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Cytokine-Specific Induction of the TGF-β Inducible Early Gene (TIEG): Regulation by Specific Members of the TGF-β Family

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Abstract Select members of the TGF-β family of cytokines play key regulatory roles in skeletal development, structure, and turnover. This laboratory has previously reported that TGF-β treatment of immortalized normal human fetal osteoblast (hFOB) cells results in the rapid induction of the mRNA levels of a TGF-β inducible early gene (TIEG) followed by changes in cell proliferation and bone matrix protein production. Previous studies have also shown that nonmembers of the TGF-β superfamily showed little or no induction of TIEG mRNA. This article further addresses the cytokine specificity of this TIEG induction by examining whether activin and select bone morphogenetic proteins, (BMP-2, BMP-4, and BMP-6), which are representative of different subfamilies of this superfamily, also induce the expression of TIEG in hFOB cells. However, TGF-β remained the most potent of these cytokines, inducing TIEG mRNA steady-state levels at 0.1 ng/ml, with a maximum induction of 24-fold at 2.0 ng/ml. The BMP-2 (16-fold), BMP-4 (4-fold), and activin (1–3-fold) also induced TIEG mRNA levels, but at reduced degrees compared to TGF-β (24-fold), and only at much higher cytokine concentrations, e.g., 50–100 ng/ml, compared to 2 ng/ml for TGF-β. BMP-6 showed no effect on TIEG mRNA levels. The TIEG protein levels generally correlated with the mRNA steady-state levels. As with TGF-β, BMP-2 treatment of hFOB cells was shown by confocal microscopy to induce a rapid translocation of the TIEG protein to the nucleus. In summary, the relative potencies of these TGF-β family members to induce TIEG expression generally follows the general osteoinductive capacity of these cytokines, with TGF-β ⩾ BMP-2 > BMP-4 > activin ⩾ BMP-6. J. Cell. Biochem. 78:380–390, 2000.

Key words: TGF-β; BMPs; TIEG; early gene; mRNA; osteoblasts

The transforming growth factor β (TGF-β) family is one subgroup in the expanding TGF-β superfamily, which includes three main families: the TGF-βs, the activins, and the bone morphogenetic proteins (BMPs). The latter can be further divided into subfamilies of BMP-2/4 and BMP-5–8 subfamilies [Massagué and Weis-Garcia, 1996; Centrella et al., 1995b]. The TGF-βs are involved in the growth and differentiation of many tissues including the skeleton, where TGF-β appears to be a major factor in the differentiation of osteoblast cells and regulates the proliferation of osteoprogenitor cells, as well as the bone-forming activities of mature osteoblast cells [Bonewald and Dallas, 1994; Centrella et al., 1994]. There are five isoforms, and TGF-$\beta_{1,2,3}$ appear to be the most important for mammalian physiology, with TGF-β_1 being the most extensively studied of the isoforms and an integral component of bone physiology.

Other members of the TGF-β superfamily also play important roles in skeletal formation. Although TGF-β influences the proliferation of osteoblasts, it does not induce ectopic bone formation as do the BMPs. Urist and coworkers [Urist et al., 1983] were the first to identify a bone-forming protein that was later found in bone as an active agent in ectopic bone forma-

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tion [Sampath et al., 1987]. There are now numerous BMP molecular species that are classified into two main subfamilies, based on the amino acid sequence of the mature protein [McPherron and Lee, 1996]. The BMP-2 and BMP-4 species are a closely related subfamily with a 92% amino acid sequence homology in the C-terminal region, and both play an important role in bone physiology. The BMP-5, BMP-6, BMP-7, and BMP-8 species comprise the second subfamily with only a 70%–89% amino acid sequence homology among them. Of all the BMP members, BMP-2 has recently been shown to have the highest capacity to form ectopic bone [Kusumoto et al., 1997].

Activin, a member of a different subfamily of the TGF-β superfamily, is also expressed in bone, stimulates BMP-induced bone formation, induces proliferation of osteoblasts, and stimulates the synthesis of type I collagen [Ogawa et al., 1992; Centrella et al., 1991]. Recently rat osteoblast cells were shown to express types I and II activin receptors, but only during periods of rapid bone formation, e.g., during early intramembranous and endochondral bone formation in rat bone fractures [Shuto et al., 1997].

This laboratory previously discovered a TGF-β inducible early gene (TIEG), which is induced by TGF-β_1 within 1 h of the growth factor treatment [Subramaniam et al., 1995]. TIEG encodes a 480 amino acid protein and has a unique N-terminal end. The C-terminal region of the gene contains 3 zinc finger motifs and several proline-rich *src* homology-3 domains. The zinc finger region of TIEG shows homology to other transcription factors such as Sp-1, Wilm's tumor, BTEB, EGR-1, and the mouse Krüppel factor. In view of TIEG's homology to other transcription factors, it is of interest that TIEG protein contains several nuclear localization signals. Recent studies from our laboratory have shown that estrogen, an important anabolic hormone for bone, also induces TIEG mRNA steady-state levels in human osteoblasts [Tau et al., 1998]. Further studies from our laboratory also have demonstrated that TIEG mRNA induction in osteoblast was growth factor specific, i.e., the TGF-β family members, TGF-β and epidermal growth factor, were the major inducers, whereas other cytokines, e.g., interleukin-6 (IL-6), insulin-like growth factor-I (IGF-I), IGF-II, tumor necrosis factor-α, IL-1β platelet-derived growth factor, and fibroblast growth factor (FGF), had no effect [Subramaniam et al., 1995]. Recent immunohistochemical studies using TIEG polyclonal antibodies in human tissues has demonstrated that TIEG protein was expressed in a cell- and tissue-specific manner [Subramaniam et al., 1998]. Finally, overexpression of TIEG protein in human osteoblasts and in pancreatic epithelial carcinoma cells not only inhibited cell proliferation in both cell types, but also induced apoptosis in the latter [Tachibana et al., 1997].

The studies presented in this article further characterize this cytokine specificity by assessing the potency among select members of the TGF-β superfamily in inducing TIEG mRNA and protein steady-state levels. These studies demonstrate a greater selective specificity of induction in human osteoblasts. Although TGF-β remains the most potent inducer, the other members of this superfamily (BMP-2, BMP-4, and activin) are also capable of inducing TIEG, but only at much higher concentrations.

METHODS

Cell Culture

All experiments used an immortalized human fetal osteoblast cell line, hFOB cells [Harris et al., 1995]. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (Sigma, St. Louis, MO) with 10% (vol/vol) fetal bovine serum (FBS) and 300 μg/ml geneticin (Gibco BRL, Grand Island, NY) in T-75 flasks (Corning). They were then subcultured in 100-mm dishes (Falcon) for the Northern analysis. For culture expansions, the cells were grown at the permissive temperature, 34°C, in a humidified atmosphere of 95% air and 5% CO_2. The recombinant human BMP-2 (rhBMP-2), rhBMP-4, and rhBMP-6 were gifts from the Genetics Institute (Cambridge, MA).

Northern Analysis

Cells were grown to confluence in T-75 flasks and were subcultured in 100-mm dishes at a density of 60,000 cells/cm^2. The cells were allowed to recover for 24 h before changing the media to serum-free media composed of DMEM/Ham's F12, 0.25% (vol/wt) bovine serum albumin (BSA), and 300 μg/ml geneticin. The cells were grown in the serum-free media

for 48 h and then treated with TGF-β, rhBMP-2, rhBMP-4, rhBMP-6, or activin for various periods before extracting the RNA. The total RNA was isolated by a modified method of Chirgwin et al. [1979]. Eight to 10 μg of total RNA were incubated with dimethyl sulfoxide: glyoxal for 1 h at 50°C, and then separated in a 1% (wt/vol) agarose gel. The RNA was transferred by capillary diffusion from each gel to nylon membranes (MSI, Westborough, MA). The membranes were then incubated at 42°C in a hybridization solution consisting of 50% (vol/vol) formamide, 10% (vol/vol) sodium dodecyl sulfate (SDS), 50 μg/ml salmon sperm DNA, and probed with [^{32}P]-labeled TIEG cDNA [Subramaniam et al., 1995] and normalized to 18s rRNA. A Kodak 1-D system was used to digitally analyze and quantitate the Northern blots.

Immunoprecipitation

The hFOB cells were plated at 50,000 cells/cm^2 in 100-mm dishes and were allowed to recover for 48 h in medium composed of DMEM/Ham's F-12, 10% FBS (vol/vol), and 300 μg/ml geneticin. After the recovery period, the cells were grown in serum-free media (DMEM/Ham's F-12, 0.25% (wt/vol) BSA, and 300 μg/ml geneticin) for 24 h. The cells were treated for 2 h with either vehicle [0.25% (wt/vol) BSA in phosphate-buffered saline (PBS)], TGF-β_1 (2 ng/ml), rhBMP-2 (100 ng/ml), rhBMP-4 (100 ng/ml), rhBMP-6 (100 ng/ml), or activin (100 ng/ml). After 2 h, the media was changed to methionine-free DMEM/Ham's F12 (Sigma), the corresponding growth factor, and 125 μCi/ml [^{35}S]-methionine was added to each dish and allowed to incubate for an additional 2 h. The unincorporated [^{35}S]-methionine was removed, and the cells were rinsed with PBS and lysed in 0.5 ml lysis buffer containing a protease inhibitor cocktail [1% (vol/vol) NP-40, 0.5% (wt/vol) deoxycholic acid, 0.1% (wt/vol) SDS, 0.001% (wt/vol) pepstatin A, 0.001% (wt/vol) chymostatin, 0.001% (wt/vol) antipain, 0.001% (wt/vol) leupeptin, 5 U/ml aprotinin, 0.2% (wt/vol) phenyl methyl sulphonyl fluoride in PBS]. The sheared lysate was incubated on ice for 15 min and centrifuged at 14,000 rpm for 20 min at 4°C. Trichloroacetic acid (TCA) precipitate counts were performed from supernatants of each sample, and aliquots of the supernatants containing equal counts per minute (CPMs) were incubated for 2 h with 4 μg PAb TIEG-228 at 4°C. A 50:50 (wt/vol) protein A/Sepharose slurry was added to all samples, and they were incubated while rocking for 2 h at 4°C. The beads were washed four times with RIPA [RadioImmuno-Precipitation Assay buffer, 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS in PBS] buffer, and then suspended in SDS sample buffer and boiled for 5 min to separate the protein aggregates. The samples were loaded on a 5%–15% (weight/weight) gradient SDS-polyacrylamide gel electrophoresis (PAGE) gel, and the gels were fixed, incubated in Autoflour (National Diagnostics) for 2 h, and dried. The gels were exposed to X-OMAT film (Kodak) at −70°C.

Immunofluorescence

The hFOB cells were plated at a density of 5,000 cells/cm^2 in Permonox 8 chamber slides and were allowed to recover for 48 h in DMEM/F-12 media containing 10% (vol/vol) FBS and geneticin, 300 μg/ml. After 48 h, the cells were placed in serum-free media, DMEM/F-12 and 0.25% (wt/vol) BSA, for 24 h and then treated with vehicle, 0.25% (wt/vol) BSA in PBS, or rhBMP-2 100 ng/ml for 2 h. Before fixation, the cells were rinsed with PBS and fixed with ice-cold acetone for 10 min. After fixation, the cells were rinsed extensively with PBS. Normal goat serum was used to block nonspecific binding, and normal rabbit IgG was used as the secondary antibody control. The cells were incubated with PAb TIEG-228, 1 μg/ml, for 30 min at room temperature in a humidified chamber. The cells were rinsed extensively with PBS and then incubated with the goat anti-rabbit secondary antibody conjugated with dichlorotriacinyl aminofluorescein (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:200. The mounting media used was a glycerol solution containing paraphenylenediamine. Slides were analyzed using a laser scanning confocal microscope (LSM 310, Carl Zeiss, Oberkochen, Germany) equipped with an argon/krypton laser, set to an excitation wavelength of 488 nm and emission filter of 530 nm. The objective lens was a Zeiss plan-neofluar 40×, oil immersion and the images were stored digitally.

RESULTS

TGF-β_1 was previously shown to induce TIEG mRNA by more than 10-fold in human

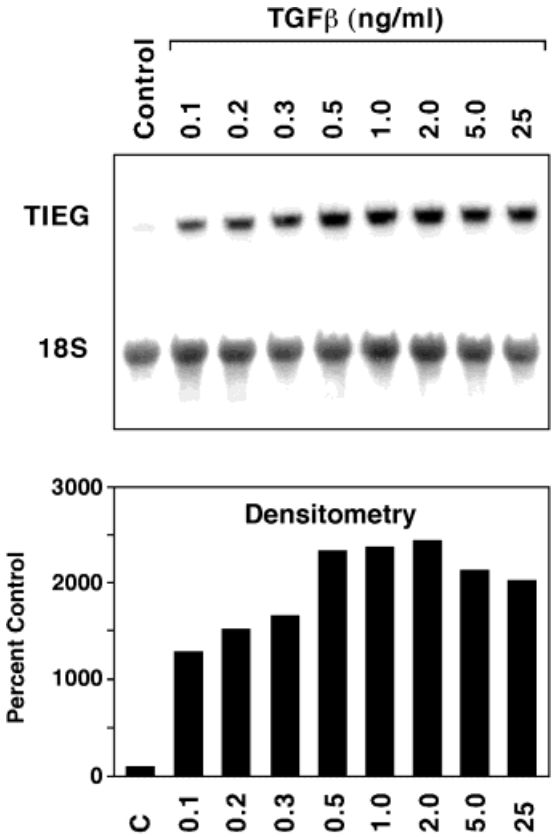

Fig. 1. Induction of TIEG mRNA in hFOB cells with increasing doses of TGF-β. TIEG mRNA steady-state levels were determined by Northern blot analysis of total RNA isolated from hFOB cells treated for 90 min with increasing doses of TGF-β, as indicated on the abscissa. The control cells were treated with vehicle, 0.25% (vol/vol) BSA in PBS. The hFOB cells were serum starved in DMEM/Ham's F-12 containing 0.25% BSA for 48 h before treatment. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control for normalization. The lower panel is a graph of densitometry data for TIEG mRNA normalized to 18S RNA and expressed as percent of vehicle control.

osteoblasts [Subramaniam et al., 1995] as well as in pancreatic epithelial cells [Tachibana et al., 1997]. Figure 1 represents a Northern blot of TIEG mRNA steady-state levels in hFOB cells treated with varying concentrations of TGF-β ranging from 0 to 25 ng/ml. The maximum induction of TIEG mRNA levels of 24-fold occurred in the range of 0.5 to 2.0 ng/ml TGF-β treatment. This marked induction remained high even at 25 to 50 ng TGF-β/ml. It was of interest to determine whether or not the various isoforms of TGF-β (TGF-β_2 and TGF-β_3) displayed the same extent and pattern of induction of the TIEG mRNA steady-state levels. All three species showed a maximal induction of TIEG at 0.5–2.0 ng/ml concentrations. Figure 2 shows Northern blot analyses of total RNA isolated from hFOB cells treated with 2 ng/ml of TGF-β_1, β_2, or β_3, and harvested after 30, 60, 120, or 240 min. The three isoforms of TGF-β exhibited a similar induction of TIEG mRNA steady-state levels. This induction was transient, with an early induction by 30 min posttreatment, a maximal induction at 1 h, and a return to control levels by 4 h of treatment.

To determine the optimal concentrations of the other superfamily members, e.g., BMP-2, BMP-4, BMP-6, and activin, for the induction of the TIEG mRNA levels in the hFOB cells, cytokine dose–response experiments were performed. Preliminary studies showed no effect of these factors at the 0.1–2.0-ng range, but did show induction at higher concentrations (data not shown). Figure 3 shows a dose–response for BMP-2 effects on TIEG mRNA levels. Compared to vehicle-treated control, a dose-dependent induction was observed, with a maximal induction of 16-fold occurring after the hFOB cells were treated at concentrations of 50–100 ng/ml of rhBMP-2. Similarly, as shown in Figure 4, TIEG mRNA levels in the hFOB cells treated with BMP-4 ranging from 2 ng/ml to 100 ng/ml for 2 h showed only a relatively small induction of fourfold of TIEG mRNA steady-state levels when compared to vehicle control. When similar experiments were performed with BMP-6 at doses of 2–100 ng/ml, no induction of TIEG mRNA was observed (data not shown). When dose–response studies were performed on hFOB cells using increasing concentration of activin from 2 ng/ml to 200 ng/ml, the mRNA steady-state levels showed a slight induction of TIEG mRNA levels of one- to threefold at 50 ng/ml (Fig. 5). To compare the relative potencies of the induction among the various family members, with regard to the degree of the induction of TIEG mRNA, serum-starved hFOB cells were treated with 50 and 100 ng/ml of each cytokine for 90 min, and the TIEG mRNA levels were measured (Fig. 6). As shown in Figure 6, BMP-2 displayed the largest induction (i.e., sixfold) of the TIEG mRNA levels, whereas activin and BMP-4 displayed a moderate induction (of two- to fourfold) and, as expected, the BMP-6 showed no induction.

To determine whether or not TGF-β and BMPs also induce TIEG protein expression,

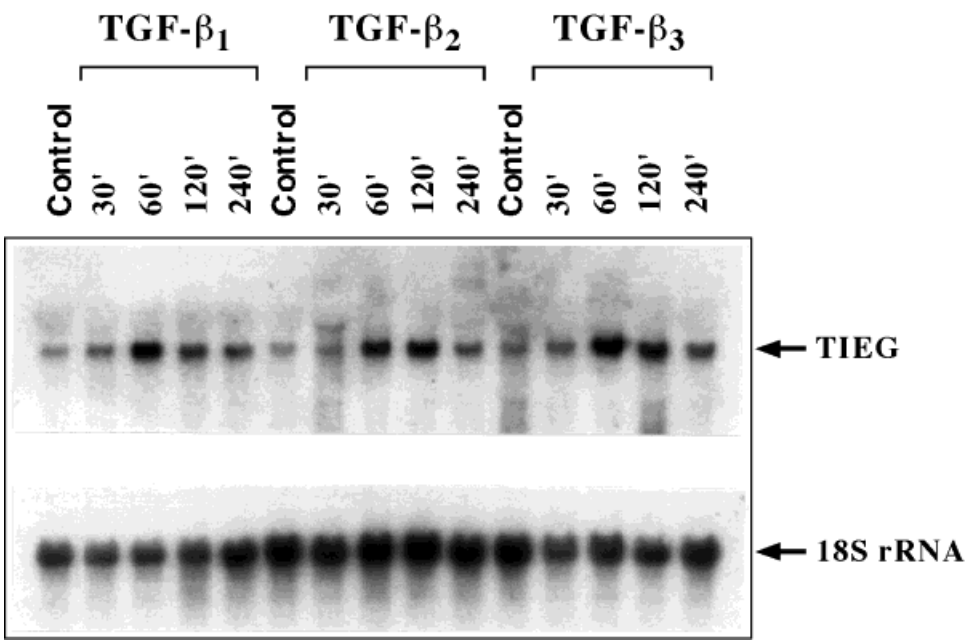

Fig. 2. Chronological patterns of the induction of TIEG expression in human osteoblasts by TGF-β isoforms. TIEG mRNA steady-state levels were determined by Northern blot analysis of hFOB cells treated for 30, 60, 120, or 240 min with either TGF-β_1, TGF-β_2, or TGF-β_3 at 2 ng/ml. The control cells were treated with vehicle, 0.25% (vol/vol) BSA in PBS. The hFOB cells were grown in DMEM/Ham's F-12 containing 0.25% BSA for 48 h before treatment. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control.

hFOB cells were treated for 2 h with vehicle (control) or optimal levels of each cytokine: TGF-β (2 ng/ml), BMP-2 (100 ng/ml), BMP-4 (100 ng/ml), or BMP-6 (100 ng/ml) and were labeled with [^{32}S]-methionine for 2 h. The [^{35}S]-labeled TIEG protein was isolated by immunoprecipitation with TIEG polyclonal antibody, followed by SDS-PAGE analyses of the precipitate, as described in the Methods section. As shown in Figure 7, TGF-β_1, BMP-2, BMP-4, and activin increased the TIEG protein levels in hFOB cells, whereas BMP-6 had little effect on the TIEG protein levels. The degree of induction of the TIEG protein are in general agreement with those of the TIEG mRNA levels as described above.

Because the predicted amino acid structure of TIEG protein contains several nuclear localization signal sequences and the C-terminal region shows homology to several transcription factors, it was of interest to examine the intracellular localization of the TIEG protein with BMP-2 treatment. Figure 8 shows the results of confocal microscopy using laser-based, fluorescence detection of immunofluorescent-labeled TIEG in hFOB cells in the absence or presence of rhBMP-2. As show in Figure 8A, cells probed with IgG-nonspecific antibody showed no fluorescence. Interestingly, Figure 8B shows that in cells probed with the TIEG polyclonal antibody, but treated with vehicle (0.25% BSA in PBS), the endogenous fluorescent-labeled TIEG protein is located mostly in the cytoplasm/perinuclear region. In Figure 8C, after 2-h treatment with 100 ng/ml BMP-2, an increased fluorescence is now found in the nucleus, with little or none in the cytoplasm.

DISCUSSION

In these studies, we have examined the response of TIEG gene expression in conditionally immortalized hFOB to members of three different families of the TGF-β superfamily: TGF-β, including its three isoforms, the activins, and two subfamily groups (BMP-2 and BMP-6) of the BMP family. All three TGF-β isoforms, β_1, β_2, and β_3, were equally potent in their induction of TIEG mRNA steady-state levels and displayed the same chronological induction pattern. This response mimics relatively similar potencies of the individual TGF-β isoforms in skeletal tissue [Centrella et al., 1995a]. All TGF-β isoforms bind with high

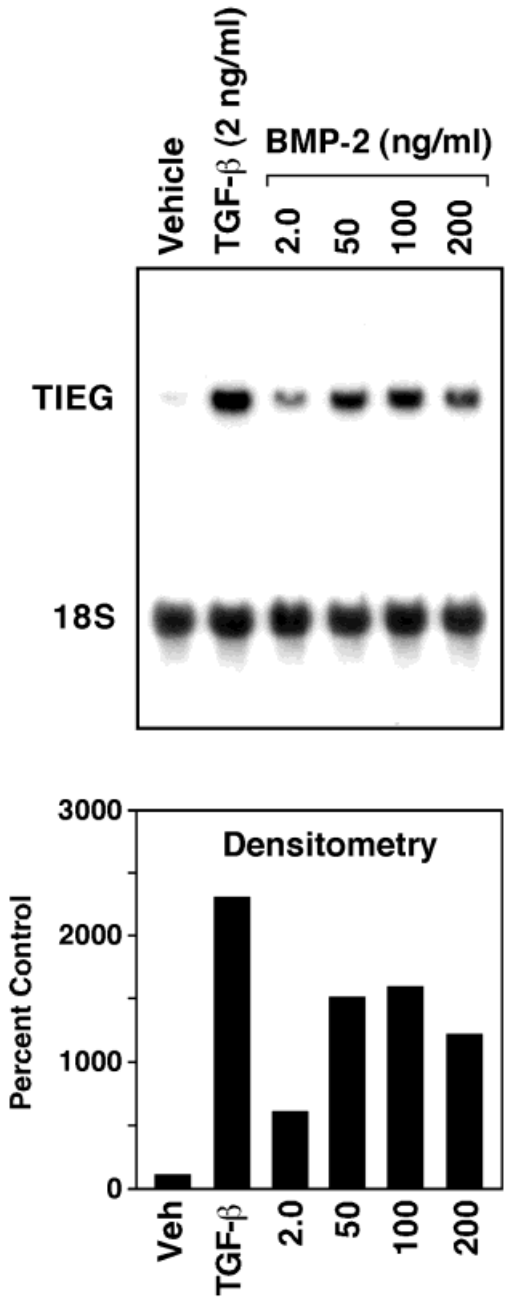

Fig. 3. Induction of TIEG mRNA in hFOB cells with increasing doses of BMP-2. TIEG mRNA steady-state levels were determined by Northern blot analysis of total RNA isolated from hFOB cells treated for 2 h with 50, 100, or 200 ng/ml rhBMP-2. The positive control represents cells treated with TGF-β_1, 2 ng/ml, for 2 h. The control cells were treated with vehicle, 0.25% (wt/vol) BSA in PBS. The hFOB cells were grown in DMEM/Ham's F-12 containing 0.25% BSA for 48 h before treatment. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control. The lower panel is a graph of densitometry data for TIEG mRNA normalized to 18S RNA and expressed as percent of vehicle control.

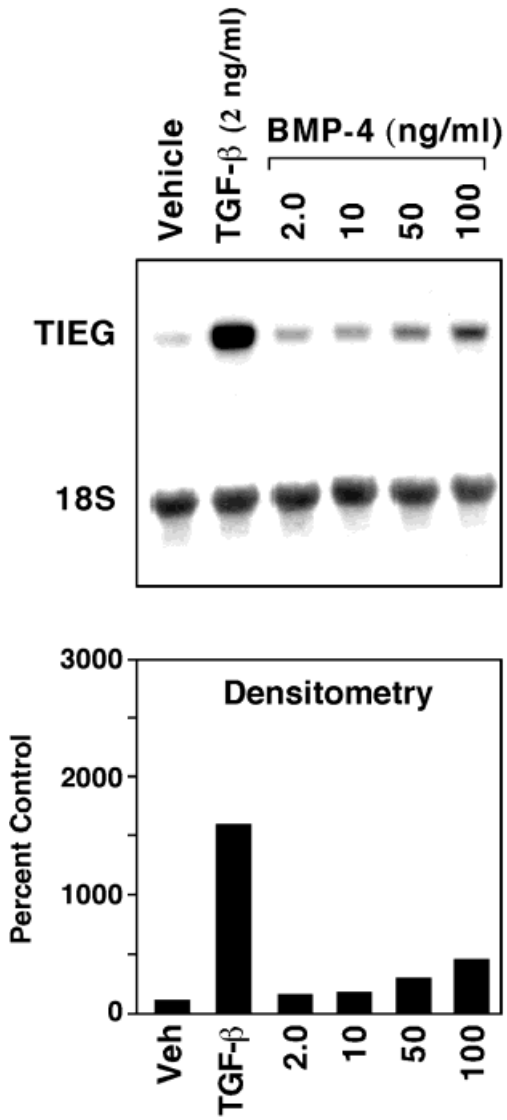

Fig. 4. Induction of TIEG mRNA in hFOB cells with increasing doses of BMP-4. TIEG mRNA steady-state levels were determined by Northern blot analysis of total RNA isolated from hFOB cells treated for 2 h with 50, 100, and 200 ng/ml rhBMP-4. The positive control is cells treated with TGF-β_1, 2 ng/ml, for 2 h. The control cells were treated with vehicle, 0.25% (wt/vol) BSA in PBS. The hFOB cells were grown in DMEM/Ham's F-12 containing 0.25% BSA for 48 h before treatment. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control. The lower panel is a graph of densitometry data for TIEG mRNA normalized to 18S RNA and expressed as percent of vehicle control.

affinity to a cell surface receptor complex of type I and type II receptors, both of which have been found in osteoblasts [Centrella et al., 1995a]. In view of the high binding affinity of the receptors for all isoforms of TGF-β, it is not

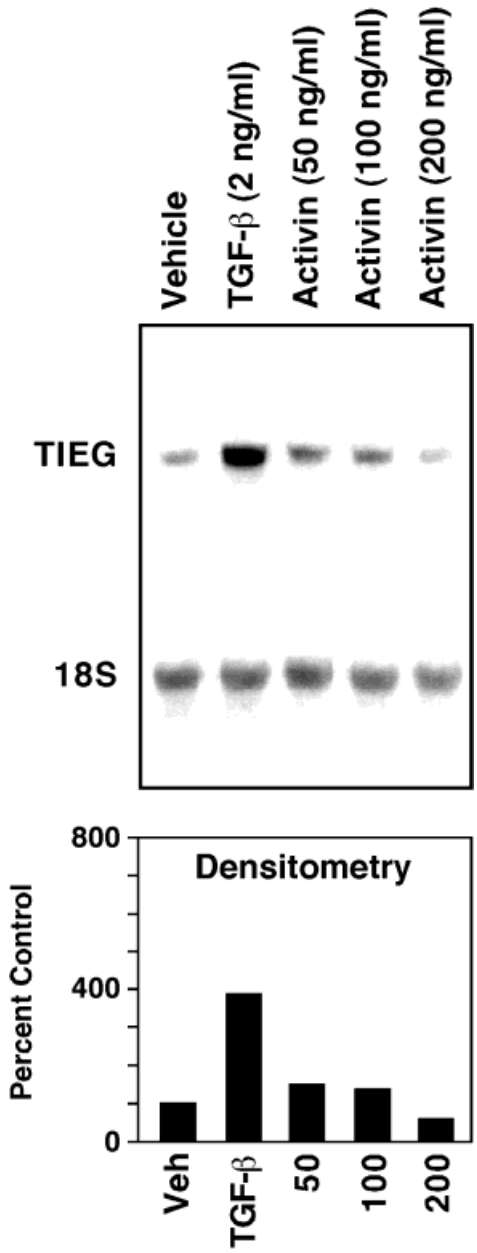

Fig. 5. Induction of TIEG mRNA in hFOB cells with increasing doses of activin. The TIEG mRNA steady-state levels were determined by Northern blot analysis of total RNA isolated from hFOB cells treated for 2 h with 50, 100, or 200 ng/ml activin. The positive control is hFOB cells treated with 2 ng/ml TGF-β_1 for 2 h and the vehicle control is 0.25% BSA in PBS. The hFOB cells were grown in DMEM/Ham's F-12 containing 0.25% BSA for 48 h before treatment. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control. The lower panel is a graph of densitometry data for TIEG mRNA normalized to 18S RNA and expressed as percent of vehicle control.

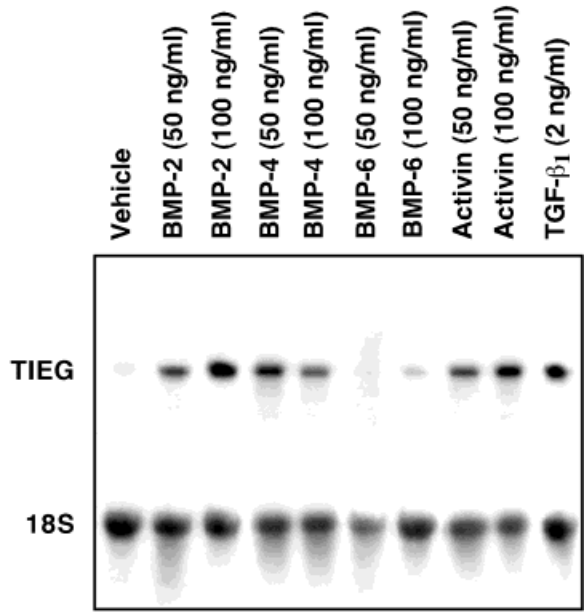

Fig. 6. Comparisons of the inductions of TIEG mRNA expression in hFOB cells by members of the TGF-β superfamily. TIEG mRNA steady-state levels were determined by Northern blot analysis of total RNA isolated from hFOB cells treated for 2 h with TGF-β_1 (2 ng/ml), or 50–100 ng/ml of rhBMP-2, rhBMP-4, rhBMP-6, or activin, as indicated in the figure. The control cells were treated with vehicle, 0.25% BSA in PBS for 90 min. The blots were probed with [^{32}P]-labeled TIEG cDNA and for 18S RNA as a control.

surprising that TIEG mRNA inductions by all isoforms of TGF-β were similar.

The treatment of the hFOB cells with the other members of the TGF-β superfamily, e.g., BMPs and activin, resulted in variable responses, with an overall decrease in inductive capacity. The BMP-2, BMP-4, and activin increased TIEG mRNA levels, but only at high concentrations, whereas BMP-6 showed little to no induction of TIEG. In addition, the optimal dose of TGF-βs was ~2 ng/ml, whereas those for BMP-2, BMP-4, and activin were 50–100 ng/ml. In general, the relative potencies of the TGF-β superfamily members for TIEG mRNA induction at their respective optimal doses were TGF-β_1, -β_2, -β_3 (>20-fold) ⩾ BMP-2 (16-fold) > BMP-4 (4-fold) > activin (1–4-fold) ⩾ BMP-6 (0-fold). Interestingly, the pattern of TIEG induction by activin was maximal at ~50 ng/ml, with a reduction in TIEG mRNA levels occurring as the dose of activin increased. The reason for this effect is not known at present. The pattern of TIEG protein induction by these TGF-β family members gen-

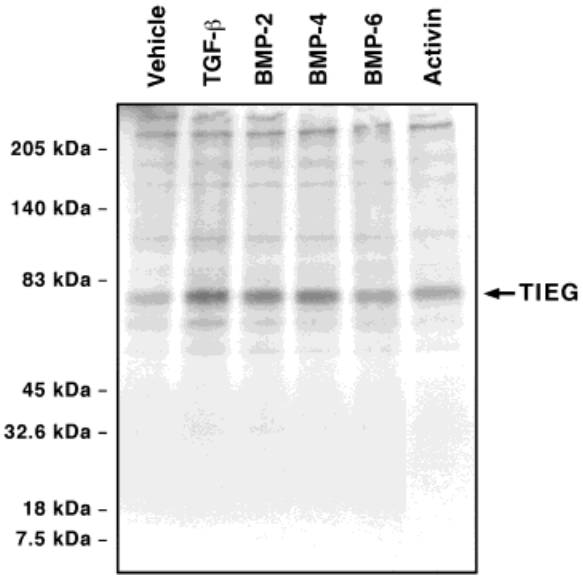

Fig. 7. Induction of TIEG protein levels/synthesis in human osteoblasts by select members of the TGF-β superfamily. The hFOB cells were treated for 2 h with either vehicle, 0.25% BSA in PBS, TGF-β_1 (2 ng/ml), rhBMP-2 (100 ng/ml), rhBMP-4 (100 ng/ml), rhBMP-6 (100 ng/ml), or activin (100 ng/ml). The cells were radiolabeled with [^{35}S]-methionine after the 2-h growth factor treatment, the total soluble protein was isolated, and the labeled TIEG was immunoprecipitated from hFOB cell lysates using a polyclonal TIEG antibody, and the precipitate analyzed by SDS-PAGE as described in the Methods section.

erally follows that of the mRNA steady-state levels. However, some regulation at the translational level and protein half-life cannot be ruled out.

In contrast to TGF-βs, the BMPs can generate ectopic bone. Historically, BMPs were initially isolated from bone; however, they are localized in many other tissues and are active during embryogenesis and differentiation. Both BMP-2 and BMP-4 are closely related to the *Drosophila* decapentaplegic (dpp) and have the ability to induce osteoblastic differentiation in vitro and ectopic bone formation in vivo. Another subgroup of BMPs—BMP-5, BMP-6, and BMP–7—differ from the BMP-2/BMP-4 family, and have a high degree of homology to the *Drosophila* protein 60A [Sampath et al., 1993; Centrella et al., 1995b]. In skeletal tissue, BMPs are critical for osteoblast differentiation, and a temporal expression of BMPs occurs that coincides with the differentiation and expression of the osteoblastic phenotype [Ghosh-Choudhury et al., 1994]. In the studies presented here, the inductions of TIEG mRNA by members of one subgroup, BMP-2 and BMP-4, were similar to each other, whereas a member of the other subgroup, BMP-6, showed no induction of TIEG. BMP-2 has a significant effect on osteoblastic cells, especially on late genes that define osteoblastic differentiation and maturation. For example, treatment of human osteoblasts and human bone marrow stromal cells with BMP-2 results in an increased alkaline phosphatase mRNA and activity [Gori et al., 1999]. In addition, other late genes, including the genes that encode for the extracellular matrix proteins, e.g., osteopontin, bone sialoprotein, osteocalcin, and type I collagen, are increased by BMP-2 treatment [Lecanda et al., 1997].

BMP-4 has been shown to mediate dental mesenchymal development and induce the expression of transcription factors Msx-1, Msx-2, and Egr-1 [Vainio et al., 1993]. BMP-6 has been localized to hypertrophic cartilage and is upregulated in the early stages of chondrocyte development [Cary and Liu, 1995]. Additionally, BMP-6 has been shown to induce endochondral bone formation [Lyons et al., 1989; Jones et al., 1991; Gitelman et al., 1994]. BMP-6 increases during osteoblastic differentiation of pluripotential mesenchymal cells [Gazit et al., 1993; Gitelman et al., 1995] and in fetal rat calvarial cultures [Harris et al., 1992; Hughes et al., 1995]. The overexpression of vrg-1/BMP-6 in pluripotent mesenchymal cells results in increased osteoblastic differentiation [Gitelman et al., 1995], whereas antisense to BMP-6 decreases the number of mineralized nodules in glucocorticoid-treated fetal rat calvarial cultures [Boden et al., 1997]. Therefore, BMP-2, BMP-4, and BMP-6 selectively mediate differentiation of mesenchymal cells to osteoblasts and enhance osteoblastic differentiation.

The differentiation state of the hFOB cells may play a role in the differential induction of TIEG mRNA by TGF-β, BMP-2, BMP-4, BMP-6, and activin. In pluripotent stem cells, BMP-2 increased the expression of osteoblastic markers [Thies et al., 1992; Wang et al., 1993; Rickard et al., 1994], and BMP-2, BMP-3, BMP-4, and BMP-7 have been shown to upregulate the markers of mature osteoblasts including alkaline phosphatase activity, type I collagen, and osteocalcin expression [Chen et al., 1991; Ohta et al., 1992; Sampath et al., 1992; Asahina et al., 1993; Zhou et al., 1993]. However, BMP-6 appears to be targeted at early-stage osteoprogenitor cells [Hughes et

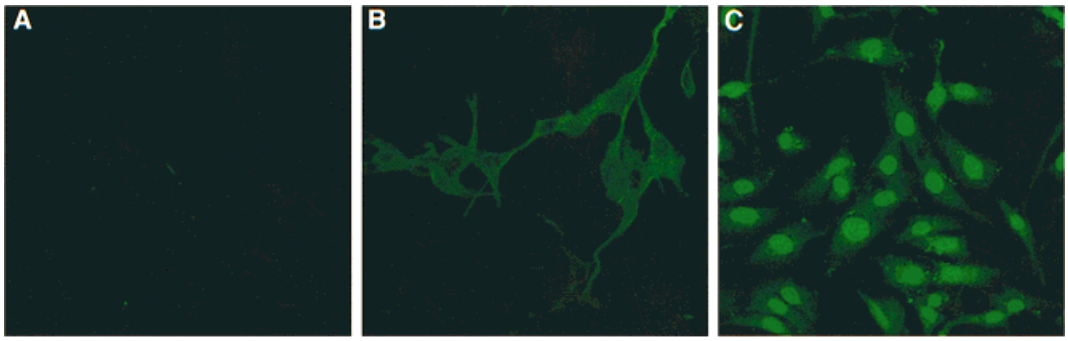

Fig. 8. Intracellular localization of TIEG protein in hFOB cells by laser-based, fluorescent antibody confocal microscopy. **A:** Cells stained without the anti-TIEG protein antibody. **B:** Cells treated with vehicle. **C:** Cells treated for 2 h with rhBMP-2. For TIEG localization experiments, hFOB cells were plated at a density of 20,000 cells/cm^2 and serum starved for 48 h. After serum starvation, the cells were treated with vehicle or rhBMP-2 for 2 h and the fixed cells were reacted with a polyclonal TIEG antibody with a fluorescein secondary antibody.

al., 1995]. The hFOB cells have been characterized as mature osteoblasts [Harris et al., 1995]. Therefore, the ability of BMP-2 and BMP-4 to upregulate TIEG expression in these cells is consistent with their stage of osteoblastic differentiation.

Activin plays an important role, not only in reproductive tissue [Woodruff, 1998], but also in bone, where periosteal and bone matrix density are increased in parietal bone of neonatal rats injected with activin A [Oue et al., 1994]. Activin receptors are present on bone cells, but appear to be only weakly linked to downstream signaling events [Centrella et al., 1991, 1995a]. However, Centrella and coworkers [1991] have found that a 500-fold-higher concentration of activin A is needed to achieve equivalent TGF-β-like activity in fetal rat bone cells. The results reported here also show that activin A is significantly less potent in the degree of TIEG mRNA induction than TGF-β, with a maximal dose for TGF-β at 2.0 ng/ml and that for activin at 100 ng/ml, which is a 50-fold difference.

The TGF-β, BMPs and activin families are important in the functioning of osteoblasts. Each subfamily has its own unique set of membrane receptors, and some use different Smad pathways [Massagué, 1998]. The differential induction of TIEG mRNA by TGF-β, BMP-2/BMP-4, BMP-6, and activins could be because of the different receptor and signaling pathways. The members of the TGF-β superfamily appear to mediate their actions through distinct receptors that contain serine/threonine kinase activity [Massagué and Weis-Garcia, 1996] and signal through Smad proteins [Heldin et al., 1997; Padgett et al., 1997] or through TGF-β activated kinase 1 [Shibuya et al., 1998]. BMP-2 and BMP-4 bind the type II receptor BMPRII and the type I receptors ALK3 or ALK6 [ten Dijke et al., 1994; Rosenzweig et al., 1995] and transduce the BMP-2 and BMP-4 response. Conversely, TGF-β binds the type II receptor TβRII and type I receptor TβRI, whereas activin binds type II receptors ActRII or ActRIIB and the type I receptor ActRIB [Wrana et al., 1992; Carcamo et al., 1994; Rosenzweig et al., 1995; Attisano et al., 1996]. After activation (binding) by TGF-β or BMP-2, the threonine kinase membrane receptors phosphorylate the Smad family of proteins (Smads 1 or 5 for BMP-2/BMP-4 and Smads 2 or 3 for TGF-β) [Heldin et al., 1997; Massagué, 1998; Padgett et al., 1998; Sakou, 1998]. These activated signal proteins then form complexes with another Smad (Smad 4), and the complex translocates to the nucleus where it interacts with other nuclear factors to regulate gene transcription.

TIEG may well be part of, or interact with, the Smad signaling pathway. In this article, we have demonstrated that TIEG protein is localized in the cytoplasm of untreated cells, but localizes to the nucleus after BMP-2 treatment of hFOB cells. As a cytonuclear or nuclear protein, TIEG could: 1) interact with Smads as an inhibitor or activator of their translocation, 2) play a role in the transcriptional regulation by the Smad 1,2–Smad 4–Fast 1 complex, or 3) serve as an immediate early gene whose protein is a transcription factor involved in further mediating late genes. Finally, there is the pos-

sibility that TIEG plays a role in a unique, undefined signal pathway of TGF-β family members.

This article describes a marked growth factor specificity for the induction of TIEG in hFOB cells. Thus, the specific induction of TIEG by TGF-β with a lower potency of the other representatives of the TGF-β superfamily members in human hFOB cells may well reflect osteoblast-specific responses. Other cell types expressing TIEG (cerebellum, pancreas, placenta, and breast epithelium) [Subramaniam et al., 1998] display a different pattern of cytokine-specific induction; therefore, other members of the TGF-β superfamily may be the predominant regulators in non–bone-cell types.

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