Production of Hydrogen Peroxide by Transforming Growth Factor-β1 and Its Involvement in Induction of egr-1 in Mouse Osteoblastic Cells

Motoi Ohba, Motoko Shibanuma,* Toshio Kuroki, and Kiyoshi Nose*
Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan; and * Department of Microbiology, Showa University School of Pharmaceutical Sciences, Shinagawa-ku, Tokyo 142, Japan

Abstract. TGF-β1 controls the expression of numerous genes, including early response and cellular matrix genes. However, the signal-transducing mechanism underlying this regulation of gene expression is not fully understood. In this study, we investigated whether redox regulation plays a role in the TGF-β1 signal transduction in the mouse osteoblastic cell line (MC3T3-E1). The overall intracellular oxidized state of the cells, when measured using 2',7'-dichlorofluorescin diacetate by laser-scanning confocal microscopy, was increased transiently after the addition of TGF-β1. This increase was abolished by the addition of oxygen radical scavengers such as catalase and N-acetyl-cysteine. In a variant cell line lacking the TGF-β1 receptor, the intracellular oxidized state was not modulated by treatment with TGF-β1. We then examined the expression of early growth response-1 (egr-1) gene, which is inducible by TGF-β1 and H2O2. Radical scavengers inhibited the induction of egr-1 by TGF-β1, but not that by 12-O-tetradecanoylphorbol-13 acetate. A nuclear run-on assay indicated that this inhibition was at the transcriptional level. From transient expression experiments using chloramphenicol acetyltransferase gene linked to serially deleted egr-1 gene 5'-upstream region, the CArG element in the 5' flanking region of egr-1 was identified as an essential sequence in the transcriptional activation for both TGF-β1 and H2O2 stimulation. These findings suggest that H2O2 acts as a mediator for the TGF-β1-induced transcription of egr-1 gene.

Transforming growth factor-β1 is a multifunctional cytokine regulating cell proliferation, differentiation, and other biological responses (Roberts and Sporn, 1988; Barnard et al., 1990). The diverse biological effects exerted by TGF-β1 depend on cell types, growth status of cells including culture conditions, and the presence of other growth factors. This factor was originally recognized as a stimulator for the anchorage-independent growth of normal cells. However, it is now widely accepted as a potent growth inhibitor of epithelial cells in vitro (Massagué, 1987; Masui et al., 1986), while it acts as a bifunctional regulator for mesenchymal-derived cells (Robey et al., 1987; Centrella et al., 1987; Shibanuma et al., 1991).

TGF-β1 modulates the expression of a variety of genes: it increases the expression of extracellular matrix proteins and protease inhibitor genes (Ignotz and Massagué, 1986; Roberts, et al., 1988; Lund et al., 1986; Thompson et al., 1988) and it decreases the expression of genes for proteases that degrade matrix proteins (Edwards et al., 1987; Kerr et al., 1988). TGF-β1 also induced the expression of immediate-early response genes such as c-jun, junB, early growth response-1 (egr-1)1, and TSC-22 (Pertovaara et al., 1989; Koskinen et al., 1991; Shibanuma et al., 1992). Pietenpol et al. (1990a; 1990b) reported that the growth inhibition caused by TGF-β1 correlated with a decrease in c-myc gene transcription. The products of the retinoblastoma gene and the p53 gene seem to be also involved in this growth inhibition and result in cell cycle arrest (Pietenpol et al., 1990b, 1991; Laiho et al., 1990). We have recently cloned a new TGF-β1-inducible gene (TSC-36) whose expression reached a peak at 6–8 h after stimulation with TGF-β1, and this gene seems to encode an extracellular matrix protein (Shibanuma et al., 1993).

Although the multifaceted gene control actions of TGF-β1 have been explored, the exact mechanisms by which TGF-β1 exerts its effects is still only partly understood. Lin et al. (1992) recently cloned TGF-β1 type II receptor with a serine/threonine kinase domain. The receptor acts as a key component in the transduction of TGF-β1–signaling by form-

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DCFH, nonfluorescent polar derivative of DCFH-DA; egr-1, early growth response-1 (gene); GSH, intracellular glutathione; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidine carbodithioic acid ammonium; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Changes in intracellular redox state of MC3T3-E1 cells after treatment with TGF-β1. (a) Cells in preconfluent state were transferred to 0.1% BSA medium, and were treated with TGF-β1 (5 ng/ml) for the indicated times (minutes). After treatment, medium was replaced with Hanks' solution containing 5 μM DCFH-DA, and fluorescence intensity was measured 5 min later with a confocal laser scanning microscope. (B) Relative fluorescence intensity of each cell was calculated relative to untreated control cells. (C) Cells were either treated with TGF-β1 (5 ng/ml) (column 2) for 75 min or with H2O2 (columns 3–7) for 10 min. About 50 cells were used for determination of each point. The results show the means ± standard deviation. The experiment was repeated three times with reproducible results.

Figure 1. Changes in intracellular redox state of MC3T3-E1 cells after treatment with TGF-β1. (a) Cells in preconfluent state were transferred to 0.1% BSA medium, and were treated with TGF-β1 (5 ng/ml) for the indicated times (minutes). After treatment, medium was replaced with Hanks' solution containing 5 μM DCFH-DA, and fluorescence intensity was measured 5 min later with a confocal laser scanning microscope. (B) Relative fluorescence intensity of each cell was calculated relative to untreated control cells. (C) Cells were either treated with TGF-β1 (5 ng/ml) (column 2) for 75 min or with H2O2 (columns 3–7) for 10 min. About 50 cells were used for determination of each point. The results show the means ± standard deviation. The experiment was repeated three times with reproducible results.

Materials and Methods

Cell Culture

The mouse osteoblastic cell line, MC3T3-E1 (Kurihara et al., 1986), and its v-Ki-ras-transformant, MC3T3-K1 (Nose et al., 1989), were cultured in Dulbecco's modified medium supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin under a humidified atmosphere of 5% CO2 in air. Cells were kept in continuous culture for <3 mo after thawing.

Chemicals

Recombinant human TGF-β1 was obtained from King Brewing Co. Ltd. (Kakogawa, Japan), and 12-O-tetradecanoylphorbol-13-acetate, N-acetyl l-cysteine (NAC) and catalase derived from Aspergillus niger were products of Sigma Chemical Co. (St. Louis, MO). Pyrrolidine carboxylic acid ammonium (PDTA) was purchased from Nakarai Tesque, Inc. (Kyoto). Potassium ferricyanide and ascorbic acid were obtained from Wako Pure Chemicals (Tokyo). NAC and ascorbic acid solutions were adjusted to pH 7.4 by the addition of NaOH. 2',7'-dichlorofluorescin diacetate (DCFH-DA) was obtained from Eastman Kodak Corp. (Rochester, NY). The kinase inhibitors H-7, H-9, and W-7 were obtained from Seikagaku Kogyo (Tokyo). Staurosporine was purchased from Kyowa Medix Co. Ltd. (Tokyo).

RNA Extraction and Analysis

At ~80% confluence, the cells were starved in serum-free DME containing...
Results

Increase of lntracellular Oxidized State in MC3T3-El Cells with TGF-β1

Our previous study showed that there was small increase in the H$_2$O$_2$ concentrations in the culture medium after treatment with TGF-β1, as detected by 3-(p-hydroxyphenyl)-propiolic acid and horseradish peroxidase assays (Shibanuma et al., 1991). In the present study, intracellular oxidized states were measured using a more sensitive fluorescent assay method with DCFH-DA and laser-scanning confocal microscopy. The dye is incorporated into cells and is converted to a fluorescent compound by oxidation. The intensity of fluorescence reflects the intracellular oxidized state (Bass et al., 1983; Robertson et al., 1990).

Quiescent cultures of MC3T3 cells were treated with TGF-β1 (5 ng/ml) for various times. As shown in Figs. 1 A and B, the intensity of DCFH fluorescence began to increase 60 min after treatment, reaching a maximal level of fivefold higher than that in unstimulated cells at 75 min after treatment. This was followed by a decline in fluorescence to the basal level by 120 min. Some cells showed high fluorescence in nuclei, possibly because of accumulation of the dye in nuclear fraction. The maximal level of intracellular fluorescence was comparable to that treated with 10-20 nM H$_2$O$_2$ (Fig. 1 C). In a ras-transformed variant of MC3T3 cells (K1) that were resistant to TGF-β1 through loss of its binding sites (Nose et al., 1989), no change in fluorescence intensity was observed after treatment with TGF-β1 (Fig. 1 B). These results suggested that an increase in the intracellular oxidized state was induced specifically by TGF-β1 through the binding to its receptors.

DCFH can be oxidized by any peroxidase and hydroperoxide, including H$_2$O$_2$ (Bass et al., 1983; Keston and Brandt, 1965). To specify the active oxygen species that were responsible for the oxidation of DCFH, we examined whether catalase, an enzyme that specifically hydrolizes H$_2$O$_2$, prevented the oxidation of DCFH. MC3T3 cells were treated with TGF-β1 in the absence (Fig. 2, top and bottom, column B) or presence (Fig. 2, top and bottom, column C) of catalase (350 U/ml). The addition of catalase completely inhibited the increase in DCFH oxidation as measured 75 min after TGF-β1 treatment. Heat-inactivated catalase had no effect on DCFH oxidation (Fig. 2, top and bottom, column E). These findings suggest that H$_2$O$_2$ participated in DCFH oxidation after the TGF-β1 treatment. Nonspecific thiol radical scavengers, NAC and PDTC, also inhibited the increase in the intracellular oxidized state induced by TGF-β1; NAC at 2.5 mM had a 25% inhibitory effect on the increase in DCFH oxidation, and PDTC at 0.1 mM inhibited this increase by 50% (data not shown).

Inhibitory Effect of Radical Scavengers on the Expression of egr-1 Gene by TGF-β1

We then investigated whether H$_2$O$_2$ generated by TGF-β1 plays a role in gene expression. Our previous study demonstrated that active oxygen species stimulated the expression of the immediate early response genes such as c-fos, c-jun, c-myc, and egr-1 (Bass et al., 1991). Of these genes, egr-1 was found to respond to both TGF-β1 and H$_2$O$_2$ in MC3T3 cells.

Fig. 3 shows Northern blotting of egr-1 gene at various times after treatment with either TGF-β1 (2 ng/ml) or H$_2$O$_2$ (0.15 mM). H$_2$O$_2$, at concentrations <0.2 mM, did not affect cellular viability as determined by dye exclusion test and plating efficiency assay (data not shown). As seen in Fig. 3, egr-1 gene expression increased within 30 min after stimulation with TGF-β1, reaching the maximal level 2 h after treatment. H$_2$O$_2$-induced egr-1 mRNA more rapidly and transiently, reaching a peak within 30 min after the treatment. Slower induction of egr-1 by TGF-β1 compared to that by H$_2$O$_2$ showed the delay of ~90 min. This delay roughly coincided with the time course of changes in the intracellular oxidized state determined by the confocal laser scanning microscopy (Fig. 1).

We examined the effects of radical scavengers, i.e. catalase, NAC, PDTC, potassium ferricyanide (Chaudhri et al.,
Figure 2. Effects of catalase on the increase in intracellular oxidized state induced by TGF-β1. Cells were treated for 75 min with either TGF-β1 (5 ng/ml) (B), TGF-β1 + catalase (350 U/ml) (C), catalase (D), or TGF-β1 + catalase (heat-inactivated) (E). Fluorescence intensity was measured and calculated as described in the legends to Fig. 1 B. The experiments were repeated four times, and the results were reproducible.
Figure 3. Time course of induction of egr-1 mRNA by TGF-β1 and H₂O₂. MC3T3 cells were either untreated (lane 1), treated with TGF-β1 (2 ng/ml) (lanes 2–6), or H₂O₂ (0.15 mM) (lanes 7–11) for indicated times. Total RNA was extracted, run on a gel, and transferred to membrane filters. The filters were hybridized with 32P-labeled probes, washed, and autoradiographed. Mouse α-tubulin probe (M α 1) was used to monitor the amount of RNA in each lane.

Figure 4. Effects of radical scavengers on the induction of egr-1 and TSC-36 mRNA by TGF-β1. (A) MC3T3 cells were either untreated (lane 1), or they were treated with TGF-β1 (2 ng/ml) in the absence (lane 2) or presence of 0.5 mM NAC (lane 3), 50 μM PDTC (lane 4), 5 mM potassium ferricyanide (K-Ferr; lane 5), 5 mM ascorbic acid (Vit.C; lane 6), or 170 U/ml catalase (cat; lane 7) for 2 h. (B) Cells were untreated (lane 1), or they were treated with TGF-β1 (2 ng/ml) in the absence (lane 2) or presence of increasing concentrations (lane 3–7, 0.1, 0.5, 1.0, 2.5, and 5.0 mM, respectively) of NAC. (C) Cells were untreated (lane 1), or they were treated with 2 ng/ml TGF-β1 in the absence (lane 2) or presence of 170 U/ml catalase (lane 3), 2.5 mM NAC (lane 4), or 50 μM PDTC (lane 5) for 8 h. RNA was analyzed using 32P-labeled probes as described in the legend to Fig. 3.

Figure 5. Effects of radical scavengers on the induction of egr-1 by TPA. Cells were untreated (lanes 1 and 4), or they were treated for 2 h with 50 ng/ml TPA in the presence (lane 2 and 5) or presence of 170 U/ml catalase (lane 3), 2.5 mM NAC (lane 6), 50 μM PDTC (lane 7), 5 mM potassium ferricyanide (lane 8), or 5 mM ascorbic acid (lane 9). RNA was analyzed as described in the legends to Fig. 3.

Figure 6. Nuclear run-on assay. Nuclei were isolated from untreated cells (lane 1), and from cells treated with TGF-β1 (2 ng/ml) for 90 min in the absence (lane 2) or presence of 2.5 mM NAC (lane 3), and were transcribed in the presence of [32P]UTP. The transcripts were hybridized with the probes immobilized on membranes. The membranes were washed and autoradiographed.
A Common Target Sequence for TGF-β1- and H₂O₂-Stimulation of egr-1 Gene Transcription

To elucidate the transcriptional regulatory mechanism responsible for redox-mediated TGF-β1 signal transduction, we attempted to identify a common cis-acting DNA element for both TGF-β1 and H₂O₂. For this purpose, the deletion mutants upstream of the transcriptional start site of the egr-1 gene were fused to a CAT reporter gene. The mouse egr-1 upstream region contains five CA(T/A)₄GG (CARG) boxes plus the incomplete CCAATATCGG sequence (position -340 to -331 bp, Tsai-Morris et al., 1988). These CARG elements were progressively deleted from pEgr1394, a plasmid containing the mouse egr-1 upstream region between -1394 to +123 bp with five CARG elements and two AP-1-like elements (Fig. 7).

The highest CAT activity was observed with pEgr417, which contained all five CARG elements, in cells treated with either TGF-β1 or H₂O₂ (Fig. 7B). Treatment with TGF-β1 or H₂O₂ led to a 2.3- and 3.5-fold increase in CAT activity, respectively, compared to that of unstimulated cells. A progressive decrease in response was observed as each CARG element was deleted. Removal of the three CARG elements from the 5'-end (pEgr111) resulted in very weak induction by both agents, indicating that TGF-β1 and H₂O₂ require at least three CARG elements from the cap site for the induction. The above findings identify the CARG elements as a common target sequence for both TGF-β1 and H₂O₂.

Effects of Protein Kinase Inhibitors on the Expression of egr-1

Protein kinases participate in signal transduction initiated by diverse stimuli. Possible involvement of protein kinases in the redox-mediated signaling pathways was examined by protein kinase inhibitors. The results of Northern blots show that 50 μM of H-7, an inhibitor of protein kinases C, A, and G, repressed almost completely the egr-1 induction by TGF-β1 and H₂O₂ (Fig. 8). However, neither staurosporine, a potent inhibitor of protein kinase C, nor H-9, an inhibitor of cAMP-dependent kinase, affected the egr-1 induction by TGF-β1 and H₂O₂. Moreover, the induction of egr-1 by TGF-β1 was retained in the protein kinase C-downregulated cells after prolonged treatment with TPA (24 h) (data not shown). Based on these observations, we postulate that an H-7-sensitive kinase, but not protein kinase C, might be involved in the gene expression of egr-1 by TGF-β1 and H₂O₂.

Discussion

In the present study, we demonstrated that TGF-β1 increased the intracellular oxidized state in mouse osteoblastic cells, but not in a TGF-β1 receptor-deficient variant. This increase was almost completely abolished by the addition of catalase, indicating that H₂O₂ was a major cause of increased oxidized state. The production of H₂O₂ was also blocked, albeit to a lesser extent, by the nonspecific thiol agents, NAC and PDTC. NAC raises the levels of intracellular glutathione (GSH), which controls the cellular redox state as a substrate of GSH peroxidase. In addition, NAC and PDTC both directly quench intracellular free radical (Staal et al., 1990). Catalase, on the other hand, cannot be incorporated into cells, thereby acting on the outside of the cells. These results suggest that H₂O₂ might be first released from the cells after treatment with TGF-β1, and then diffused into the cells.

In the previous study (Shibanuma et al., 1991), we measured levels of H₂O₂ in the medium by using 3-(p-hydroxyphenyl)-propionic acid and horseradish peroxidase, and we detected H₂O₂ in the medium of cells stimulated by TGF-β1 only in cells at G₁ phase and not in G₀ phase cells. In the present study, however, the method using DCFH-DA and confocal laser microscopy made it possible to detect the increase of the intracellular oxidative state. Consistent with the previous work, the increase in the intracellular oxidative state was more pronounced and sustained in cells in late G₁ phase (data not shown).

The pathways for generation of free radicals in MC3T3 cells are not fully understood. NAD(P)H oxidase, which produces superoxide anion from O₂, is known to be localized in the outer cell membrane (Ramasarma, 1982). In neutrophils and macrophages, burst of oxygen radical occurs within seconds after stimulation (Sagone et al., 1976; Fridovich, 1983). In contrast, the change in redox state in response to TGF-β1 was slow and transient. In addition, radical scavengers did not inhibit egr-1 induction by TPA, which is known to activate NAD(P)H oxidase (Maridonneau-Parini, 1982).
The generating system of H$_2$O$_2$ activated by TGF-β1 in MC3T3 cells seems to be distinct from the NAD(P)H oxidase system, probably because of a specific, still-unknown generating system.

Changes in the redox state of cells are thought to induce modifications of cellular signaling molecules, including protein kinases, protein phosphatases, and transcription factors. Regulation by redox of a portion tyrosine kinase (Bauskin et al., 1991; Devary et al., 1992) and a protein phosphatase (Guy et al., 1993) has also been reported. In the present study, possible biological roles of oxidized state in gene expression was studied with the egr-1 gene, which was transcriptionally activated by both TGF-β1 and H$_2$O$_2$. The results of Fig. 4, A and B, show that scavengers for active oxygen specifically inhibited the induction of egr-1 by TGF-β1, indicating that the action of TGF-β1 is regulated by redox, at least in term of egr-1 gene transcription.

CAT assay using the egr-1 promoter region indicated that the CArG element is a common target for the transcriptional activation of egr-1 by both TGF-β1 and H$_2$O$_2$. A recent finding (Datta et al., 1993) demonstrated that the transcription induction of egr-1 caused by ionizing radiation also requires CArG elements. The involvement of active oxygen in this transcriptional activation was implied from the experiment with NAC. The CArG element is known to bind to serum response factor, whose binding activity is enhanced by its phosphorylation (Treisman, 1993).

Recent observations provide evidence that redox regulation is a general mechanism for the posttranslational control of transcription factor function. Changes in reduction-oxidation potential have been shown to influence the DNA binding activity of several transcription factors, such as Oxy R (Storz et al., 1990), FOS/JUN (Abate et al., 1990) and NFκB (Schreck et al., 1991). Oxy R is a best example of this redox regulation in the direct activation of a transcription factor in bacterial systems. DNA-binding activity of mammalian FOS/JUN is reported to be modified by the oxidation of their cysteine residues (Abate et al., 1990). Most of these works are, however, limited to in vitro experiments, and our present results indicate that redox regulation of transcription exists in vivo as well.

Active oxygen species are produced by various types of cultured cells under the influence of numerous stimuli (Meier et al., 1989; Robertson et al., 1990). They are generally regarded as harmful to cells, because they modify cellular macromolecules, including lipids, proteins, carbohydrates, and nucleic acids. Cells have several different types of systems to protect them against such free radical-induced damages; systems involving intracellular glutathione, catalase, superoxide dismutases, and glutathione peroxidase (Fridovich, 1978; Meister, 1983). Most active oxygen can be deleterious to cells, but cytokines, especially suppressors of growth such as tumor necrosis factor-α and interleukin-1, are known to stimulate cells to produce active oxygen (Beutler and Cerami, 1986; Meier et al., 1989).

We previously proposed that active oxygen species can work as a competence factor for growth in quiescent fibroblasts (Shibanuma et al., 1990). It now seems reasonable to believe that active oxygen at certain levels could work as biosignals in physiological conditions. The molecular mechanisms responsible for such signals may include direct oxidation of protein factors by sulfenylating (Storz et al., 1990) and S-thiolation (Gilbert, 1984). Our results with the inhibitors of protein kinases indicate that the activation of H-7-sensitive protein kinase(s) is necessary for the induction of egr-1 by either TGF-β1 or H$_2$O$_2$, and such kinase(s) could be a direct target of redox regulation. Protein kinases that are stimulated by interleukin-1 and tumor necrosis factor (Guesdon et al., 1993) could be regulated by redox.

The best example of the second messenger actions of active oxygen species is nitric oxide. Nitric oxide is one member of active oxygen species, and is well known as a major messenger molecule regulating immune function and blood vessel dilatation. It also serves as a neurotransmitter in the brain and peripheral nervous system (Lowenstein and Snyder, 1992). We propose that hydrogen peroxide could be another member of active oxygen that operates as a second messenger in growth factor signalings.

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