Overexpression of a Nuclear Protein, TIEG, Mimics Transforming Growth Factor-β Action in Human Osteoblast Cells*

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Although transforming growth factor-β (TGF-β) is a growth factor with many known regulatory activities in many different cell types, its intracellular signaling pathway is still not fully understood. A TGF-β-inducible early gene (TIEG) was discovered and shown by this laboratory to be a 3-zinc finger transcription factor family member; its expression is rapidly induced in cells treated with TGF-β. To ascertain whether TIEG plays a major role in the TGF-β pathway, human osteosarcoma MG-63 cells were stably transfected either with an expression vector containing a TIEG cDNA or with the vector alone. Clones that contain only the vector express normal levels of TIEG mRNA and protein and display the same patterns of gene expression and levels of cell proliferation as the nontransfected, non-TGF-β-treated parental cells. However, transfected cells that overexpress TIEG mRNA and protein (TIEG-6 and TIEG-7) display changes that mimic those of MG-63 cells treated with TGF-β, i.e. increased alkaline phosphatase activity, decreased levels of osteocalcin mRNA and protein, and decreased cell proliferation. The degree of these changes correlated with the level of TIEG expressed in the cell lines. TGF-β treatment of the overexpressed cells showed no added effects. These findings and other published reports support a primary role of TIEG as a transcription factor in the TGF-β signaling pathway.

TGF-β1 has been shown to regulate many diverse tissue and cell processes in a multitude of cell types (1, 2). The cellular processes affected by TGF-β include inhibiting proliferation, inducing cell differentiation and apoptosis, and altering gene expression. These and additional properties have resulted in the labeling of TGF-β and members of its signaling pathway as tumor suppressors (3, 4).

In the skeleton, TGF-β is involved in bone growth, cell differentiation, and overall bone metabolism. In rodent and human OBs, TGF-β is involved in the regulation of 1) proliferation of OB cells, which appears to occur at the G1 phase of the cell cycle; 2) the proliferation and differentiation of OB osteogenitor cells; and 3) OB bone matrix production and mineralization (5–7). Interesting studies by Derynck and co-workers (8, 9) using transgenic mice have shown that TGF-β regulates a rapid, age-dependent loss of bone mass, which resembles oteoprosis. TGF-β appears to directly increase OB activity and differentiation, to uncouple OB and OCL activities, and to result in bone loss.

Our laboratory has previously identified a TGF-β-inducible early gene (TIEG) in normal human OB cells (hFOB) in which protein is 1) rapidly translocated from the cytoplasm to the nucleus and 2) rapidly induced (as is its mRNA) by TGF-β within 60 min after treatment in both primary and immortalized hFOB in culture (10, 11). TIEG mRNA was induced equally by all three isoforms of TGF-β, which is not surprising in view of the high binding affinity of the receptors for all isoforms of TGF-β. Our laboratory has reported that TIEG encodes a 480-amino acid protein (72 kDa) and has a unique N-terminal end, which distinguishes it from an early growth response-α (EGR-α) gene. We have previously reported that these two proteins are encoded in the same gene, but EGR-α is transcribed at much lower levels than TIEG in all tissues (12, 13). The zinc finger region of TIEG shows homology to the 3-zinc finger family of transcription factors such as Sp-1, Wilm’s tumor, BTEB, EGR-1, and the Krippel-like factors. The TIEG gene is localized on chromosome 8, q22.2, the same locus that contains genes related to myeloma and osteopetrosis (11). TIEG protein has been identified in many human tissues and cell types in addition to osteoblasts, including certain cells in the breast, uterus, brain, pancreas, and muscle (11). A mouse TIEG, termed mGIF for murine glial cell-derived neurotrophic factor (GDNF)-inducible factor, has also been shown to be distributed in several regions of mouse brain and is rapidly induced by GDNF in a neuroblastoma cell line and in primary cultures of rat embryonic cortical neurons (14, 15).

TIEG has been shown to play a role in TGF-β-induced inhibition of cell proliferation and apoptosis in human osteoblast cells and pancreatic carcinoma cells and more recently in epithelial and liver cancer cells (16–19). The induction of apoptosis appears to be the same for TGF-β and TIEG when the latter is overexpressed (18, 19).

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§ The abbreviations used are: TGF-β, transforming growth factor-β; TIEG, TGF-β-inducible early gene; OB, osteoblasts; hFOB, human immortalized fetal osteoblasts; AP, alkaline phosphatase; OC, osteocalcin; OCL, osteoclasts; TIEG-6/TIEG-7, MG-63 cell lines, stably transfected with the cDNA expression vector; GDNF, glial cell-derived neurotrophic factor; mGIF, murine GDNF-inducible factor; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BMP, bone morphogenetic protein; EGR, early growth response.
To gain information on the role of TIEG in the actions of TGF-β, we developed and characterized two stably transfected human osteoblastic MG-63 cell lines (TIEG-6 and TIEG-7), demonstrating that they overexpress the TIEG protein by 200 and 300%, respectively. This paper also describes the phenotypic properties of these two lines to show that they mimic those of the TGF-β-treated parent osteosarcoma MG-63 compared with vector control cells or with untreated, nontransfected, parent MG-63 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Northern Analyses**

MG-63 human osteosarcoma cells were routinely maintained in DMEM/F-12 (1:1) (Sigma) containing 10% (v/v) FBS. For Northern blot analysis, the cells were grown to near confluency in 100-mm culture dishes. Total RNA was isolated from these cells using the guanidinium/cesium chloride method. Northern analyses were performed using total RNA (8–15 μg) as described previously (10). The blots were probed with 32P-labeled human TIEG cDNA, GAPDH cDNA, and 18S ribosomal RNA as a control. The Northern blots were exposed to Kodak X-Omat AR film, and densitometry was determined using the NIH Image 1.47 program.

**Isolation of Stable Cell Lines with Vector Only or with TIEG Overexpression Constructs**

The 1.4-kilobase pair TIEG cDNA was cloned in-frame into the multiple cloning site of a pEBV His (Invitrogen) expression vector, which was thereafter named pEBV His-TIEG. MG-63 cells were stably transfected with either 10 μg of pEBV His-TIEG DNA or pEBV-His control vector using an electroporation method. The transfected cells were seeded onto 100-mm plates and allowed to grow for 48 h at 37 °C. The cells were then grown in selection medium containing hygromycin (100 μg/ml). The medium was replaced with selection medium every 2–3 days until antibiotic-resistant colonies developed. The colonies were then ring-cloned and propagated separately. The TIEG expression clones were characterized by analyzing the expression of TIEG mRNA and protein by Northern and immunoprecipitation, respectively.

**Immunoprecipitation of TIEG Protein**

The cells were seeded onto 100-mm plates and allowed to grow to near confluency. Prior to labeling, the medium was removed, and the cells were washed twice in methionine-free medium. The cells were preincubated in methionine-free medium for 1 h. Following preincubation, 750 μCi of [35S]methionine (Amersham Pharmacia Biotech) was added to each 100-mm plate, and the cells were labeled for 2 h at 37 °C. To harvest the cells, they were washed twice with phosphate-buffered saline and lysed in 1 ml of radioimmuno precipitation buffer containing 0.1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, and 5 mM Na/KPO4, pH 7.4. A protease inhibitor mixture was added to give final concentrations of the following protease inhibitors: 0.10 ng/ml phenylmethylsulfonyl fluoride, 30 μM of aprotinin, and 0.1 mM sodium orthovanadate. The cells were sheared using a 20-gauge needle, incubated on ice for 20 min, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was preincubated with protein A-Sepharose beads. The preclarified lysates were incubated with an equal amount of radioactivity with 5 μg of affinity-purified TIEG polyclonal antibody for 16 h at 4°C. The immunocomplex was pelleted using protein A-Sepharose beads. The beads were resuspended in SDS-sample buffer and boiled, and the proteins were separated by 10% (w/v) SDS-polyacrylamide gel electrophoresis. The gels were soaked in Autorfluor (National Diagnostics) and exposed to Kodak X-Omat AR film. Densitometry was performed using the NIH Image 1.47 program.

**Cell Proliferation**

The cells were plated in 12-well plates at a density of 10,000 cells/cm² in growth medium for 24 h. The cells were then serum-starved for 24 h by replacing the medium with DMEM/F-12 (1:1) containing 0.25% (w/v) BSA. The parent MG-63 control cells were then treated with vehicle or 2 ng/ml TGF-β. The TIEG-6 and TIEG-7 cells were maintained in serum-containing medium. All cells were incubated with 1 μCi/ml [3H]thymidine for 24 h; [3H]thymidine incorporation was determined in the cells by trichloroacetic acid precipitation (20, 21). The data are presented as the percent of control values, i.e. those from the untreated, nontransfected MG-63 cells.

**RESULTS**

TGF-β-treated and untreated MG-63 cells and vector control cells, as well as stably transfected TIEG-6 and TIEG-7 cells, were analyzed for TIEG mRNA steady state levels by Northern blotting and for TIEG protein levels by immunoprecipitation and SDS-gel electrophoresis. As reported previously in normal human osteoblast cells (10) and shown in Fig. 1A for osteosarcoma cells, TGF-β induces TIEG mRNA levels in parent MG-63 cells to a maximum of 5-fold within 1 h post-treatment. Fig. 1B shows the Northern blot of transfected cells wherein TIEG mRNA is increased in TIEG-6 and TIEG-7 cell lines, with only background levels detected in untreated, nontransfected, parental MG-63 cells or in vector-transfected control cells. To estimate TIEG protein levels, soluble protein was isolated from [35S]-labeled cell extracts and immunoprecipitated with anti-TIEG protein antisera. Fig. 2A shows that TGF-β treatment of the nontransfected MG-63 cells rapidly increases TIEG protein levels by 1.8-fold. In Fig. 2B, TIEG protein levels are shown to
be 2- and 3-fold higher in TIEG-6- and TIEG-7-transfected cells, respectively, compared with the untreated, nontransfected, MG-63 parent control cells.

The effect of overexpressing TIEG protein on the MG-63 cell proliferation was then examined. As shown in Fig. 3A, the parent MG-63 cells display a 50% inhibition of cell proliferation when treated with TGF-β. Similarly, Fig. 3B shows that the overexpressing TIEG-6 and TIEG-7 cells display a more than 50% decrease in the rate of proliferation compared with the rates in the untreated, parent MG-63 cells, whereas the vector-transfected control cells show only a moderate reduction. The effect of overexpressing TIEG in the MG-63 cells on the expression of bone markers for the OB phenotype was then examined. As shown in Fig. 4A, TGF-β-treated control MG-63 cells display a 2-fold induction of AP activity. In Fig. 4B transfected TIEG-6 and TIEG-7 cells are shown to contain -1.4- and 1.8-fold enhanced AP activity, respectively, compared with the AP levels in the untreated, parent MG-63 cells or the vector control MG-63 cells. To determine whether TGF-β-induced endogenous TIEG protein acts differently or in addition to the overexpressed TIEG protein, we treated the TIEG-7 cells with TGF-β and assayed for AP levels. As shown in Fig. 4B, although TGF-β showed strong effects on the AP levels in the nontransfected control or vector control MG-63 cells, no additional TGF-β-induced changes were detected in TIEG-7 cells.

Regarding the expression of the OC gene, the TGF-β treatment of the parent MG-63 cells results in a modest (25%) inhibition of OC production (Fig. 5A). Similarly, as shown in Fig. 5B, the TIEG-6 and TIEG-7 cells display a significant decrease, i.e. 94 and 97%, respectively, in the levels of OC production compared with the parent MG-63 cells and the vector only control cells. To determine whether TIEG overexpression actually regulates OC protein at the level of gene expression, and as further support that TIEG overexpression mimics TGF-β action on osteoblasts, we performed RT-PCR analysis of the MG-63, TIEG-6, and TIEG-7 cells. As shown in Fig. 6, the TIEG-6 and TIEG-7 cells show reduced OC mRNA steady state levels compared with MG-63 cells, which correlates to the protein levels.

Preliminary studies to identify the mechanistic pathway for TIEG involved co-precipitation experiments using the anti-TIEG polyclonal antibodies and anti-Smad 3 antibodies, under conditions similar to those described above for measuring TIEG protein. The precipitated proteins were analyzed by Western blotting using anti-Smad 3 antibody on the samples precipitated by anti-TIEG antibody and vice versa. These studies failed to detect any interactions between TIEG and Smad 3.

DISCUSSION

The data presented in this paper support the hypothesis that TIEG may serve an important role as a primary participant in the TGF-β signaling pathway and/or as an early response gene for TGF-β in osteoblast cells. The data show that cell proliferation is inhibited, as are OC mRNA and protein levels, whereas the AP activity is increased in TIEG-6 or TIEG-7 cells compared with the vector control cells. These responses mimic the effects of TGF-β in MG-63 cells. Importantly, the overexpres-
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Fig. 5. Analyses of the OC protein levels in MG-63 (A) and TIEG-6/7 cells (B). OC concentrations were measured as described under “Experimental Procedures.” In A, CONTROL represents OC protein levels in untreated parental MG-63 cells, and TGF-β represents OC levels in TGF-β-treated MG-63 cells. The latter were incubated with 2 ng/ml TGF-β for 72 h before harvesting. The data are presented as % of control, i.e. values from the untreated, nontransfected MG-63 cells and vector-transfected control cells. The mean and S.D. from three separate experiments are presented. * represents p < 0.05 represents the significance of the differences between the control cell lines and the TGF-β-treated cells. In B, MG-63 represents the OC levels in untreated MG-63 cells, Vector Control represents the OC levels in the untreated vector control cells, and TIEG-6 and TIEG-7 represent the OC levels in the untreated TIEG-6 and TIEG-7 overexpressing cell lines, respectively. ** indicates p < 0.001, significantly different from the MG-63 control.

Fig. 6. RT-PCR for OC mRNA in MG-63, TIEG-6, and TIEG-7 cells. Total RNA was isolated from MG-63, TIEG-6, and TIEG-7 cells and reverse transcribed, and PCRs were performed in triplicate for osteocalcin and GAPDH mRNA. The PCR products were separated on 1.5% (w/v) agarose gels and visualized with ethidium bromide.

Sation displays a dose dependence in that the effects are increased as the concentrations of TIEG protein increases by 200 and 300% in stably transfected TIEG-6 and TIEG-7 cells, respectively. It should also be noted that the concentrations of TIEG protein in the TIEG-6 and TIEG-7 cells are lower, but more constant, compared with the higher, but transient, induction of TIEG protein levels in the TGF-β-treated (nontransfected) parental MG-63 cells. Interestingly, TGF-β treatments of TIEG-7 cells show no further responses in the AP levels, supporting the idea that TIEG levels in the transfected lines were saturating and that overexpressed TIEG in the latter can fully substitute for endogenous TGF-β-induced TIEG protein.

The fact that TIEG mRNA and protein levels were previously shown to be rapidly induced after TGF-β treatment further supports a role for TIEG as an early response gene in the TGF-β signaling pathway (15, 16). TIEG has previously been implicated in tumor suppressor functions by inhibiting cell proliferation and inducing apoptosis in pancreatic carcinomas, hepatocarcinoma, and epithelial cells. In this regard, even the transient overexpression of TIEG seems to mimic the actions of TGF-β (16–19). The data presented here suggest a function for TIEG in osteosarcoma cells.

Previous studies (12, 13) from our laboratory have demonstrated that the TIEG gene codes for two proteins (TIEG protein and EGR-α), which are members of a 3-zinc finger family of transcription factors. However, TIEG represents the predominant species (>95%) in many tissues and cell types examined (12, 13). Interestingly, TIEG is a cytonuclear protein in which the constitutive levels are rapidly translocated to the nucleus following TGF-β treatment. This translocation is followed by a rapid induction of the TIEG gene expression (11, 16). In addition, the induction of TIEG mRNA steady state levels in OB cells is specific for TGF-β and not a variety of other growth factors and cytokines (10). Treatments with other cytokines, e.g. BMP-6, inteleukin-6, insulin like growth factor-I and -II, tumor necrosis factor-α, interleukin-1β, platelet-derived growth factor, and fibroblast growth factor, had no effect on TIEG expression (10). Selected other TGF-β family members, e.g. BMP-2, BMP-4, and activin, as well as epidermal growth factor, also showed some induction but only at much higher concentrations than required for TGF-β (11). Interestingly, other members of the TGF-β family regulate TIEG in other cell types, e.g. GDNF rapidly induces TIEG levels in neuroblastoma cells (14, 15). TIEG has been shown to be highly conserved and is homologous with the previously reported mGIF in mouse neuroblastoma cells (14, 15). We have previously reported a tissue- and cell type-specific distribution of TIEG, with TIEG protein localized to specific cell types in the cerebellum, pancreas, placenta, uterus, muscle, bone, bone marrow, and breast epithelium (11, 15, 24). Because TGF-β is known to regulate cell functions in many of these tissues, it is probable that TIEG is utilized by TGF-β, or possibly some of its family members, in these other cell types. These and other studies support a role for TIEG in the TGF-β signaling pathway as a possible tumor suppressor gene (16–19).

Members of the TGF-β superfamily appear to mediate their actions through distinct receptors that have serine/threonine kinase activity (25) and utilize signaling pathways involving either Smad proteins (1, 26), TGF-β-activated kinase 1 (TAK-1) (27), or possibly other, yet undefined, pathways. After binding and activation by TGF-β or BMPs, the serine kinase membrane receptors phosphorylate receptor-regulated Smads 1 and 5 in response to BMPs and Smads 2 and 3 for TGF-β and activin (25–29). The activated Smads complex with Smad 4 and translocate to the nucleus, where the complex can interact with other nuclear factors and modulate target gene expression. Smads 6 and 7 are negative regulators of this pathway and function by binding and inhibiting the actions of the type I TGF-β receptor kinase domain or the receptor-regulated Smads. It is unclear whether TIEG plays a role in this signaling pathway. As a cytonuclear protein that is induced to rapidly translocate to the nucleus in response to TGF-β treatment, and as a TGF-β rapidly induced (early response) gene, TIEG could: 1) interact with Smads 2, 3, or 4, travel to the nucleus, and play a role as a co-activator/repressor in transcriptional regulation; 2) play a direct role in a unique and heretofore undefined signaling pathway of TGF-β family members; or finally 3) not play a signal pathway role but function as an early response gene in which the protein product would be involved in further mediating TGF-β effects on late genes, including those involved in maintaining cell proliferation.

If TIEG is a participant in the Smad pathway, the lack of any further TGF-β response on AP activity in the TIEG-7 cells suggests that the Smads are already occupied by the overexpressed TIEG protein and unavailable for further ligand-mediated activation of Smads. Regarding the early response gene

2 At present we are not sure whether TIEG interacts only with the Smad binding elements or interacts directly with the Smad proteins. Two lines of evidence suggest an interaction of TIEG with Smads (S. A. Johnson, M. Subramaniam, and T. C. Spelsberg, unpublished data). First, preliminary studies attempting a co-transfection of the TIEG expression vector with the Smad binding element reporter construct into AKR2B cells resulted in an enhanced reporter response to TGF-β. Second, when the cells were co-transfected with Smads 3 and 4 expression vectors, TIEG co-activation was further enhanced in the presence of TGF-β treatment.
role, recent studies have reported that TIEG represses TATA-containing and TATA-less promoters in reporter gene assays (14) and contains repression domains that are known to repress transcription in a heterologous GAL-4-based transcriptional assay (30). The latter studies, combined with the results presented in this paper, support the idea that TIEG acts as a transcription factor in the TGF-β action pathway. This view is supported by similar roles of other members of the 3-zinc finger protein family members, e.g. Sp-1, Krüppel, and EGR-1 factors.

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