Suppression of Antioxidative Enzyme Expression by Transforming Growth Factor-β1 in Rat Hepatocytes*

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We have investigated the effect of transforming growth factor-β1 (TGF-β1) and three cytokines on expression of antioxidative enzymes, manganese-superoxide dismutase, copper, zinc-superoxide dismutase, and catalase in cultured hepatocytes of rat. While interleukin-1β and interleukin-6 induced manganese-superoxide dismutase gene expression, they slightly suppressed catalase gene expression in rat hepatocytes. TGF-β1 suppressed expression of all these antioxidative enzymes in time- and cell density-dependent manners. Furthermore, we examined the effect of TGF-β1 on expression of glutathione peroxidase and glutathione-S-transferase, which exhibit glutathione-dependent peroxidase activity in rat hepatocytes. Expression of two major classes of the rat glutathione-S-transferase subunits 1 and 2 was also reduced by TGF-β1, although expression of glutathione peroxidase was not affected. Flow cytometric analysis indicated that production of hydrogen peroxide and its intermediate through superoxide radical was increased in hepatocytes treated with TGF-β1. These data suggest that augmented production of hydrogen peroxide and its intermediate through suppression of antioxidative enzyme expression may participate in cellular injury or growth inhibition promoted by TGF-β1.

Reactive oxygen species have been implicated in the development and progression of cancer, inflammation, radiation injury, and aging (1, 2). As a defense system, cells possess specific enzymes that act directly on the reactive oxygen species. In mammals, three types of SOD, CuZn-SOD, Mn-SOD, and extracellular SOD, exist in three different regions of cells. These enzymes play key roles in protecting cells against direct and indirect oxidative damage by catalyzing dismutation of the superoxide radical O2− to O2 and H2O2. The generated hydrogen peroxide can convert to hydroxy radical, which is a more dangerous oxidant in cells by Fenton-type reaction. Catalase, a peroxisomal enzyme enriched in hepatocytes and erythrocytes, catalyzes the dismutation of hydrogen peroxide, forming as neutral products O2 and H2O. Moreover, rat hepatocytes have glutathione peroxidase and glutathione-S-transferase, other antioxidative enzymes which exhibit peroxidase activity. Glutathione peroxidase is a seleno-enzyme present in the cytosol and also the mitochondrial matrix and metabolizes organic peroxides as well as H2O2. The reaction H2O2 + 2GSH → GSSG + 2H2O converts hydrogen peroxide to water with the oxidation of GSH (3). Glutathione-S-transferase comprises several kinds of subunits, and glutathione peroxidase activity lies in subunits 1 and 2, which are major components present in the cytosol of rat hepatocytes (4). Glutathione-S-transferase, however, is able to catalyze organic peroxides only.

Our group previously showed that serum levels of Mn-SOD protein increased markedly in patients with epithelial ovarian cancer (5), neuroblastoma (6), and acute leukemia (7). On the contrary, Mn-SOD activity was reported to be low in human tumor cells from colon cancer (8) and hepatocellular carcinoma (9). Several humoral factors are suspected to cause induction of the Mn-SOD gene. TNF-α, IL-1, and lipopolysaccharide have been demonstrated to directly induce expression of the gene in many cells (10–13). The effectiveness of these substances seems to be cell-type specific, because IL-1 and IL-6 do, but TNF-α does not increase Mn-SOD expression in hepatocytes (14). Abnormally high levels of TNF and IL-1 were reported in ovarian cancer (15), suggesting a causative relationship between these cytokines and the high expression of Mn-SOD in this disease (16). There is, however, no information about what causes the suppression of Mn-SOD gene expression in colon cancer or hepatocellular carcinoma. Catalase activity was also found to be remarkably reduced in many tumor tissues including hepatocellular carcinoma. Loss of this activity was assumed to be due to depression of enzyme biosynthesis.

TGF-β1 is a multifunctional polypeptide, promoting embryogenesis (17) and collagenase (18), while inhibiting hematopoiesis (19) and growth of epithelial cells (20, 21) and hepatocytes (22). TGF-β1 also induces fibrosis and angiogenesis in vitro although it suppresses the growth of endothelial cells in vitro (23, 24). It is now recognized that TGF-β1 plays a central role in negative regulation of cell growth. TGF-β1 is expressed at the later stage of liver regeneration (25), which suggests that it has an important role in terminating the process. It is secreted by nonparenchymal cells but not by hepatocytes in normal liver and suppresses the growth of hepatocytes but not hepatoma cells (26). In the progression from chronic hepatitis to hepatic cirrhosis, expression of TGF-β1 increases, reaching the maximum level in patients with hepatocellular carcinoma (27). Recently we presented immunohistochemical data showing the expression of TGF-β1 in human hepatoma tissues (28).

In this study, we showed that TGF-β1 directly suppressed expression of antioxidative enzyme genes including Mn-SOD, CuZn-SOD, catalase, and glutathione-S-transferase genes in...
cultured rat hepatocytes. Flow cytometric analysis using DCFH-DA, which is a fluorescence indicator sensitive to a variety of peroxides, showed that TGF-β1 augmented production of peroxides probably through suppressing antioxidative enzyme genes.

MATERIALS AND METHODS

Isolation and Culture of Rat Hepatocytes—Hepatocytes were isolated from male Wistar rats (150–250 g, being fed ad libitum) by in vitro collagenase perfusion, filtration, and low-speed centrifugations (29). The viability of the hepatocytes was greater than 90%, as determined by the trypan blue exclusion method. Cell suspensions were washed in cold Dulbecco’s modified Eagle’s medium and plated on collagen-coated plates at a density of 5 × 10^5 cells/ml in Willim’s E medium supplemented with 7% fetal bovine serum, 0.1 mM dexamethasone, 0.1 mM insulin, 100 μg/liter streptomycin, and 10^4 units/liter penicillin G. Aseptic procedures were used throughout. Cells were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After incubation for 5 h, the cultures were washed with phosphate-buffered saline to remove dead cell aggregates only loosely attached to the dish. The medium was changed to serum-free William’s E medium with 5 units/ml aprotinin, a protease inhibitor. After further incubation for 20 h, the hepatocytes were grown to 70–80% confluence. Then cells were changed to new medium containing 10 ng/ml TGF-β1 (Nacalai Tesque Inc.), 10 ng/ml IL-1β (Otsuka Pharmaceutical Co.), 50 units/ml human IL-6 (a kind gift from Dr. T. Hirano, Osaka University), or 10 ng/ml TNF-α (Ube Industries), and were incubated for an additional 24 h.

RNA Isolation and Northern Analysis—After incubation with the agents indicated above, cells were washed twice with phosphate-buffered saline, scraped off the plates in the presence of 5 mM EDTA, and precipitated by centrifugation. Total RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform (30) and quantified by measuring the absorbance at 260 nm. Twenty μg of total RNA were heat-denatured at 65°C for 15 min in the presence of 50% formamide and 1× running buffer (40 mM Mops, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0) and electrophoresed on a 1% agarose gel containing 2.2% formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane (Bio-Rad) for 20–40 h by capillary action, and the blotted RNAs were immobilized on the membrane by incubation at 80°C for 60 min. After hybridization with a 32P-labeled rat Mn-SOD cDNA (31), rat CuZn-SOD cDNA (32), rat catalase cDNA (33), rat glutathione peroxidase cDNA (34), rat glutathione-S-transferase subunit 1 cDNA (35), or glutathione-S-transferase subunit 2 cDNA (35) probe at 42°C in the presence of 50% formamide, the membranes were washed twice for 30 min at 55°C with 30 mM sodium citrate, 300 mM NaCl, pH 7.0, and 0.1% sodium dodecyl sulfate. Kodak XAR films were exposed for 1–3 days with an intensifying screen at −80°C. In order to normalize mRNA content, blots were stripped and reprobed with a β-actin cDNA using the hybridization and washing conditions described above. All other DNA and RNA manipulations were conducted according to Maniatis et al. (36).

Measurement of Intracellular Peroxides by Flow Cytometry—Intracellular peroxide levels were assessed using an oxidation-sensitive fluorescent probe DCFH-DA (37). In the presence of a variety of intracellular peroxides, DCFH is oxidized to a highly fluorescent compound, 2',7'-dichlorofluorescein. Cells, treated with TGF-β1 or IL-1β, were incubated with 5 μM DCFH-DA. The cellular fluorescence intensity, which was directly proportional to levels of intracellular peroxides after 30 min DCFH-DA oxidation, was measured using FACScan (Becton Dickinson). For each analysis, 10,000 events were recorded. For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system.

RESULTS

Effect of Cytokines on SODs and Catalase mRNA Expression in Cultured Rat Hepatocytes—Since it is known that HCC cells, but not normal hepatocytes, produce TGF-β1 (28), a negative regulator of cell growth, and the expression of Mn-SOD and catalase genes were reduced in HCC cells (38), we have investigated the effect of TGF-β1 as well as TNF-α, IL-1β, and IL-6 on the expression of three antioxidative enzymes, Mn-SOD, CuZn-SOD, and catalase mRNAs in cultured rat hepatocytes (Fig. 1). Mn-SOD mRNA levels were increased after 24 h incubation with either IL-6 or IL-1β, but not after incubation with TNF-α, a known inducer of Mn-SOD in many cells (10). TGF-β1 alone reduced the level of Mn-SOD mRNA but had virtually no effect on the induction by IL-1β and IL-6. Levels of CuZn-SOD mRNA were not affected by the cytokines except for TGF-β1, which reduced the mRNA level to about half of the control level at 10 ng/ml. Catalase gene expression was suppressed by TGF-β1, IL-1β, and IL-6. Expression of β-actin was not affected by these cytokines, indicating that their functions were specific to these genes. Thus TGF-β1 directly suppressed the expression of Mn-SOD, CuZn-SOD, and catalase in rat hepatocytes under the condition of culture.

Effect of TGF-β1 on Glutathione Peroxidase and Glutathione-S-transferase mRNA Expression in Cultured Hepatocytes—Since hepatocytes have glutathione peroxidase and glutathione-S-transferase as peroxide-scavenging enzymes and subunits 1 and 2 are major components of glutathione-S-transferase in rat hepatocytes, effects of TGF-β1 and IL-1β on expression of subunits 1 and 2 genes, as well as glutathione peroxidase gene, were investigated (Fig. 2). TGF-β1 again suppressed expression of both glutathione-S-transferase subunits genes but had no effect on glutathione peroxidase expression. IL-1β did not affect expression of these genes. Taking Figs. 1 and 2 together, TGF-β1 suppressed all the antioxidative enzyme genes except for the glutathione peroxidase gene.

Characteristics of the Suppression of Antioxidative Enzymes Gene Expression by TGF-β1—As shown in Fig. 3, incubation with TGF-β1 led to a time-dependent decrease in the levels of
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Mn-SOD, CuZn-SOD, and catalase mRNAs, while the level of β-actin mRNA remained constant (data not shown). The function of TGF-β1 on cells is dependent on cell cycle (22). To investigate whether the suppression of these gene expressions are specific to the cell cycle, effects of TGF-β1 on the levels of Mn-SOD mRNA in rat hepatocytes were cultured at different cell densities (Fig. 4). In confluent conditions (4 x 10⁶ cells/dish), in which hepatocytes stay at G₀ phase, no change in the level of Mn-SOD mRNA was observed with or without TGF-β1. The suppression was cell density-dependent and was prominent at lower cell density (70% suppression at 1 x 10⁶ cells/dish) in which more hepatocytes entered into the G₁ phase from the G₀ phase. Thus the effect of TGF-β1 on Mn-SOD mRNA synthesis was also dependent on the cell density in the culture, being consistent with other functions of TGF-β1.

Flow Cytometric Analysis of Intracellular Peroxides Produced by IL-1β and TGF-β1—Cells treated with TNF-α and IL-1β produce hydrogen peroxide which is assumed to mediate cytotoxic effects of these cytokines. Using flow cytometry we examined production of peroxides by rat hepatocytes treated with IL-1β. The cells treated with IL-1β for 24 h markedly increased the intracellular content of peroxides (Fig. 5A). When cells were treated with phorbol 12-myristate 13-acetate, more peroxide was produced (data not shown). Production of peroxides essentially similar to that by IL-1β was observed in the cells treated with TGF-β1 (Fig. 5B). This is the first direct evidence that TGF-β1 stimulated cells to produce peroxides.

**DISCUSSION**

The present study was undertaken to examine the correlation between expression of TGF-β1 and reduced expression of Mn-SOD in cancerous lesions of the liver. In situ hybridization studies showed that HCC cells were expressing TGF-β1 but normal hepatocyte were not (28). It is well known that catalase activity and the protein level are reduced in the HCC cells (38). While Mn-SOD staining by a specific antibody was strong in normal hepatocytes, it was weak in HCC cells. In cultured rat hepatocytes, TGF-β1 suppressed expression of the Mn-SOD, CuZn-SOD, and catalase genes (Fig. 1). It is, therefore, conceivable that TGF-β1 secreted by the hepatoma cells down-regulated antioxidative enzyme expressions, such as Mn-SOD and catalase, in these cells by an autocrine mechanism in vivo. We also found that the level of plasma TGF-β1 increased in patients with colon cancer and hepatoma. Hence, TGF-β1 may also participate in suppression of Mn-SOD expression in colon cancer as well as in hepatoma.

TGF-β1 inhibits growth of hepatocytes and prevents rat liver regeneration after intravenous injections (39). Following partial hepatectomy, TGF-β1 mRNA increased only after the major wave of hepatocyte division, suggesting that TGF-β1 was produced to limit excessive regeneration (25). This function would likely be carried out by lengthening or blocking the cell cycle between the early G₁ and late G₁ phase (22). In this study, we found that TGF-β1 suppressed Mn-SOD mRNA expression in cultured hepatocytes at low cell density, but not at high cell density.
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accumulated peroxides. Reactive oxygen species, including superoxide anion, hydrogen peroxide, and hydroxyl radicals, are known to induce biological damage by reacting with DNA and proteins as well as various membrane components (46–48). These intracellular reactive oxygen species also bring about several different types of DNA damage, including chromosomal aberrations, sister chromatid exchanges, and mutations (49, 50). These reports suggest that reactive oxygen species are involved in the process of tumor promotion and carcinogenesis. Moreover, subsequent production of TGF-β1 by the tumor cells would inhibit normal cell regeneration and decrease the antioxidant enzyme content in the tumor cells, causing malignant tumor formation through the accumulation of further mutations including tumor suppressor genes such as p53 and RB.

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