TGF- β Attenuates the Transactivation Activity of Ets-1 despite its Induction via the Inhibition of DNA Binding

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IWASAKA-YAGI, C., ABE, M. and SATO, Y. *TGF- β Attenuates the Transactivation Activity of Ets-1 despite its Induction via the Inhibition of DNA Binding.* Tohoku J. Exp. Med., **193** (4), 311–318 — We examined whether TGF- β affects the transactivation activity of Ets-1. TGF- β augmented *ets-1* mRNA expression and Ets-1 protein synthesis in ECV304 cells to the level equivalent to bFGF. When the DNA binding activity of Ets-1 protein was examined, bFGF was found to enhance DNA-Ets complex formation, whereas TGF- β attenuated basal as well as bFGF-enhanced DNA-Ets complex formation. As a result, TGF- β attenuated the promoter activity driven by Ets-1. The DNA binding of Ets-1 protein was enhanced by the initial 4-hour bFGF treatment and the subsequent 8-hour cycloheximide treatment. When TGF- β replaced cycloheximide in the subsequent 8-hour treatment, TGF- β inhibited this bFGF-enhanced DNA-Ets complex formation. When TGF- β and cycloheximide were simultaneously added in the subsequent 8-hour treatment, the inhibitory effect of TGF- β on bFGF-enhanced DNA-Ets complex formation was completely abolished. These results suggest the possibility that TGF- β attenuates the transactivation activity of Ets-1 by inducing a protein that interferes with the binding of Ets-1 to the DNA binding site.

——— *ets-1*; TGF- β ; DNA binding; transactivation

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The Ets family transcription factor has a DNA-binding domain in common that binds to a core GGA(A/T) DNA sequence, and Ets-1 is a prototype of the ETS family of transcription factors. Ets-1 was first described as the cellular homologue of v-ets, which is translated as a 135-kDa gag-myb-ets fusion protein from the

replication-deficient retrovirus E26 in chickens (Leprince et al. 1983; Nunn et al. 1983). A large number of proteins have now been identified that contain an ETS-binding site in the promoter region of their genes, indicating that the ETS family of transcription factors has profound effects on cellular activity (Wasylyk

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et al. 1993).

Ets-1 is expressed in various cell types. Especially, the expression of Ets-1 in endothelial cells (ECs) was observed during embryonic development (Kola et al. 1993; Pardanaud and Dieterlen-Lievre 1993; Maroulakou et al. 1994) and tumor vascularization in adult (Wernert et al. 1992, 1994). We previously reported that Ets-1 was induced in ECs by the stimulation with angiogenic growth factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) (Iwasaka et al. 1996; Tanaka et al. 1999), or in response to a denuding injury (Tanaka et al. 1998). Overexpression of the *ets-1* gene induced matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, and integrin $\beta 3$, and changed ECs to the angiogenic phenotype (Oda et al. 1999). Moreover, dominant negative molecule of Ets-1 inhibited angiogenesis in vivo (Nakano et al. 2000).

Transforming growth factor- β (TGF- β), a family of 25-kDa homodimeric peptide, has profound effects on cell growth, motility, gene expression and differentiation of various cell types including vascular cells (Pepper 1997). Here, we examined the effect of TGF- β on the transactivation activity of Ets-1. The present results suggest that TGF- β inhibits the transactivation activity of Ets-1 by inducing the synthesis of a protein that may interfere with the binding of Ets-1 protein to its DNA binding site.

MATERIALS AND METHODS

Materials

The materials used and their respective sources were the following: bFGF, from Becton Dickinson, Inc. (Bedford, MA, USA); human recombinant TGF- $\beta 1$, from R & D Systems (Minneapolis, MN, USA); rabbit polyclonal anti-human Ets-1 antibody and AP-1 consensus oligonucleotides, from Santa Cruz Biotechnology (Santa Cruz, CA, USA); T4 polynucleotide kinase, from Takara (Ohtsu); poly(dI-

dC) • poly(dI-dC), Sephadex G-50 (DNA grade), and type I collagen-coated dishes, from Iwaki Glass Co., Ltd. (Chiba); Opti-MEM 1 Reduced Serum Medium and LipofectAMINE PLUS Reagent, from Gibco BRL (Gaithersburg, MD, USA); [α - 32 P] dCTP and [γ - 32 P] ATP, from Amersham (Buckinghamshire, UK); horseradish peroxidase conjugated-protein G, from Bio-Rad Laboratories (Hercules, CA, USA); PGL3 vector, pRL-TK vector, and Dual-luciferase reporter assay system, from Promega (Madison, WI, USA).

Cell culture

ECV304 cells (Takahashi et al. 1990) were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo) containing 5% fetal bovine serum (FBS), 4 mM glutamate, 100 μ g/ml Kanamycin, and 2.2 mg/ml NaHCO₃ as described previously (Iwasaka et al. 1996).

Northern blot analysis

Northern blot analysis for *ets-1* mRNA was performed as described previously (Iwasaka et al. 1996). Briefly, confluent ECV304 cells were preincubated for 24 hours in serum-starved medium and were then stimulated with various concentrations of TGF- β with or without 1 nM bFGF. After the incubation, total RNA was extracted, fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond N⁺ filter (Amersham Life Sciences Inc., Buckinghamshire, UK). The filter was hybridized with [32 P]-labeled probe in hybridization solution overnight at 42°C. The filter was washed in 2×SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature, and then in 0.2×SSC and 0.1% SDS at 65°C. Autoradiography was carried out with a BAS2000 Image Analyzer (Fuji, Tokyo).

Western blot analysis

Western blot analysis for Ets-1 protein was performed as described previously (Iwasaka et

al. 1996). Briefly, confluent ECV304 cells were serum-starved and stimulated with desired additives for 12 hours. Thereafter, the cells were extracted, and the resultant samples were separated on 10% SDS polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences Inc., Buckinghamshire, UK). The filter was incubated with rabbit polyclonal anti-human Ets-1 antibody for 1 hour at room temperature, followed by incubation for 1 hour with horseradish peroxidase-conjugated protein G. Immunoreactive bands were developed with an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Life Sciences Inc.) and were visualized with an LAS-1000 Image Analyzer (Fuji, Tokyo).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (Iwasaka et al. 1996). Briefly, subconfluent ECV304 cells were preincubated in DMEM containing 1% FBS for 24 hours. Thereafter, the cells were stimulated with various concentrations of TGF- β with or without 1 nM bFGF in DMEM containing 1% FBS for further 12 hours. After the incubation, whole cell extracts (WCEs) were prepared by the freezing-thawing method. The synthetic oligonucleotides (a control Ets-1 motif, AATTCACCGGAAGTATTCGA and a mutant Ets-1 motif, AATTCACCTTAAGTATTCGA) (Shapiro 1995) were annealed and their 5 ends were labeled by T4 polynucleotide kinase using [γ - 32 P] ATP. AP-1 oligonucleotides (a control AP-1 motif, CGCTTGATGAGTCAGCCG-GAA and a mutant AP-1 motif, CGCTTGAT-GACTTGCCCGAA) were prepared by the same method. Two μ l of DNA was incubated in a final volume of 20 μ l with 1.5 μ g of poly(dI-dC).poly(dI-dC), 10 mM Tris-HCL, 50 mM NaCl, 1 mM ethylenediamine tetra acetic acid (EDTA), 5% glycerol, 1 mM dithiothreitol (DTT), and equal amounts of WCEs for 30

minutes at room temperature. The mixture was separated on a native 4% polyacrylamide gel in 1 $\times$ TAE for 90 minutes. Autoradiography was carried out with an imaging plate and analyzed with a BAS2000 Image Analyzer.

Luciferase assay

Human genomic DNA fragments containing portions of the putative MMP-1 promoter were fused to the firefly luciferase gene and subcloned into the promoterless pGL3-basic vector. Based on the sequence of genomic DNA, PCR primers were designed to incorporate a Kpn site into the forward primers and an Xma site into the reverse primer to amplify the fragment, which contains Ets and AP-1 binding sites. The PCR product was ligated into PGL3 plasmids, cleaved with the same restriction enzymes, and used to transform competent cell; and clones containing the plasmids with inserts were verified by DNA sequencing. The control plasmids were obtained by point-mutating at Ets or AP-1 binding sites. A second reporter construct was generated by inserting five head-to-tail ligated copies of oligonucleotides that contained the PEA3 Ets binding site from the polyoma virus enhancer into the PGL3 plasmid. A control reporter plasmid that contained an equal length insert lacking Ets binding sites was also generated.

ECV304 cells were plated in 24 well tissue culture dishes at 2×10^4 cells/well and incubated for 2 days. By use of LipofectAMINE PLUS Reagent, subconfluent ECV304 cells were transfected for 3 hours with 0.2 μ g of luciferase constructs and pRL-TK plasmid, which contained a herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene, and then incubated for 12 hours in DMEM containing 5% FBS. Transfected cells were preincubated in DMEM containing 0.1% BSA for 24 hours, and then incubated with phorbol 12-myristate 13-acetate (PMA) (200 ng/ml), TGF- β (40 pM), or PMA (200 ng/ml) plus TGF- β (40 pM) in DMEM containing 0.1%

BSA for another 24 hours. After the incubation, cells were washed with PBS, and lysed with Passive lysis buffer (Promega). Firefly luciferase and renilla luciferase activities were measured sequentially by using a Dual-Luciferase Reporter assay system and a Luminesencer JNR (ATTO, Tokyo). After measuring the firefly luciferase signal (FLS) and the renilla luciferase signal (RLS), the relative luciferase activity (RLA) was calculated as FLS/RLS.

RESULTS

We first examined the effect of TGF- β on the expression of *ets-1* mRNA in ECV304 cells. We previously reported that bFGF induced the expression of *ets-1* mRNA in ECV304 cells (Iwasaka et al. 1996). We observed that TGF- β increased the expression of *ets-1* mRNA to the level equivalent to bFGF (Fig. 1A). The peak of *ets-1* mRNA expression was observed between 2 to 4 hours after the stimulation (data not shown). When the cells were stimulated with bFGF and TGF- β simultaneously, the expression of *ets-1* mRNA was further enhanced (Fig. 1A). Next, we examined the effect of TGF- β on the synthesis of Ets-1 protein. We previously reported that bFGF increased Ets-1 protein level in ECV304 cells (Iwasaka et al. 1996). Here we observed that TGF- β either

with or without bFGF increased the levels of Ets-1 protein (Fig. 1B). Ets-1 protein synthesis was peaked between 8 and 12 hours after the stimulation (data not shown), and the Ets-1 protein levels correlated well with the pattern of induction of *ets-1* mRNA by these factors.

As *ets-1* mRNA expression and Ets-1 protein levels were augmented by bFGF and TGF- β by in ECV304 cells, we examined whether the DNA binding activity of Ets-1 protein was equally augmented. Specificity of the band was confirmed by the competition with normal but not mutant competitor (data not shown). To our surprise, whereas bFGF increased the DNA binding activity of Ets-1, TGF- β reduced the basal as well as bFGF-stimulated DNA binding activity of Ets-1 (Fig. 2A). This inhibitory effect of TGF- β was dose dependent (Fig. 2B).

To test whether TGF- β affected the transactivation activity of Ets-1, we performed luciferase assay using human MMP-1 promoter with single AP-1 site and Ets-binding site. bFGF stimulated this promoter activity (Fig. 3A). TGF- β alone had no effect on the promoter activity, but apparently repressed the bFGF-augmented promoter activity (Fig. 3A). This repressing effect of TGF- β was also observed when cells were co-transfected with the MMP-1 reporter construct and human *ets-1* cDNA (data not shown). Since the MMP-1

Fig. 1. Effects of bFGF and TGF- β on *ets-1* mRNA expression and Ets-1 protein synthesis. ECV304 cells were treated with bFGF and/or TGF- β . (A) The expression of *ets-1* mRNA was examined by Northern blotting. lane 1, basal; lane 2, bFGF (1 nM); lane 3, TGF- β (40 nM); lane 4, bFGF (1 nM) plus TGF- β (40 nM). (B) The level of Ets-1 protein was examined by Western blotting. lane 1, basal; lane 2, bFGF (1 nM); lane 3, TGF- β (40 nM); lane 4, bFGF (1 nM) plus TGF- β (40 nM).

Fig. 2. Effects of bFGF and TGF- β on the DNA binding activity of Ets-1.

ECV304 cells were treated with bFGF and/or TGF- β . Thereafter, DNA binding activity was examined by EMSA. (A) Lane 1, basal; lane 2, TGF- β (40 nM); lane 3, bFGF (1 nM); lane 4; TGF- β (40 nM) plus bFGF (1 nM). (B) Lane 1, bFGF (1 nM); lane 2, bFGF (1 nM) plus TGF- β (0.4 nM); lane 3, bFGF (1 nM) plus TGF- β (4 nM); lane 4, bFGF (1 nM) plus TGF- β (40 nM).

Fig. 3. Effects of bFGF and TGF- β on the transactivation activity of Ets-1.

ECV304 cells were transfected with promoter-luciferase construct, and treated with bFGF and/or TGF- β . Thereafter, the promoter activity was examined. (A) human MMP-1 promoter-luciferase construct. (B) luciferase construct with five multimerized Ets binding sites.

promoter contained Ets and AP-1 binding sites, we constructed the Ets-specific luciferase vector with five multimerized Ets binding sites and performed the promoter assay. When ECV304 cells were treated with 200 ng/ml PMA, Ets-

driven luciferase activity was increased. TGF- β repressed this PMA-stimulated transactivation, but had no effect on the basal promoter activity (Fig. 3B).

In order to clarify the mechanism under-

Fig. 4. Protein synthesis is required for the TGF- β -mediated attenuation.

ECV304 cells were incubated for an initial 4 hours with or without bFGF. Thereafter, the cells were washed and treated with TGF- β with or without CHX or with CHX alone for 8 hours. After the treatment, WCE was obtained and EMSA was performed. (A) EMSA for Ets, (B) EMSA for AP-1. Lane 1, no supplementation during entire experimental period; lane 2, bFGF for 4 hours and no supplementation for an additional 8 hours; lane 3, bFGF for 4 hours and TGF- β for an additional 8 hours; lane 4, bFGF for 4 hours and CHX for an additional 8 hours; lane 5, bFGF for 4 hours and TGF- β plus CHX for an additional 8 hours.

lying TGF- β inhibition of DNA complex formation with Ets-1 protein, we used cycloheximide (CHX) to inhibit new protein synthesis and performed the EMSA. ECV304 cells were incubated with or without bFGF for 4 hours. Thereafter, the cells were washed with PBS and incubated with the indicated supplements for an additional 8 hours. DNA binding activity of Ets-1 protein was augmented by the initial 4-hour stimulation with bFGF and no supplementation (Fig. 4A, lane 2) or with CHX (Fig. 4A, lane 4) for the subsequent 8-hour incubation. On the other hand, the subsequent 8-hour stimulation with TGF- β almost completely inhibited the DNA-Ets complex formation that was augmented by the initial 4-hour bFGF treatment. Interestingly, when TGF- β and CHX were added at the same time and incubated for the subsequent 8 hours after the bFGF stimulation, the inhibitory effect of TGF- β was completely abolished (Fig. 4A, lane 5). The same experiments were done for the DNA-AP-1 complex formation. However,

TGF- β did not exhibit such inhibitory effects on the DNA-AP-1 complex formation at all (Fig. 4B).

DISCUSSION

The effect of TGF- β on the activity of Ets-1 has not been extensively examined. Gilles et al. (1996) have shown that TGF- β had marginal effect on the expression of Ets-1 in fibroblasts. However, they did not examine the effect of TGF- β on the transactivation activity of Ets-1. Here, we demonstrated for the first time that TGF- β increased *ets-1* mRNA expression and Ets-1 protein synthesis but attenuated the transactivation activity of Ets-1 by inhibiting the DNA binding of Ets-1. CHX experiment suggested that this inhibition required new protein synthesis. Therefore, we propose the possibility that TGF- β induces the synthesis of a protein that interferes with the binding of Ets-1 to the Ets-binding site on DNA.

We previously reported that angiogenic growth factors such as VEGF and bFGF induced Ets-1 in ECs (Iwasaka et al. 1996) and that Ets-1 potentiated the angiogenic activity of ECs by inducing MMP-1, MMP-3, MMP-9, and integrin $\beta 3$ as target genes (Oda et al. 1999). TGF- β is proposed to down-regulate the angiogenic activity of ECs and to make the neo-vessels mature (Folkman and D'Amore 1996). The present observations that TGF- β inhibits the transactivation activity of Ets-1 supports this proposed role of TGF- β in angiogenesis.

At least two proteins that repress the transcriptional activity of Ets-1 protein have been reported to date. MafB, a member of the Maf family protein, binds to the DNA binding domain of Ets-1 via the basic region or leucine-zipper domain and represses the transcriptional activity of Ets-1 (Sieweke et al. 1996). EAP1/Daxx has been recently identified as another repressor of Ets-1 (Li et al. 2000). EAP1/Daxx binds to the N-terminal 139 amino acids of Ets-1 and represses the transcriptional activity of Ets-1. However, since this region of Ets-1 does not contain the DNA-binding Ets domain, it is not likely that EAP1/Daxx represses the transcriptional activity of Ets-1 by interfering with the binding of Ets-1 to its binding site. We did not see any induction of *Kleisler* gene, a human homologue of MafB, by TGF- β (data not shown). It remains to be elucidated whether TGF- β affects the expression of EAP1/Daxx. Further study is required to clarify the identity of the protein that TGF- β was shown to induce in this study.

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