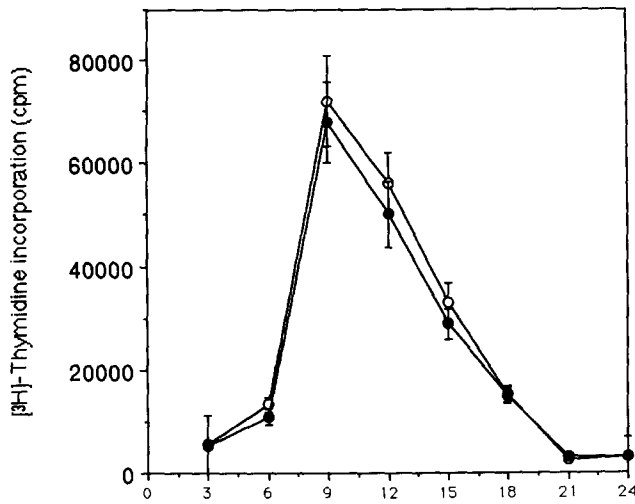


Figure 6. Effect of the neutralizing anti-TGF- β mAb, ID11.16 on [3 H]thymidine incorporation. ID11.16 (10 μ g/ml) was added from the start of the cultures. \circ and \bullet represent control and ID11.16 treated cultures, respectively. The results represent the mean + SD of quadruplicate cultures of one representative experiment.

Discussion

Mouse bone marrow cells synthesize bone proteins including ALP, collagen and osteocalcin, and form a mineralized extracellular matrix when cultured in the presence of β -glycerophosphate and vitamin C. In a previous report we showed that these phenomena were inhibited in the presence of IL-10 (Van Vlasselaer et al., 1993). The goal of this study was to determine the mechanism of action by which IL-10 elicits these suppressive effects. Work by others showed that IL-10 inhibits the production of a variety of cytokines which have

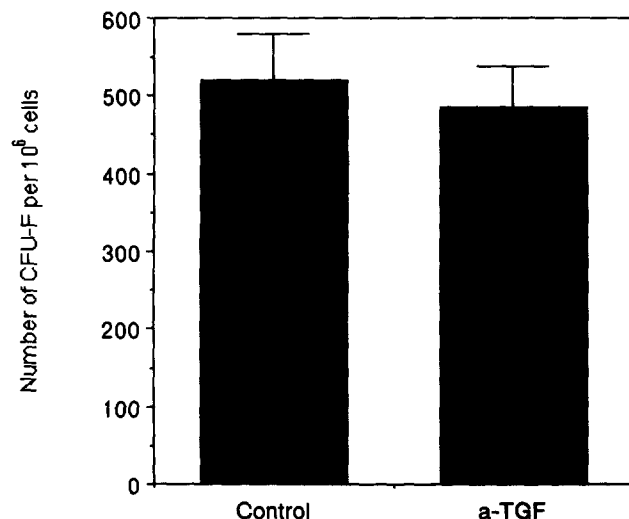


Figure 7. Effect of the neutralizing anti-TGF- β mAb, ID11.16 on CFU-F frequency. Marrow cells were plated at 5×10^4 cells per plate and grown for 10 d in the absence or presence of ID11.16 (10 μ g/ml) (α -TGF). Subsequently they were stained with May-Grunwald-Giemsa and the number of fibroblast colonies was counted. The results represent the mean + SD of quadruplicate cultures of one representative experiment.

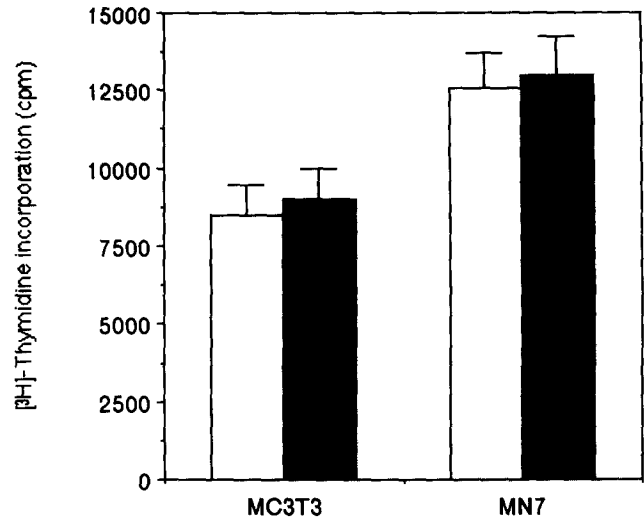


Figure 8. Effect of the neutralizing anti-TGF- β mAb, ID11.16 on the proliferation of the osteoprogenitor cell lines MC3T3 and MN7. ID11.16 (10 μ g/ml) was added from the start of the culture. White and black bars represent control and ID11.16 (10 μ g/ml) treated cultures, respectively. The cells were plated at 5×10^3 cell per well and cell proliferation was determined 24 h later. The results represent the mean + SD of quadruplicate cultures of one experiment.

been implicated in bone modulation, e.g., IL-1, IL-6, TNF- α , and GM-CSF (Fiorentino et al., 1991; Bodgan et al., 1991). In addition, this report illustrates that IL-10 inhibits endogenous TGF- β synthesis in osteogenic bone marrow cultures. This appears to be especially the case for TGF- β 1 since no detectable levels of biologically latent or active TGF- β 2 could be observed in the supernatants of the osteogenic cultures. Consequently this suggests that TGF- β 2 plays no crucial role during in vitro osteogenic differentiation of mouse marrow cells. The necessity of endogenous TGF- β 1 for in vitro osteogenic differentiation was furthermore illustrated using the TGF- β neutralizing mAbs ID11.16 and 3C7.14. ID11.16 neutralizes the biological activity of both TGF- β 1 and TGF- β 2 (Dasch et al., 1989). Both forms are abundant in bone tissue and elicit multiple comparable effects on skeletal cells in vitro (Wrana et al., 1988). However in this culture system it appears only to be TGF- β 1 which is of importance since the TGF- β 2 specific neutralizing system it appears only to be TGF- β 1 which is of importance since the TGF- β 2 specific neutralizing mAb 3C7.14 was unable to block in vitro osteogenic differentiation. This is not surprising since no detectable amounts of either biologically latent or active TGF- β 2 were observed in supernatants from osteogenic cultures in the first place. In addition, TGF- β 1 mRNA levels were reduced in IL-10 treated as compared to untreated cultures. At this point it is unclear whether this is the result of a direct inhibitory effect of IL-10 on TGF- β 1 gene transcription or merely the result of a reduction of the number of TGF- β 1 secreting cells. Although each of these possibilities needs further investigation, IL-10 was shown not to induce quantitative changes in the stromal compartment (CFU-F) but rather to direct stromal differentiation away from bone development (Van Vlasselaer et al., 1993). Although IL-10 reduces TGF- β 1 mRNA levels for only 40%, at the protein level this repre-

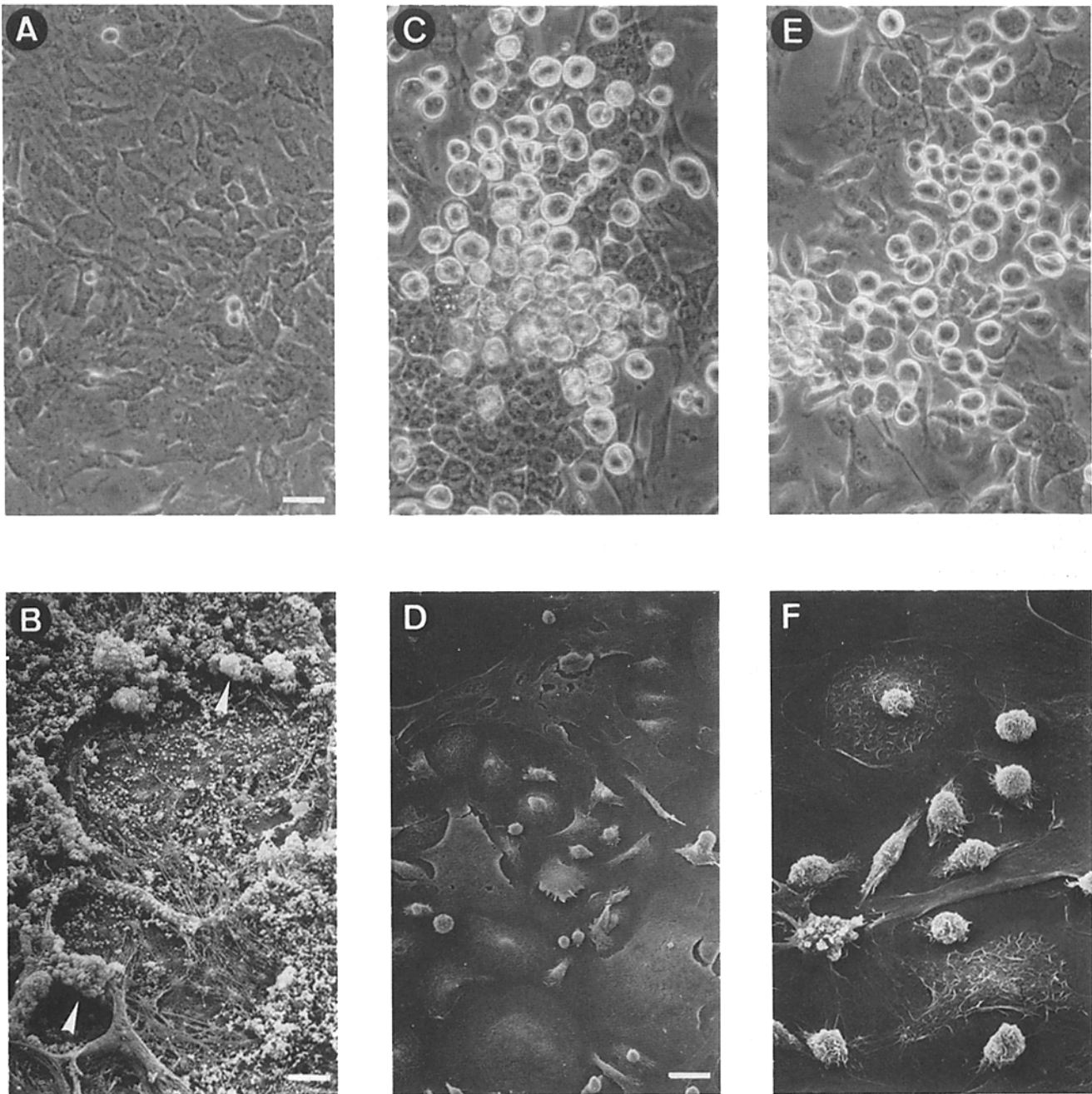


Figure 9. Effect of IL-10 and the neutralizing anti-TGF- β mAb, 1D11.16 on stroma morphology. Marrow cells were grown in the absence or presence of IL-10 (2.5×10^3 U/ml) or 1D11.16 ($10 \mu\text{g/ml}$) and examined in light microscopy on day 15 (A, C, and E) and in scanning electron microscopy on day 27 (B, D, and F). A, C, and E represent phase contrast micrographs of control, IL-10 and 1D11.16 treated cultures, respectively. B, D, and F represent scanning electron microscopy of control, IL-10 and 1D11.16 treated cultures, respectively. The mineralized collagenous extracellular matrix in the control culture (B) is indicated by white arrow heads. Bars: (A, C, and E) $22 \mu\text{m}$; (C and F) $9 \mu\text{m}$; (D) $11 \mu\text{m}$.

sents the complete lack of biologically active TGF- β 1 in the culture system. This illustrates once more that mRNA levels may provide incomplete information about the amount of biologically active material that is present in a given culture system. Direct proof for the role of TGF- β 1 in IL-10 activity, appeared from the observation that the IL-10-induced suppression of bone protein synthesis and mineralization could be reversed by adding exogenous biologically active TGF- β 1. This illustrates the crucial role of TGF- β 1 during *in vitro* osteo-

genic differentiation and correlates with previous findings illustrating the enhancing effect of TGF- β 1 on extracellular matrix formation by osteoblasts (Centrella et al., 1986).

Of particular interest is the observation that biologically latent as well as active TGF- β 1 are present in the culture supernatants. It is not clear whether TGF- β 1 is synthesized as an active form or whether latent TGF- β 1 is activated after secretion. Since osteoblasts synthesize latent TGF- β (Pfeilschifter et al., 1987; Bonewald et al., 1991) which incor-

porates in large amounts in the bone matrix (Jennings and Subburaman, 1990), we like to support the latter possibility. Hence, this implies an endogenous activation mechanism present in the mouse bone marrow culture system. More than one mode of activation of latent TGF- β has been demonstrated including acidification, heat, and protease treatment. Among others, plasmin was found to convert latent TGF- β into a 25-kD form representing active TGF- β (Lyons et al., 1990). Osteoblasts produce plasminogen activator and consequently are able to convert latent TGF- β into its active form (Hamilton et al., 1985).

TGF- β 1 is present throughout the culture period but is maximally synthesized around days 6 and 27. A relevant question is whether in vitro osteogenic differentiation requires TGF- β 1 to be present continuously or only during a restricted time frame of the culture. In a previous report we emphasized that IL-10 suppressed bone protein synthesis and mineralization only when added at the beginning of the culture (Van Vlasselaer et al., 1993). In the context of the present data this implicates a role for TGF- β 1 during the early steps of osteogenic differentiation. The latter was illustrated by the observation that 1D11.16 elicited a suppressive effect on bone protein synthesis and mineralization when added before day 10 of the culture but not when added at later time points. Hence, especially the first increase of TGF- β 1 secretion around day 6 appears to be necessary. Looking at the kinetics of bone protein synthesis this implicates that endogenous TGF- β 1 induces osteogenic differentiation at a time point which precedes the expression of the earliest bone related marker, ALP. Considering that all components of the skeleton are derived from undifferentiated mesenchymal cells, we like to envisage TGF- β 1 as a commitment factor which drives these "naive", "uncommitted" cells towards osteogenic differentiation.

The progression of mesenchymal cells through the distinct steps of osteogenic differentiation is characterized by three distinct periods: proliferation, extracellular matrix maturation, and mineralization (Owen et al., 1990; Falla et al., 1993). The present data show that TGF- β 1 is active before [3 H]TdR uptake increases. Consequently, the role of TGF- β 1 may merely be the stimulation of cell proliferation. However, TGF- β 1 appears not to function as a mitogenic trigger for osteoprogenitor cells since [3 H]TdR incorporation was comparable between control and 1D11.16 treated cultures. In addition, 1D11.16 did not affect the number of CFU-F of which ~30% represent cells with osteogenic potential (Falla et al., 1993). Moreover, [3 H]TdR uptake by the pre-osteoblastic cell lines MN7 (Mathieu et al., 1992) and MC3T3 (Sudo et al., 1983) was not altered in the presence of 1D11.16. At first sight this may contradict previous data illustrating TGF- β 1's stimulatory effect on osteoblast proliferation (Hill et al., 1986; Gehron-Robey et al., 1987). However, we like to emphasize that TGF- β elicits both stimulatory and suppressive effects on osteoblast proliferation depending on the differentiation stage of the particular cells (Centrella et al., 1987a). Hence, differential effects of TGF- β 1 on the proliferation of mature osteoblasts and immature mesenchymal cells of the bone marrow cannot be ruled out at this point.

Apart from their suppressive activity, IL-10 and 1D11.16 induced comparable changes in the morphology of cultivated marrow. Whereas control cultures contained mainly flat adherent cells with a fibroblastoid morphology, IL-10 and

1D11.16 treated cultures were characterized by the presence of significant numbers of semi- and non-adherent cells. In addition, scanning electron microscopy revealed that in contrast to the control cultures, no mineralized matrix was present in the IL-10 and 1D11.16 treated cultures. This illustrates once more that inhibiting TGF- β 1 activity results in a suppressive effect on bone protein synthesis and mineralization and underlines the importance of TGF- β 1 during extracellular matrix formation (Hill et al., 1986; Centrella et al., 1987b). In addition, IL-10 and 1D11.16 treated cultures revealed the presence of "blanket stroma" and cell aggregates resembling the "cobblestone areas" described by Allen and Dexter (1984) suggesting hematopoietic activity in these cultures. Recent studies in our laboratory support the latter idea and showed the long term generation of myeloid cells in IL-10 and 1D11.16 treated osteogenic cultures (Van Vlasselaer, P., R. Van Den Heuvel, B. Borremans, J. R. Dasch, and R. de Waal Malefyt, manuscript submitted for publication). Collectively these data indicate that IL-10 inhibits bone protein synthesis and mineralization in mouse bone marrow cultures by blocking endogenous TGF- β 1 production which is required for the osteogenic commitment of undifferentiated mesenchymal cells.

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