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Expression and Activation of Transforming Growth Factor-Beta 2 in Cultured Bone Cells

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Transforming growth factor-β (TGF-β)s are multifunctional small polypeptides synthesized in most cell types. TGF-β exerts pivotal effects on both bone formation and resorption. In addition, increasing lines of evidence implicate TGF-β as a potential coupling factor between these two processes during bone remodeling. In the present study, the expression form and the activation mechanism of latent-TGF-β were investigated using specific antibodies for each isoform. TGF-βs were observed to be synthesized and accumulated in a large amount in cultured osteoblastic cells. The estimated molecular weights of intracellular TGF-β2 and -β3 were 49 and 55 kDa, respectively. Based on proteolytic digestion study and immunofluorescence observation, these precursor forms seemed to be accumulated in distinct intracellular compartments. To examine whether the internal pool of TGF-β was possiblely regulated by external signals, their biological activites were examined in a conditioned media of this cell. Although the intact conditioned media did not contain detectable TGF-β activity, heat-treatment or acid-activation of the conditioned media revealed significant TGF-β activity. Furthermore, in the presence of estrogen, this activity was dramatically diminished. It is known that activation of latent TGF-β can be achieved by different chemical and enzymatic treatments, or by incubation with certain cell types. This extracellular activation was suggested as a key step in the regulation of TGF-β activity. In addition to these extracellular activation, this study suggests that the synthesis and intracellular processing are important regulation steps for TGF-β action. In addition, this regulation is specific for TGF-β type 2, because the change was not observed in TGF-β3 in osteoblastic cell line.

TGF-βs are important regulators of various physiological events. A distinct feature of TGF-βs is that they are usually produced in a latent form, which are subsequently activated to functional regulator. Several studies have suggested that both post-transcriptional and post-translational regulation are important in the regulation of TGF-β activity. For example, unstimulated and activated monocytes have similar level of TGF-β mRNA, but active TGF-β is secreted only by the latter (Schalch et al., 1991).

Previous reports have indicated that the secreted form consists of an active dimeric 25 kDa polypeptide noncovalently associated with TGF-β latency associated protein (LAP) domain (Wakefield et al., 1988; Bonewald et al., 1991; Bonewald, 1999). The secreted latent TGF-β appears to be bound to extracellular matrix or to cell surface. Because plasmin is known to cleave the propeptide fragment of TGF-β but not to affect TGF-β itself, it is possible that plasmin digests the pro-segment to release TGF-β from the extra cellular matrix. Several proteases other than plasmin, such as cathepsin D and plasminogen activator, have been also suggested to be responsible for TGF-β activator *in vivo*. Together with the protease study, cellular component of the TGF-β activation has been studied in various systems. As results, macrophage (Twardzik et al., 1990; Chong et al., 1999), mesenchymal cell (Rowley, 1992), cocultured endothelial cell with smooth muscle (Sato and Rifkin, 1989), and osteoclast (Oreffo et al., 1989) have been shown to be responsible for the cellular activation of latent TGF-β. Those cells may activate latent TGF-βs through secreting or exposing protease on their cell surface. Recent knockout mice studies suggested thrombospondin-1 to be the enzyme responsible for the activation *in vivo*. They proposed that the activation mechanism involved the conformational changes instead of proteolytic removal of LAP and LAP-binding protein (LTBP) (Crawford et al., 1998).

Several forms of latent TGF-β have been identified in various tissues (Bonewald et al., 1991; Lioubin et al., 1991). For example, platelet contains a 235 kDa latent complex composed of mature TGF-β1 noncovalently associated with the remainder of the TGF-β1

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precursor region, which in turn is bound to a binding protein of 135 kDa through disulfide bonds. Therefore, the release of active TGF-β from its proregion would be the key steps for the activation of TGF-β. Prior to the extracellular processing, the latent-TGF-β must be cleaved between the active form and pro-region intracellularly. KEX/furin-like proteases has been suggested to be a responsible protease (Barr, 1991; Nakayama, 1997). This intra cellular processing was regarded as an automatic, non-regulatable process. In addition, previous investigators argued that there were no differences between the isoforms during the activation process. Recent work, however, showed a large internal pool of latent TGF-βs (Miyazono et al., 1992; Roth-Eichhorn et al., 1998). The intracellular pool of latent TGF-βs may suggest another regulatory step in the activation of latent TGF-βs (Roth et al., 1998).

In this study, I attempted to identify the TGF-β forms in osteoblastic cells using Western blot analysis and immunohistochemical methods. I found a major 49 kDa precursor especially for TGF-β2 in a perinuclear region of ROS17/2.8 cell. In addition, secretion of the latent TGF-βs also seemed to be regulated by external signals such as estrogen challenge.

Materials and Methods

Materials

The antibodies against TGF-β1, -β2, and -β3 were purchased from Santa Cruz. The neutalizing antibody against TGF-β2 is the product of R&D System. Secondary antibody that is conjugated with alkaline phosphatase. Fetal bovine serum (FBS) and all cell culture reagents were purchased from GIBCO/BRL. CHO cell expressing recombinant human TGF-β1 and E. coli expressing TGF-β2 were purchased from Boehringer Mannheim. NBD C6-ceramide was purchsed from Molecular Probe/Inc., and all other chemicals were purchased from Sigma Chem. Com.

ROS 17/2.8 cell was kindly provided by Dr. Bonewald (Univ. of Texas, USA) and mink lung epithelial cell (Mv1Lu) was purchased from the American Type Culture Collection.

Cell culture and TGF-β assay

The cells were cultured and maintained as follows: they were plated at 10^5 cells/ml in 96 well plates for 24 h in Dulbelcco's Modified Eagle Midium (DMEM) containing 10% FBS at 37℃ in 5% CO_2. The culture medium was then changed to 2% FBS/DMEM for treatments with TGF-βs, PTHrP, 1,3,5(10)-estratriene-3,15β-diol (estrogen), and 1,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$].

The protease inhibitors PMSF and o-phenanthroline were solubilized in ethanol or dimethylsulfoxide and used at final concentrations not exceeding 0.1% (v/v).

NIH3T3 cells were used for the growth stimulatory assay and MTT assay was performed as described in the manual from Boehringer Menheim.

Alkaline phosphatase activity assay

The activity of alkaline phosphatase (ALP) was measured using p-nitrophenyl phosphate as a substrate. After 48 h incubation with the factors, the cells were washed with PBS three times and solubilized in 0.5% Triton X-100 by repeated freeze-and-thawing. The protein content was measured by the micro-Lowry method with a slight modification for microplate reader. The ALP reaction was performed in the presence of 10 mM p-nitrophenyl phosphate, 5 mM magnesium chloride buffered with 150 mM amino-methyl propanol, pH 10.0, for 5 min or longer. During incubation, the absorbance was measured every 15 sec at 405 nm and from which the reaction rate was calculated.

Western blotting

The cultures were washed three times with PBS and harvested by solubilizing in 1% Triton X-100 in 10 mM Tris/HCl, pH 7.4. The cell lysate was homogenized by passing through -28 Gauge syringe needle for 20 times at 4℃. The protein amount was estimated by the micro-Lowry method with some modification for microplate reader. SDS polyacrylamide gel electrophoresis was performed in 10% or 7%-14% gradient gel in the presence or absence of 2-mercaptoethanol using tall mighty electrophoresis unit (SE260) from Hoefer Scientific Instruments. After the electrophoresis, the proteins were transferred to nitrocellulose paper in semidry-transfer unit (TE70) for 50 min. The primary antibody was reacted with blotted nitrocellulose paper overnight, and secondary antibody binding was subsequentry performed for 3 h at room temperature. The chemiluminescence detection of alkaline phosphatase conjugated second antibody was performed using disodium 3-(4-methoxyspiro1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.13,7] decan-4-yl)phenyl phosphate (CDP star) as a substrate.

Transfection of antisense oligonucleotide against TGF-β2

Lipofectin Reagent (Life Technologies Inc.) was used for transfection of antisense oligonucleotide designed to inhibit TGF-β2 mRNA (AACATGCACTACTGT) into ROS17/2.8 cells. The transfection was performed as described in supplier's manual with some modification to optimize the efficiency. In brief, the cells were grown until 40-60% confluency in 35 mm plate. The cells were washed with serum-free DMEM, then incubated with transfection solution (1.5 ug/ml DNA, 0.5% Lipofectin in DMEM) in CO_2 incubator for 7 h. The cells were then harvested and subjected to Western blot analysis for TGF-β2. As a control, scrambled (ACT CGTCGGTGATCG) and sense (ACAGTAGTGCATGTT) sequences were transfected in the same way.

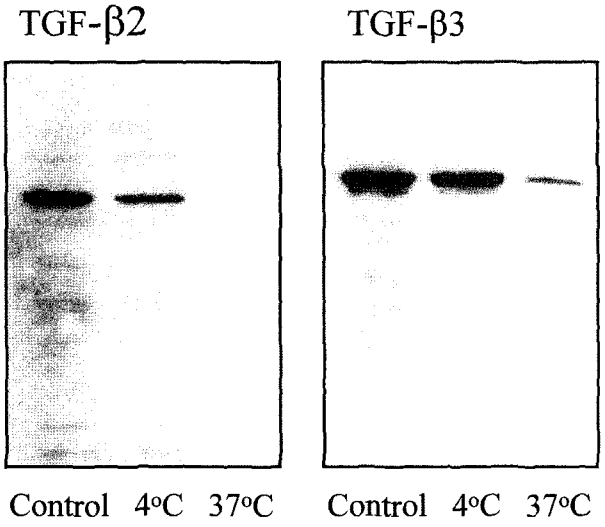

Fig. 1. Western blot analysis of TGF-β2 and 3 in ROS17/2.8 cells. For the protease-treatment, the cells were incubated with 0.1% collagenase, 0.25% trypsin in Ca^{2+}, Mg^{2+} -free DME for 1 h at 4℃ or 37℃ respectively.

Immunofluorescence staining

ROS17/2.8 cells were grown on a cover slip, and were fixed with 4% formaldehyde in PBS for 30 min. After permeabilization using chilled methanol for 2 min, the cells were overlayed with 30 μl of anti- TGF-β2 rabbit antibody solution (1% I-block solution with 0.4% sodium azide), and incubated for more than 4 h in a humidified condition. The primary antibody solution was then completely removed, and the cells were rinsed four times with PBS. Using the secondary antibody labeled with fluorescine, the precise location of antigen was visualized under a fluorescence microscope.

Results

In the homogenate of ROS17/2.8 call, Western blot analysis revealed 49 kDa and 55 kDa protein bands for TGF-β2 and -β3, respectively in a reducing condition (Fig. 1). Together with those high molecular weight forms, although in small amounts, 12-17 kDa bands were also observed. This observation is in good accordance with the expected molecular weight of TGF-β2 precursor deduced from its gene structure. In case of TGF-β3, the apparent molecular size was somehow bigger than expected, which may be explained by glycosylation and some other post-translational modifications.

Reports have indicated that TGF-β1 might be present as a bound form on the cell surface or on extracellular matrices (Zhu et al., 1999; Bonewald, 1999). To examine whether TGF-β2 and -β3 are also present on extracellular region in ROS17/2.8 cells, the cultures were digested with high concentration of collagenase and trypsin, and the remaining cellular components were analyzed by Western blot (Fig. 1). Since the

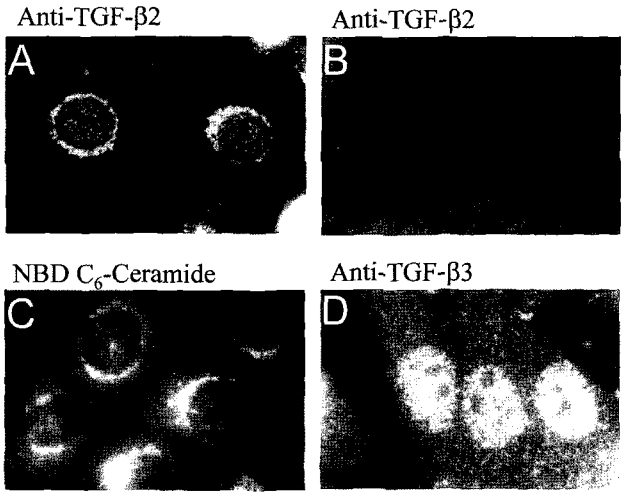

Fig. 2. Immunofluorescence localization of TGF-βs (A, B, D) in the cultured ROS17/2.8 cell. Golgi apparatus was specifically visualized by adding NBD C6-ceramide in culture medium (C). A, C, D: the cells were fixed and permeabilized with chilled methanol, B: the permeabilization step was omitted.

major bands of TGF-β remained constant even in prolonged protease-treatment at 4℃, TGF-βs appeared to be unexposed on the outer surface or extracellular matrix. On the other hand, protease-treatment at 37℃ showed dramatic reduction of TGF-βs within 1 h. This indicated that a large amount of TGF-β might be stored inside of the cell as a high molecular weight form. The precise location for TGF-β2 was visualized using immunofluorescence stain (Fig. 2). Without permeabilization, the intensity of staining was very weak (Fig. 2B). However, methanol-permeabilization revealed the perinuclear location of TGF-β2 in ROS17/2.8 cell (Fig. 2A). The TGF-β2 antibody was highly restricted in the perinuclear region that would overlap with Golgi complex. Although the precise molecular forms of intracellular TGF-β2 is not revealed in this study, the electrophoresis analysis in non-reducing condition implied that it is covalently linked with other cellular components (data not shown). TGF-β3 showed distinct distribution and somehow accumulated in the nuclear region (Fig. 2D). In this study, however, TGF-β2 was focused for further study, because TGF-β2 was suggested as a main participant in bone metabolism (Erlebacher and Derynck, 1996).

To confirm that the 49 kDa band is TGF-β2, we employed antisense oligonucleotide inhibition method. Using the antisense oligonuclotide designed to bind against the translation starting region of TGF-β2 mRNA, we were able to significantly reduce the 49 kDa band (Fig. 3). Transfection of other oligonucleotides of scrambled and sense sequences had little effect (Fig. 3). Therefore, the 49 kDa protein band seemed to be a specific gene product of TGF-β2.

Since the Western blot study suggested that ROS17/2.8 cell may constantly produce TGF-β2, we examined the biological activity of TGF-β2 in a conditioned me-

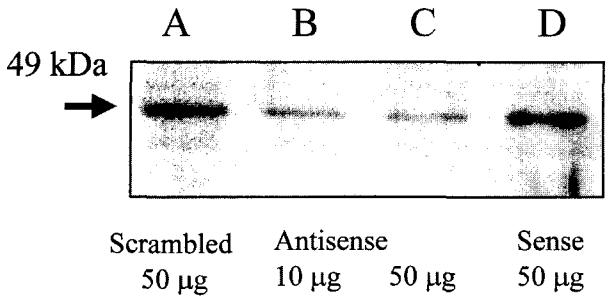

Fig. 3. Western blot analysis of TGF-β2 precursors after transfection with oligonucleoties of scrambled sequence (50 μg, A), antisense sequence against the starting region of TGF-β2 mRNA (10 μg and 50 μg, B and C), and the sense sequence of the same region (50 μg, D).

dium. As described by previous investigators (Huang et al., 1999), TGF-β2 has a strong growth stimulatory activity in NIH3T3 cells at concentrations as low as 0.05 ng/ml. Since other growth factors may have a similar growth stimulatory effect on these cells, we specifically measured the TGF-β2 activity by substracting the growth promoting activity in the presence of anti-TGF-β2 neutralizing antibody. In pilot experiments, this antibody inhibited the growth stimulation induced by TGF-β2 but not by other growth factors including TGF-β1. The 24 h-conditioned medium of ROS17/2.8 was combined with fresh 2% FBS/DMEM to supply any depleted components during the conditioning, and then was used to measure the TGF-β2 activity. In the intact conditioned medium, as mentioned by previous investigators (Dallas et al., 1995), I was not able to detect any TGF-β activities. The heat-treatment of the conditioned medium, however, revealed TGF-β2 activity that could be neutralized by anti-TGF-β2 specific antibody (Fig. 4). Furthermore, the activity in the medium seemed be down regulated by the presence of

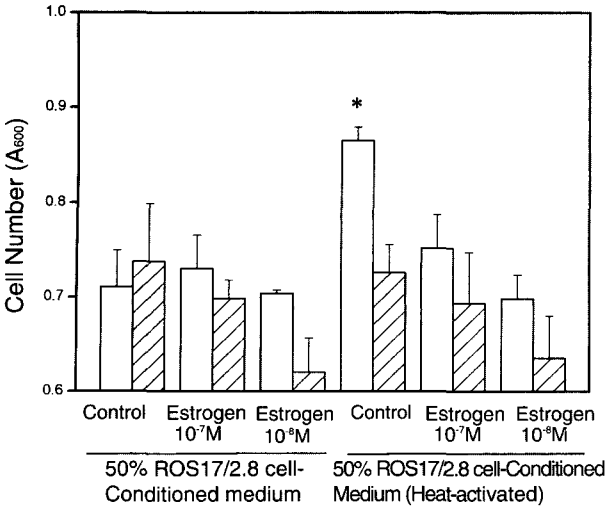

Fig. 4. TGF-β2 activity in the conditioned media of ROS17/2.8 cells. The presence of estrogen signficantly reduced TGF-β2 activity that is specifically inhibited by 5 ug/ml anti-TGF-β2 neutralizing antibody (▨). The fresh media containing the same amount of estrogen was used as a control (□) for each group (*p<0.01).

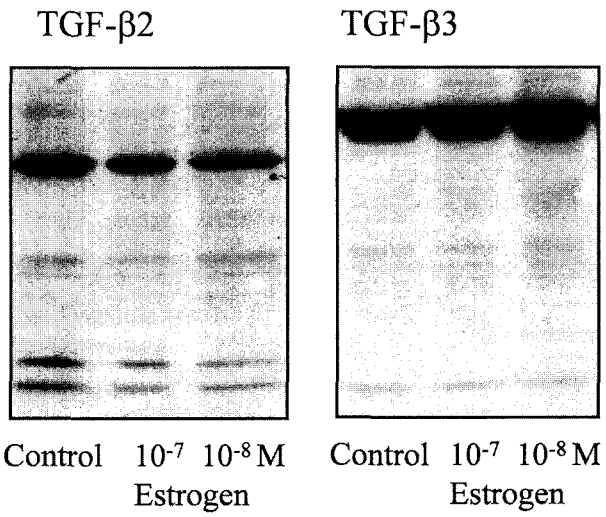

Fig. 5. Western blot analysis of TGF-β2 and -β3. The band patterns of TGF-β2, but not TGF-β3, were changed in the presence of estrogen. The cells were challenged for 24 h with indicated concentration of estrogen, then harvested for SDS-PAGE and subsequent Western blot analysis using specific antibodies.

estrogen (Fig. 4). Therefore, it could be inferred that the process and secretion of the stored latent-TGF-β is under the control of external signals. Correspondingly, the internal pool of TGF-β2 was changed in the presence of estradiol in the culture medium (Fig. 5). The changes mainly occurred in the low molecular weight form of TGF-β2. In contrast, TGF-β3 showed no changes in Western blot patterens by the presence of estrogen.

Discussion

The TGF-β isoforms consists of TGF-β1 to -β3 in mammals. Although they are differentially expressed in various tissues, they may share same biological activities. In various systems, including bone cell culture, those three isoforms have same effects, and, furthermore, these isoforms bind to the same surface receptors. Therefore, the differences among the isoforms were investigated primarily for their expression patterns or extra-cellular activation processes. The activation process was studied in detail for TGF-β1 (Taipale et al., 1992), however, little is known how TGF-β2 and -β3 are processed and activated. In this study, we analyzed the intracellular processing of TGF-βs using specific antibodies.

Since the half-life of TGF-β is remarkably short in blood, TGF-β would work as a paracrine autocrine factor rather than as a systemic effector. Previous study suggested that latent TGF-β1 accumulates in the extracellular matrix (Zhu et al., 1999), and some proteases, which are secreted by TGF-β1 activating cells such as activated macrophage and endothelial cells cocultured with smooth muscle cells, may release and activate the stored latent TGF-β1 (Grainger et al.,

1994; Chu and Kawinski, 1998). More recently, thrombospondin-1 was suggested to be a major activator of TGF-β1 (Crawford et al., 1998). The authors also suggested that its conformational change is a principal activation mechanism. However, those studies were basically concerned with TGF-β1, and it is still not clear whether other types of TGF-β undergo the same activation pathway. Recently the existence of latent TGF-β in the perinuclear region of hepatic stellate cell was visualized by confocal microscopy using type-nonspecific polyclonal antibody (Roth-Eichhorn et al., 1998), and subcellular fractionation using standard differential centrifugation showed colocalization of TGF-β1, LAP, and LTBP in the Golgi microsomal fraction. Although the interpretation of the data is difficults due to the use of type-nonspecific antibody, it seems clear that at least one type of TGF-β present in the perinuclear region of this cell type. In this study, I used antibody that specifically binds to the indicated type and demonstrate clearly that the different types of TGF-β are present in different subcellular regions. A significant amount of TGF-β2 and -β3 appears to be accumulated in the cell in latent forms not only in osteoblastic cell, but also in other cell types, such as NIH3T3, MC3T3-E1 (data not shown). Therefore, the different types of TGF-β may be present in specific regions of subcellular structure regardless of cell types.

Because the considerable amount of the latent forms is depleted by the protease treatment at 37℃, they seem to be secreted rapidly from the cell in response to the protease treatment (Fig. 1). In addition, administration of estrogen on ROS17/2.8 cell clearly altered the secretion of TGF-β2 (Fig. 5). Accordingly, there is a report indicating that tissue-injury and obligational cellular damage may cause the release of TGF-βs from neighboring cells (Roth et al., 1998). Although the precise relationship of extracellular signals causing secretion and intracellular processing of TGF-β2 are not known, it seems that the regulation of intracelluar processing could be a key step in controlling TGF-β 2-related physiological changes.

It is known that the activation of latent TGF-β can be achieved by different chemical and enzymatic treatments, or by incubation with certain cell types. This extracellular activation was suggested as a crucial step in the regulation of TGF-β activity (Grainger et al., 1994; Crawford et al., 1998). In addition to these extracellular activation, recent studies showed injury-dependent release of TGF-β from liver cells (Roth et al., 1998).

In this study, I suggest the intracellular processing of TGF-β to be an important regulatory step in the action of TGF-β. Little is known about the intracellular processing of TGF-βs. However, it could be inferred that the latent form of TGF-β might be stored in the cytoplasm. In response to external signals, it is subjected to intracellular proteolytic cleavage, and the subsequent secretion may complete the intracellular processing of TGF-β2 necessary for further extracellular activation and/or exerting its potent biological activities.

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