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TGF-β Regulation of Nuclear Proto-Oncogenes and TGF-β Gene Expression in Normal Human Osteoblast-Like Cells

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Abstract Transforming growth factor-β (TGF-β) is present in high levels in bone and plays an important role in osteoblast growth and differentiation. In order to dissect the molecular mechanisms of action of TGF-β on osteoblasts, the effects of TGF-β on the steady state mRNA levels of c-fos, c-jun, and jun-B proto-oncogenes on normal human osteoblast-like cells (hOB) and a transformed human osteoblast cell line (MG-63) were measured. Treatment of hOBs with 2 ng/ml of TGF-β_1 resulted in a rapid increase in c-fos mRNA levels as early as 15 min post-treatment. A maximum (10-fold) increase was observed at 30 min after TGF-β treatment followed by a decrease to control values. Similar responses were measured whether the cells were rapidly proliferating or quiescent. TGF-β_1 induced jun-B mRNA levels more gradually with steady increase initially observed at 30 min and a maximum induction measured at 2 h post-TGF-β treatment. In contrast, TGF-β treatment caused a time dependent decrease in the c-jun mRNA levels, an opposite pattern to that of jun-B mRNA. Treatment of hOBs with TGF-β_1 in the presence of actinomycin-D abolished TGF-β_1 induction of c-fos mRNA, suggesting that TGF-β action is mediated via transcription. In the presence of cycloheximide, TGF-β causes super-induction of c-fos mRNA at 30 min, indicating that the c-fos expression by TGF-β is independent of new protein synthesis. Further, transfection of 3 kb upstream region of jun-B promoter linked to a CAT reporter gene into ROS 17/2.8 cells was sufficient to be regulated by TGF-β_1. Interestingly, TGF-β treatment also increased the mRNA levels of TGF-β_1 itself at 4 h post TGF-β treatment, with a maximum increase observed at 14 h of treatment. TGF-β_1 treatment for 30 min were sufficient to cause a delayed increase in TGF-β protein secretion within 24 h. These data support that TGF-β has major effects on hOB cell proto-oncogene expression and that the nuclear proto-oncogenes respond as rapid, early genes in a cascade model of hormone action.

Key words: TGF-β, c-fos, jun-B, promoter regulation, osteoblasts

Transforming growth factor-β (TGF-β) is a multifunctional growth regulator involved in growth and differentiation of cells. TGF-β is known to act as growth-stimulating factor for mesenchymal cells and growth inhibitor for epithelial cells [Moses et al., 1990]. TGF-β stimulates cell proliferation in bone organ cultures of rat calvariae [Centrella et al., 1986; Pfeilschifter and Mundy, 1987]. Robey et al. [1987] have demonstrated that osteoblast-like cells synthesize and respond to TGF-β. Moreover, functional TGF-β cell surface receptors have been identified in adult human trabecular bone cells [Kells et al., 1992]. Furthermore, TGF-β has been shown to influence the transient increase in cell spreading associated with enhanced polymerization and synthesis of cytoskeletal proteins in mouse osteoblastic cells from the endosteal bone [Lomri and Marie, 1990]. Since TGF-β has been implicated to play an important anabolic role in prostate cancer [Steiner and Barrack, 1992], its presence in large quantities in bone might play a role in the metastasis of these tumor cells to bone.

TGF-β has multiple influences on bone cells in culture. TGF-β regulation of gene expression (i.e., mRNA levels) is usually measured at 18–24 h after TGF-β treatment. Using a variety of osteoblast-like cells, TGF-β has been shown to increase type I collagen, osteopontin, alkaline phosphatase, and plasminogen activator inhibitor syntheses [Noda and Rodan, 1987; Pfeilschiefter et al., 1987; Wrana et al., 1988; Noda et

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al., 1988]. In contrast, TGF-β also decreases osteocalcin synthesis and reduces vitamin D induction of osteocalcin [Noda, 1989; Bonewald et al., 1992]. An increase in the inhibitor protein of plasminogen activator and a corresponding decrease in plaminogen activator activity by TGF-β was observed in rat calvarial osteoblasts and UMR 106-01 cells [Allan et al., 1991]. TGF-β has been known to increase PTH receptors, Vitamin D receptors, and G proteins in UMR-106-06 cells [Schneider et al., 1992]. Lastly, insulin-like growth factor II and TGF-β_1 have been shown to modulate the IGF-1 secretion in mouse bone cells [Tremollieres et al., 1991].

In order to more fully understand the influence of TGF-β on bone cell metabolism, it was the focus of this investigation to study the early events that take place during growth factor stimulation. In this investigation we have measured the early responding steady state mRNA levels of c-fos, c-jun, and jun-B mRNA levels and the late responding TGF-β mRNA and protein levels following TGF-β treatment of normal human osteoblast-like cells.

METHODS

Cell Culture

Normal human osteoblast-like cells were cultured using a modification of the procedure of Robey and Termine [1985] as described previously [Eriksen et al., 1988]. Briefly, trabecular bone explants obtained during bone grafting procedures were minced in phosphate buffered saline and digested with crude bacterial collagenase (Gibco, Grand Island, NY) at 1 mg/ml in Dubecco's modified Eagles medium (DMEM, Gibco, Grand Island, NY) for 2 h at 37°C in a shaking water bath. The bone fragments were then cultured in phenol red-free medium approximately equivalent to a Ca^{++} free mixture (1:1) of Ham's F.12 (Gibco, Grand Island, NY) and DMEM plus 10% (v/v) heat inactivated fetal calf serum (FCS). The cells were maintained at 37°C in a 5% (v/v) CO_2 atmosphere. Cells derived from the explants were passaged once and used for experiments at the end of the first passage. Through the duration of the culture period, these cells maintain the many aspects of the mature osteoblast phenotype. To assess the effect of TGF-β on these hOB cells, the cells were plated at a density of 1×10^6 cells/T75 flask and grown in media (without phenol red) containing 10% (w/w) fetal calf serum. The cells were allowed to grow until reaching confluence. Confluent cells were treated with either TGF-β_1, β_2, or β_3 (R and D, Minneapolis, MN) suspended in 0.25% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) or an equal volume of 0.25% BSA in PBS for different time periods and the cells were harvested for RNA isolation. One T75 flask of hOB cells was used for each time point.

cDNA Probes

Human c-fos cDNA was a kind gift from Dr. Inder Verma, Salk Institute, La Jolla, CA. Human c-jun cDNA was provided by Dr. Robert Tjian, University of California at Berkley, CA. Mouse jun-B cDNA was obtained from Dr. Walter Eckhart, Salk Institute. The cDNA for human TGF-β_1 was provided by Dr. M. Sheppard, Genentech, Inc. (South San Francisco, CA).

Northern Blot Analysis

Total RNA was isolated from single cell strains that had been treated with either vehicle or TGF-β using a modified method of Chirgwin et al. [1979] as described previously [Lau et al., 1991]. Total RNA preparations were denatured using glyoxal-DMSO and resolved by electrophoresis in a 1% (w/v) agarose gel. The RNAs were transferred overnight to a Magna 66 nylon membrane (MSI, Fisher Scientific, Pittsburgh, PA) by capillary action in 20 × SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0). The filters were baked for 2 h at 80°C, hybridized with [^{32}P]-labeled cDNA probes, and the blots washed, as previously described [Lau et al., 1991]. The cDNA probes were labeled with [^{32}P] by random primer extension using the Multiprime DNA labeling system from NEN Research Products, Boston, MA. The [α-^{32}P]dCTP with a specific activity of approximately 3,000 Ci/mmol (New England Nuclear Research Products, Boston, MA) was used to radiolabel the cDNA to achieve specific activities approximately 10^9 cpm/mg.

Transient Transfection Assays

Rat osteosarcoma (ROS 17/2.8) cells were routinely maintained in DMEM + F12 (1:1) with 10% fetal calf serum. Twenty-four hours before transfection the cells were plated in 100 mm plates and the cells were transfected with 0.5 μg of JB 3000-CAT DNA and 1 μg of CMV-luciferase plasmid DNA as an internal control to monitor transfection efficiency. The JB3000-CAT is a mouse jun-B promoter containing 3,000 bp up-

stream of the gene linked to chloramphenicol acetyltransferase gene (CAT gene). Transient transfections were performed using the DEAE-dextran mediated transfection technique [Lopata et al., 1984]. The transfected cells were grown in 10% serum containing media for 24 h and then serum starved for 48 h by growing them in 1% FCS serum containing media. The serum starved cells were treated with TGF-β_1 (5 ng/ml) for 24 h and the cells were harvested for CAT enzyme activity. CAT enzyme assays were performed by the method of Gorman et al. [1989]. Quantiation of total protein in each cell extract was performed by the Bradford method and equal amounts of protein from each cell extract were included in each CAT enzyme reaction. The luciferase assay was performed using the luciferase assay kit obtained from Promega (Madison, WI).

TGF-β Assay

Treatment of hOB cells. Confluent hOB cells were treated with 2 ng/ml TGF-β or an equal volume of 0.25% BSA in PBS for 30 min. The media was aspirated off and the cell layer washed three times with PBS. Fresh DMEM + F12 media with 0.25% BSA was added and incubated for various time periods.

Sample preparation. Conditioned media was harvested and centrifuged at 10,000*g* for 5 min at 4°C to remove cell debris. Supernatants were divided into 4 ml aliquots and stored at −70°C until assayed. For activation of latent TGF-β, media was dialyzed against McCoy's 5A, pH 1.5, for 18 h at 4°C, and then against fresh McCoy's 5A, pH 7.4, for 18 h. For determination of the specificity of the assay, 10^{-8} M of neutralizing antibody which recognizes TGF-β_1, TGF-β_2, TGF-β_3, or all isoforms of TGF-β (R and D, Minneapolis, MN) combined with antirabbit IgG (Sigma Chemical Co., St. Louis, MO) each at 1 μg/ml (w/v) were mixed with an aliquot of activated samples for 60 min at 37°C, the samples were microcentrifuged at 3,000*g* for 30 min at 4°C, and the resultant supernatant assayed for TGF-β activity.

TGF-β bioassay. DNA synthesis inhibition assay using the mink lung cells with and without TGF-β and conditioned media was carried out as described by Danielpour et al. [1989].

Statistical analysis. The effect of treatment was compared with controls by the Student's two-tailed paired *t* test. Values are expressed as ±SEM.

RESULTS

TGF-β_1 treatment of normal human osteoblast-like (hOB) cells results in a several-fold increase of c-fos mRNA levels at 30 min post-treatment with the levels returning to control values by 60 min (Fig. 1). A similar response pattern is observed when the cells are grown in serum containing medium or serum-free media (data not shown). Since 0.25% BSA was used as a carrier in TGF-β, the control cells were treated with 0.25% BSA for 30 min to rule out the possibility that the effect is due to TGF-β and not due to BSA. In an independent study when hOB cells treated with 0.25% BSA for various time periods did not have any effect on c-fos mRNA levels (data not shown). TGF-β_1 treatment in the presence of actinomycin-D (1 μg/ml) for 30 min abolishes the TGF-β_1 induced c-fos mRNA induction (Fig. 2). In order to examine whether the increase in c-fos mRNA by TGF-β_1 requires new protein synthesis, cycloheximide (10 μg/ml) was added in the presence of TGF-β_1 for 30 min. As shown in Figure 2, super-induction of c-fos mRNA is observed.

To determine whether osteoblast-like cell lines respond similarly to TGF-β_1, MG-63 (human osteosarcoma) cells were treated with TGF-β_1

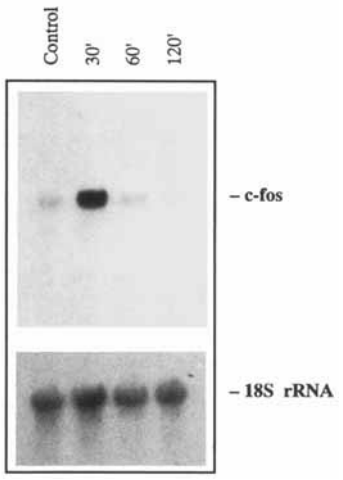

Fig. 1. Northern blot analyses of c-fos mRNA levels in hOB cells after TGF-β_1 treatment. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml) for various time periods as indicated at the top of each lane. After the indicated time periods, total RNA was isolated, processed for Northern analyses, and probed for c-fos mRNA levels. The bottom panel shows the same blot as shown above which was stripped and reprobed for 18S rRNA levels. Control represents the cells that were treated with vehicle (0.25% BSA in PBS) for 30 min. This experiment was done more than five times and the results of a representative experiment are shown in this figure.

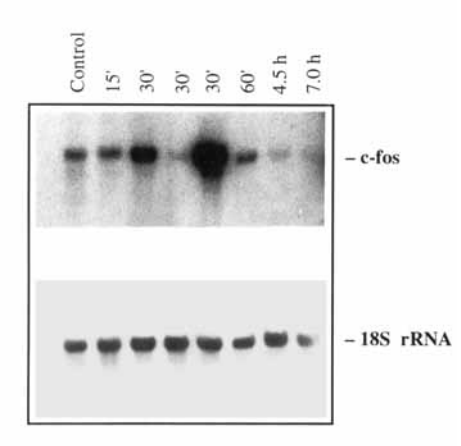

Fig. 2. Northern blot analyses of c-fos mRNA levels in hOB cells after TGF-β_1, actinomycin-D, and cycloheximide treatment. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml), actinomycin-D (1 μg/ml), and cycloheximide (10 μg/ml) as indicated for various time periods as shown on top of each lane. Total RNA was isolated from control and treated cells and processed for Northern analyses. The blots were probed for c-fos mRNA levels. The bottom panel shows the same blot shown above which was stripped and reprobed for 18S rRNA levels. This experiment was repeated at least twice and the representative results are shown.

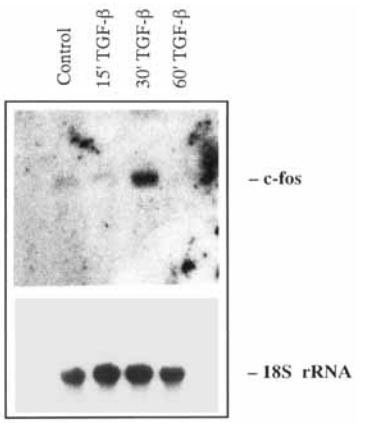

Fig. 3. Northern blot analyses of c-fos mRNA levels in MG-63 cells. Confluent MG-63 cells were washed twice in serum-free media and the cells were incubated in DMEM + F12 with 0.25% BSA containing media for 24 h. The cells were treated with TGF-β_1 (2 ng/ml) for various time periods as indicated at the top of each lane. After the indicated time periods, total RNA was isolated and processed for Northern analyses and probed for c-fos mRNA levels. The bottom panel shows the same blot as shown above which was stripped and reprobed for 18S rRNA levels. This experiment was repeated more than two times and the representative results are shown.

for various time periods and analyzed for c-fos mRNA levels (Fig. 3). A similar increase in the c-fos mRNA levels is observed at 30 min post-treatment. Since the c-fos and c-jun gene products form the AP-1 transcription factor complex, it was of interest to investigate whether TGF-β_1 has any effect on jun-B and c-jun mRNA levels in normal hOB cells. Interestingly, TGF-β_1 treatment increased jun-B mRNA levels more than 10-fold over the control levels, with a maximum increase occurring after 2 h of TGF-β_1 treatment (Fig. 4). Treatment of osteoblasts with TGF-β_1 past 2 h showed a decrease in jun-B mRNA levels (data not shown). When the Northern blot shown in Figure 4 is probed with c-jun cDNA, there was a 50% decrease in c-jun mRNA levels observed which was followed by a 90% decrease within 2 h. When TGF-β_1 was added in presence of cyclohexamide for 2 h, a super-induction of Jun-B and c-jun mRNA levels were observed (Fig. 5).

In order to determine whether TGF-β_1 has similar effects on jun-B mRNA levels in other osteoblasts, ROS 17/2.8 cells were treated with TGF-β_1 for various time periods and analyzed for jun-B mRNA. As shown in Figure 6, the TGF-β_1 treatment increased jun-B mRNA at 60 min of treatment and the maximal increase was

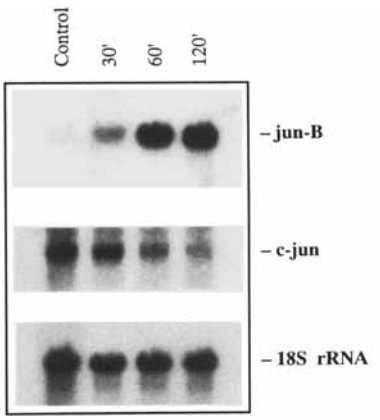

Fig. 4. Northern blot analyses of jun-B and c-jun mRNA levels after TGF-β_1 treatment. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml) for various time periods as shown on top of each lane. Total RNA was isolated and processed for Northern analyses and the same blot was probed with [^{32}P]-labeled jun-B mRNA levels (top panel), c-jun mRNA levels (middle panel), and 18S rRNA levels (bottom panel). This experiment was repeated more than four times and the representative result is shown.

observed at 120 min of treatment. This response was similar to that of normal human osteoblast-like cells.

To investigate whether different classes of the TGF-β family have any differential effect on the early gene expression, hOB cells were treated with either TGF-β_1, β_2, or β_3 for different intervals and analyzed for c-fos and jun-B mRNA

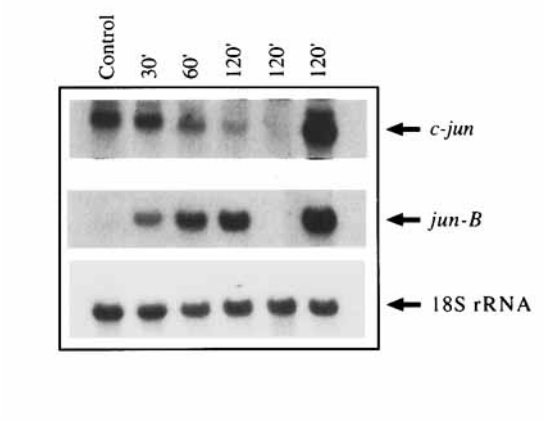

Fig. 5. Northern blot analysis of jun-B and c-jun mRNA levels after TGF-β, actinomycin-D, and cycloheximide treatment. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml), actinomycin-D (1 μg/ml), and cycloheximide (10 μg/ml) as indicated for various time periods as shown on top of each lane. Total RNA was isolated from control and treated cells and processed for Northern analyses. The blots were probed for jun-B, c-jun mRNA, and 18S RNA levels.

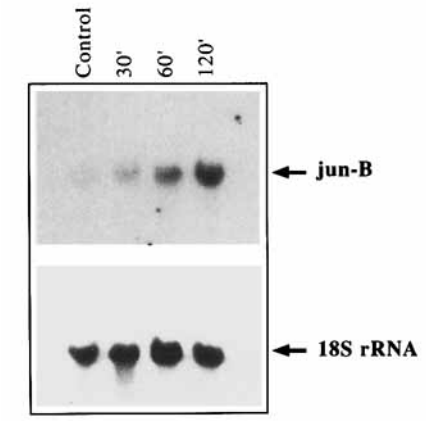

Fig. 6. [illegible] .n ROS/2.8 cells. Confluent rat osteosarcoma (ROS 17/2.8) cells were serum starved with DMEM + F12 media containing 1% fetal calf serium (FCS) for 48 h. The serum starved cells were treated with TGF-β_1 (2 mg/ml) for various time periods as shown on top of each lane. Total RNA was isolated and processed for Northern analyses and probed for Jun-B mRNA levels (top panel) and 18S rRNA levels (bottom panel). This experiment was repeated several times and the representative results are shown.

levels. All the isoforms displayed a similar mRNA induction pattern in the hOB cells (see Fig. 7). Further, to verify the transcriptional regulation of jun-B in osteoblasts, the jun-B promoter containing 3 kb upstream of the gene in the presence of CAT reporter gene was transiently transfected into ROS 17/2.8 cells. Interestingly, when the transfected cells were serum starved and then treated with TGF-β_1 for 24 h, a 4–5-fold increase in CAT activity was observed indicating the jun-B promoter is regulated by TGF-β_1 (Fig. 8). When the ROS 17/2.8 cells were transfected with PBLCAT-3 (promoterless CAT reporter) DNA and when treated with TGF-β_1 it did not show an increase in CAT activity suggesting that the effect is due to jun-B 5′-flanking sequences and not due to the vector sequences (Fig. 8).

Since TGF-β has been known to auto-induce its own mRNA and protein levels in other cell/tissue types [Obberghen-Schilling et al., 1988], we examined the influence of TGF-β on TGF-β expression in osteoblast cells. A time dependent increase in TGF-β_1 mRNA levels was observed following 8 h of treatment with a maximum reached at 16 h (Fig. 9). The earliest increase in the mRNA steady state levels was detected at 4 h post-TGF-β_1 treatment (data not shown). Since 30 min of treatment with TGF-β_1 was sufficient to induce maximal c-fos gene expression, we investigated the influence of this brief treatment on subsequent TGF-β protein secretion. To accomplish this, hOB cells were treated for 30 min with 2 ng/ml TGF-β_1 or vehicle. Following treatment, the media was aspirated off, the cells were rinsed thoroughly to remove any re-

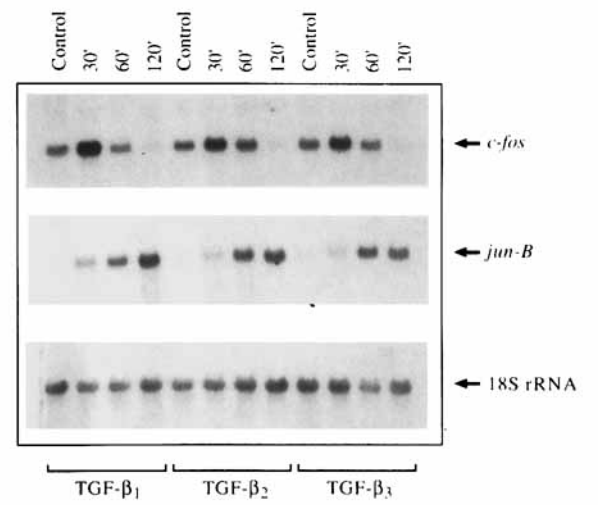

Fig. 7. Northern blot analyses of c-fos and jun-B mRNA levels after TGF-β_1, β_2, and β_3 treatment. Confluent hOB cells were treated with TGF-β_1, β_2, or β_3 (2 ng/ml) for various time periods as shown on top of each lane. Total RNA was isolated and processed for Northern analyses and the same blot was probed for c-fos mRNA levels (top panel), jun-B mRNA levels (middle panel), and 18S rRNA levels (bottom panel). This experiment was repeated at least three times and the representative result is shown.

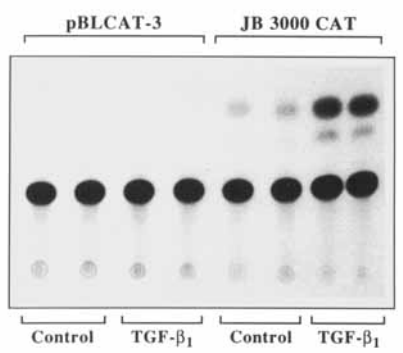

Fig. 8. TGF-β_1 regulation of jun-B promoter activity in ROS 17/2.8 cells. Jun-B promoter construct containing 3 kb upstream of the gene which is linked to a CAT reporter gene (0.5 μg) of DNA and 0.5 μg of PBLCAT-3 DNA were independently transfected in duplicates into rat osteosarcoma (ROS 17/2.8) cells using DEAE-dextran mediated transfection method. The transfected cells were serum starved for 48 h and then treated with TGF-β_1 for 24 h and the cell lysates were processed for CAT assays. CAT assays were analyzed by thin layer chromatography and the results are shown.

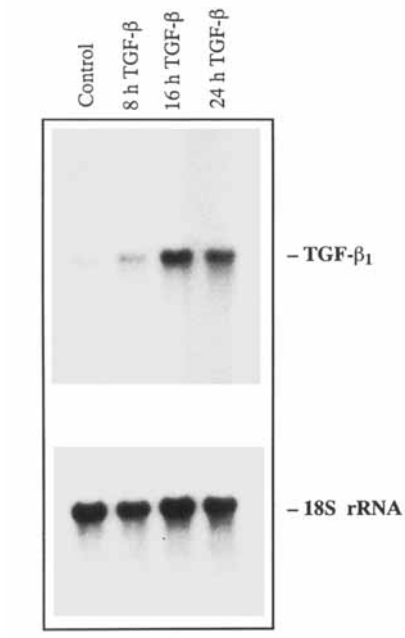

Fig. 9. Northern analyses showing the auto-induction of TGF-β_1 mRNA leves in hOB cells. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml) for various time periods as indicated at the top of each lane. Total RNA was isolated and processed for Nortern analyses. The blots were probed for TGF-β_1 mRNA levels. The bottom panel shows the same blot as shown above which was stripped and reprobed for 18S rRNA levels. This experiment was repeated at least three times and the representative result is shown.

sidual TGF-β_1, and fresh serum-free media was added.

There was no measurable TGF-β protein present 60 min following removal of the recombinant TGF-β (Fig. 10A). Within 24 h following treatment with TGF-β_1, there was an increase in TGF-β protein. The level of TGF-β protein continued to rise and reached a 5 to 6-fold increase by 48 h post-treatment. Immunoprecipitation with an antibody to all TGF-β isoforms confirmed that the assay was measuring TGF-β activity (Fig. 10B). Moreover, immunoprecipitation with isoform-specific antibodies revealed that the hOB cells mainly secreted TGF-β_1 with less, but some, TGF-β_2 and no detectable β_3 secreted.

DISCUSSION

These studies show that TGF-β treatment to hOB cells induces an increase in the steady state mRNA levels of c-fos within 30 min and jun-B mRNA within 2 h. In contrast, a decrease in c-jun mRNA is observed in these cells after TGF-β treatment. Since actinomycin D treatment completely blocked this response, the increase in c-fos mRNA by TGF-β might be regulated in part by transcription. The observation that cycloheximide treatment resulted in a superinduction of the mRNA levels suggests that TGF-β-induced nuclear proto-oncogene responses in these cells are independent of protein synthesis. This superinduction might also suggest that the transcription of this gene is under the control of a repressor protein [Sassone-Corsi et al., 1988; Subramaniam et al., 1989]. A similar type of response of the c-fos gene by serum growth factors and cycloheximide has been observed by others in mouse fibroblasts [Greenberg and Ziff, 1984]. Studies by Pertovaara et al. [1989] have demonstrated that TGF-β induces proto-oncogenes c-jun and jun-B in the human lung adenocarcinoma cell line A549 which are growth inhibited by TGF-β_1, in ARK-2B mouse embryo fibroblasts which are growth stimulated by TGF-β, and, interestingly, in k562 human erythroleukemia cells that are not affected by TGF-β. They have concluded from their studies that the measured increase in the proto-oncogenes c-jun and jun-B are the early genomic responses to TGF-β in these cells. Furthermore, Li et al. [1990] have shown that TGF-β treatment to BC_3H1 myocytes induces jun-B mRNA 20-fold over the control levels at 2 h post-treatment, suggesting that this might be the mechanism by which TGF-β mediates intercellular signalling. Recent studies by Hashimoto et al. [1993] have shown that activin, a member of TGF-β family, induced jun-B mRNA within 1 hour in human

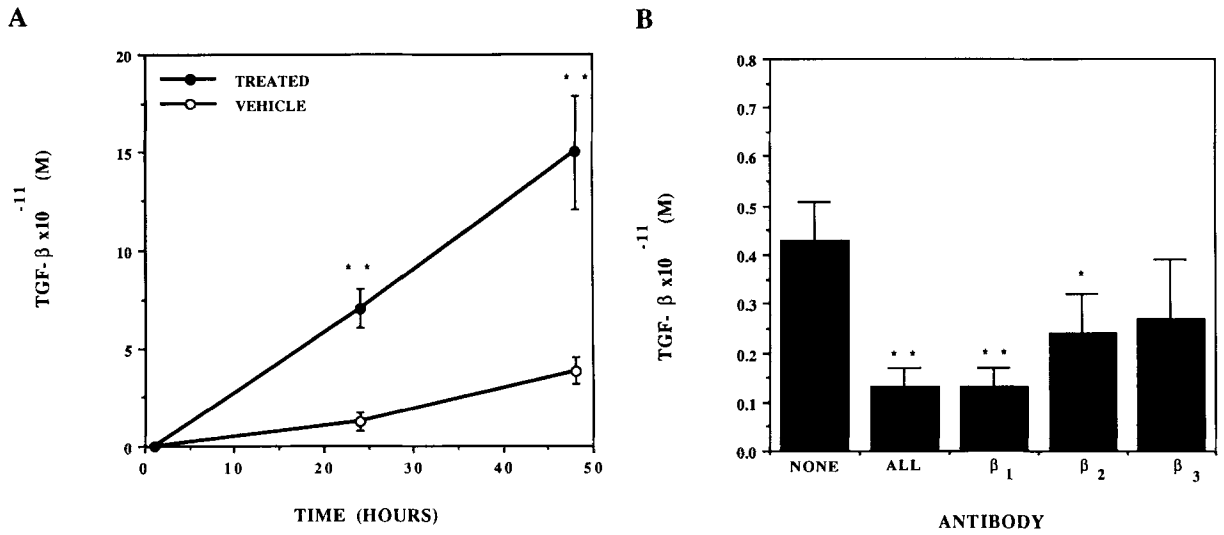

Fig. 10. Autoinduction of TGF-β protein in hOB cells. Confluent hOB cells were treated with TGF-β_1 (2 ng/ml) for 30 min and the cells were washed three times with PBS, and incubated in 10 ml of DMEM + F12 media with 0.25% BSA for various time periods. **A:** After the indicated time periods, the conditioned media was harvested and used for TGF-β bioassay. **B:** An aliquot of diluted conditioned media from 48 h TGF-β_1 treated cultures was acid activated and the TGF-β removed as described in the Methods Section with the following antibodies: none; no anti-TGF-β antibody; all, anti-TGF-β antibody to all isoforms; β_1, anti-TGF-β_1 antibody; β_2, anti-TGF-β_2 antibody; or β_3, anti-TGF-β_3 antibody. The amount of remaining TGF-β activity was then determined by bioassay. $^*P < 0.05$; $^{**}P < 0.01$. Data are the average ±SD of quadruplicate samplings. This experiment was done three times and these data are representative of the results.

K562 myelogenous leukemia and rat PC12 cells. In the same study they have shown that activin did not induce c-fos or c-jun mRNA levels in these cells. Thus, the early responses of jun and fos to TGF-β_1 may be a prerequisite event in TGF-β action but other factors/processes are probably required for the complete biological responses of cells to TGF-β.

Our results demonstrate that TGF-β down-regulates c-jun mRNA levels in a time-dependent manner. This inhibition of c-jun gene expression can be readily explained in terms of the increase jun-B expression by TGF-β. Chiu et al. [1989] have shown that jun-B is a negative regulator of c-jun, a similar mechanism may be involved in the findings reported here.

Several isoforms of TGF-β (β_1, β_2, β_3, β_4, β_5, and $\beta_{1.2}$) which differ slightly in their amino acid sequence have been identified, but the differences between the isoforms with regard to the biological effects remains unresolved [Roberts et al., 1991]. Although all isoforms will bind to the three known classes of TGF-β receptors, the relative affinities of the various isoforms for the different receptors vary [Roberts and Sporn, 1990]. The primary differences between the isoforms resides in the upstream regulatory regions of their genes and their sites of synthesis and localization in vivo differ [Jakowlew et al., 1991; Pelston et al., 1991; Thorp et al., 1992]. Using the mouse osteoblast-like cell line MC3T3-El, there appeared to be little difference between the potency of TGF-β_1 and TGF-β_2 in the inhibition of alkaline phosphatase activity [Ibbotson et al., 1989]. Interestingly, TGF-β_3 appears to be several-fold more potent than TGF-β_1 in rat calvarial cell cultures when measuring mitogenic activity, collagen synthesis, and alkaline phosphatase activity [Dijke et al., 1990]. This increased potency of TGF-β_3 correlates with an increased binding affinity of TGF-β_3 for these mixed cell cultures. The differences between these data and ours is unresolved but may be the result of species differences or the use of a cultured cell population containing multiple phenotypes in the previous work.

TGF-β treatment to hOB cells not only induces early responding genes but also induces its own mRNA at a later time point. A brief (30 min) exposure of TGF-β was sufficient to cause an increase in TGF-β protein secretion into the medium 24 to 48 h later. In a similar manner, studies by Ranganathan and Getz [1990] have shown that TGF-β_1 treatment of AKR-2B cells for 10 min was sufficient to bring about a delayed EGF-dependent transcription of fibronec-

tion, β-actin, and γ-actin genes in these cells. The response appeared specific for TGF-β since a similar short term treatment with PDGF or FGF had little or no effect. Furthermore, MG-63 cells pre-incubated with TGF-β for 30 min followed by treatment with dihydroxy Vitamin D 1,25 in the absence of TGF-β was sufficient for maximal induction of alkaline phosphatase activity [Bonewald et al., 1992].

In this report, we have demonstrated that TGF-β not only induces jun-B mRNA levels but also regulates the promoter activity in normal human osteoblast cells in culture. It is also evident that the TGF-β responsive element resides within the 3 kb upstream region of the jun-B gene. Further studies are in progress to identify the sequences involved in the rapid regulation of jun-B by TGF-β in osteoblasts. The presence of TGF-β responsive elements has been demonstrated in other systems. The demonstration for the presence of TGF-β responsive element was shown in mouse α2 (I) collagen gene by Rossi et al. [1988] who demonstrated that TGF-β induces this gene through a NF-1 binding site (GCCAAT). A TGF-β responsive element has also been demonstrated in plasminogen activator inhibitor Type I gene by Westerhausen et al. [1991]. By promoter deletion analysis it was demonstrated that two TGF-β responsive elements: one between −791 to −323 and another between −328 to −186 bp, upstream of PAI-I cap site. Interestingly, these two regions were different from AP-1 and NF-1 binding sites. Studies by Ritzenthaler et al. [1993] have shown that TGF-β regulation of α1(I) collagen gene in human lung fibroblasts is mediated through TGF-β activating element (TAE) sites by protein complexes independent of NF-1 or AP-2 protein. In another independent study, de Groot and Kruizer [1990] have demonstrated that TPA responsive element and dyad symmetry element were found to be activated by TGF-β_1 in 3T3 and ML-CCL64 cells. A study by Riccio et al. [1992] have shown that, in the α2(I) collagen promoter, CTF/NF-1 binding sites and a USF binding site are necessary for a TGF-β response. From the above studies, it is clear that the TGF-β responsive elements from different genes vary and are complex.

Since TGF-β rapidly induces the oncogenes c-fos and jun-B in normal hOB cells, this may be one mechanism by which TGF-β mediates its effect on these cells. The oncoproteins of Fos and Jun form a heterodimeric complex and bind to regions of the gene that contains AP-1 binding sequences, thus mediating gene transcription [Ransone and Verma, 1990; Vogt and Bos, 1990]. The jun family encodes a component of AP-1 transcription factor. The murine c-jun and jun-B have been shown to be rapidly induced by serum growth factors in murine cells. In the same system, jun-D was shown to be differently regulated than c-jun and jun-B [Ryder et al., 1989]. The c-jun, jun-B, and jun-D share a significant homology among themselves [Ryder et al., 1989; Schutte et al., 1989]. Studies by Chiu et al. [1989] have shown that c-jun is an efficient activator of genes that contain even a single AP-1 binding sequence whereas jun-B is an efficient activator only when genes contain multiple AP-1 binding sequences.

The AP-1 binding sites have been shown to be involved in TGF-β regulation of the TGF-β_1 promoter [Kim et al., 1990]. Since the TGF-β_1 promoter contains at least two AP-1 binding sites and jun-B has been shown to efficiently regulate genes which contain multiple AP-1 binding sites [Chiu et al., 1989], it is possible that the jun-B protein might participate in the TGF-β_1 regulation in these cells. The mechanism whereby TGF-β induces c-fos and jun-B genes while down-regulating c-jun is not known at present. These studies and those from other laboratories suggest that TGF-β might exert its early nuclear events by inducing the nuclear proto-oncogenes Fos and Jun-B which, in turn, might induce TGF-β gene and other gene products involved in the growth and differentiation of osteoblasts. A similar mechanism has been proposed for TGF-β action in AKR-2B cells by Leof et al. [1986] where TGF-β induces c-fos, c-myc, and other PDGF-inducible genes which then leads to the mitogenicity of these cells.

These data are consistent with a cascade model of hormone action that involves rapid modulation of early regulatory genes, such as those coding for transcription factors including the Fos and Jun complex (i.e., AP-1). These transcription factors then regulate the transcription of more slowly responding structural genes such as those coding for growth factors. This process may explain many processes in the TGF-β regulation of bone physiology, especially osteoblasts.

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