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Transforming Growth Factor β Is a Modulator of Platelet-Derived Growth Factor Action in Vascular Smooth Muscle Cells: A Possible Role for Catalase Activity and Glutathione Peroxidase Activity

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Transforming growth factor-β (TGF-β) has been implicated in mediating the growth of vascular smooth muscle cells (VSMCs) after vascular injury. In this study, we examined the mechanism underlying the growth-modulating effects of TGF-β in confluent VSMCs. Stimulation of rat VSMC by TGF-β decreased both their catalase activity and glutathione peroxidase activity in a dose-dependent manner. In mitogenesis assays using the confluent cells, TGF-β was not a direct mitogen for VSMC, but potentiated the stimulatory effect of platelet-derived growth factor (PDGF)-BB. This enhancement of mitogenesis was blunted by the addition of the scavenging enzyme catalase or the chemical antioxidant N-acetyl-L-cysteine. In summary, TGF-β enhances the mitogenic effect response of PDGF-BB, largely depending on the dysregulation of catalase activity and glutathione peroxidase activity by TGF-β.

The proliferation of VSMC plays a major role in the vascular reparative response after mechanical injury. There is evidence that a variety of factors regulate this VSMC growth, including PDGF (1), TGF-β (2), angiotensin-II (3) and basic fibroblast growth factor (4).

TGF-β is a 25KD growth factor released during the acute phase of injury by degranulating platelets (5) and activated macrophages (6). TGF-β is a multifunctional regulating peptide that can inhibit or promote the proliferation of cultured VSMC depending on cell density (7). TGF-β is also reported to stimulate growth of cultured VSMC primarily through autocrine production of PDGF-AA (8).

Recent increasing evidence suggests that reactive oxygen species (ROS) may function as second messengers in cytokines (interleukin-1 and tumor necrotizing factor-α), and in some growth factor mediated intracellular signal transduction pathways (9). In particular, ROS is reported to stimulate VSMC growth and protooncogene expressions (10). Sundaresan *et al.* demonstrated that a signal transduction induced by PDGF-BB requires the generation of hydrogen peroxide (H_2O_2) (11). Therefore, there may be a relation among TGF-β stimulation, H_2O_2 and VSMC proliferation. To test this hypothesis, we studied the effects of H_2O_2 on VSMC growth, and the effect of TGF-β on the catalase and glutathione peroxidase activity in cell, which can metabolize H_2O_2 to H_2O. In this work, we have shown that the addition of TGF-β to the cells decreases their catalase and glutathione peroxidase activity. In addition, it was suggested that an enhancement of PDGF-induced mitogenesis by TGF-β is partly caused by the decrease in catalase and glutathione peroxidase activity by TGF-β.

MATERIALS AND METHODS

Materials. Aminotriazole (ATZ) and nitroarginine were obtained from Sigma. 2′-7′-Dichlorofluorecin diacetate (DCFH-DA) from Molecular Probes, and [^{3}H] thymidine from Amersham. N-acetyl-L-cysteine (NAC) was from Aldrich. Catalase was from Boehringer Mannheim. TGF-β was from Funakoshi Pharmaceutical, Tokyo. All cell culture materials were from Life Technologies.

Cell culture. Aortic VSMCs were obtained from thoracic aorta of the rat by the method described previously (12). The cells(1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. The cells were used between the third and fifth passage. Cells were grown to confluence, at which time they were rendered quiescent by serum deprivation and maintained in serum-free medium for 36h before experiment.

[^{3}H]Thymidine incorporation. VSMCs were seeded at a density of 5×10^4 cells/dish and synchronized at the G_0/G_1 phase of the cell cycle by incubation for 3 days in DMEM containing 0.5% (v/v) fetal bovine serum. The medium was then removed and the cells were stimulated to proliferate in DMEM containing TGF-β with or without

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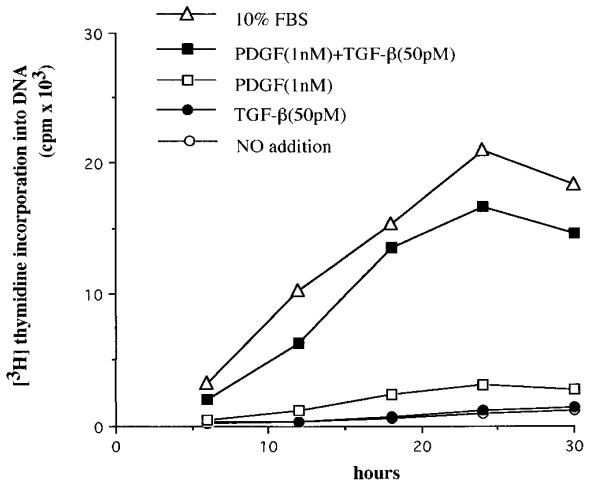

FIG. 1. Determination of the mitogenic response of cultured VSMC to TGF-β, PDGF, serum, and TGF-β+PDGF. Confluent SMC were incubated with growth factors or serum for the indicated times. Each time point represents [^{3}H] thymidine incorporated into DNA. Data represent the mean of four independent replicates in duplicate.

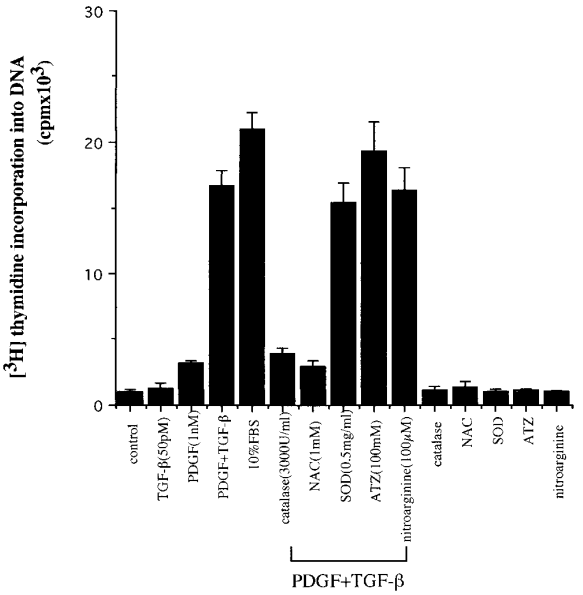

FIG. 2. Antioxidants inhibit mitogenesity by PDGF-BB and TGF-β together in confluent SMC culture. Confluent SMC cultures were growth-arrested by serum deprivation (0.5% for 72hr). Then they were exposed to the indicated reagents for 24hr. The amount of [^{3}H] thymidine incorporated into DNA was determined by scintillation counting. Results represent the mean± S.D. of four independent replicates in duplicate.

PDGF-BB, and [^{3}H] thymidine was added (5μCi/ml). After 24hr incubation, the incorporation of [^{3}H] thymidine into acid-insoluble materials was measured (13).

Enzymatic assays. For determination of enzymatic activities, monolayers of VSMC on 100mm-diameter cell culture plates were washed twice with ice-cold Krebs-Ringer solution (10ml), resuspended in Krebs-Ringer solution (20mM HEPES, 10mM dextrose, 127mM NaCl, 5.5mM KCl, 1mM $CaCl_2$, and 2mM $MgSO_4$, pH7.4, 1ml) by scraping with a sterile disposable cell lifter, and spun at 120g for 10min in Eppendorf tubes. VSMCs were resuspended in 400μl of phosphate buffer (50mM sodium phosphate and 0.5% TritonX-100, pH7.5) and sonicated for two 15s bursts. Sonicates were spun for 10min at 15,000g and the supernatants were used immediately for protein content determinations by Bradford assay. Enzymatic assays were performed immediately or following storage at −70°C. Catalase activity of the extracts (20μl, 5-10mg/ml) was measured by monitoring the disappearance of hydrogen peroxide at 240nm. Glutathione peroxidase activity of crude extracts (20μl, 5-10mg/ml) was determined used a couple assay in which the rate of t-butyl hydroperoxide-dependent NADPH oxidation at 340nm was monitored (14).

Detection of intracellular H_2O_2. Intracellular levels of H_2O_2 were analyzed by fluorescence-activated cell sorting (FACS) using DCFH-DA as a probe (15). Experiments were performed under dim light. Confluent, serum-deprived SMCs were incubated in DMEM containing 5mM DCFH-DA for 24hr with additional TGF-β and PDGF then chilled on ice and washed with cold PBS. Washed cells were detached from culture plates by trypsin digestion. The activity of trypsin was quenched with 0.05% BSA in PBS. The fluorescent intensities of DCFH-DA for samples of 10,000 cells each were analyzed by flow cytometry using a FACScan flow cytometer equipped with an air-cooled argon laser.

RESULTS AND DISCUSSION

TGF-β Was Not a Direct Mitogen for Rat VSMC but Was Able to Enhance the Mitogenic Response of VSMC to PDGF

The role of TGF-β in intimal lesion formation after injury has been confounded by in vitro observations

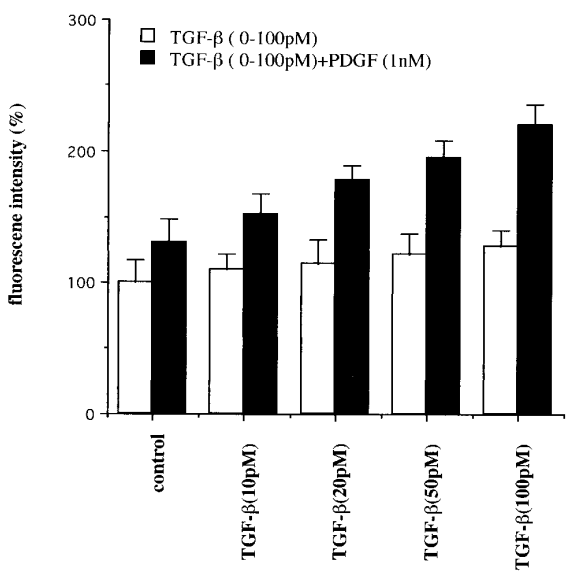

FIG. 3. The effect of TGF-β and PDGF on the content of H_2O_2 in VSMC. VSMCs were incubated for 24hr with TGF-β or PDGF or TGF-β and PDGF. The levels of intracellular peroxides were estimated by FACS analysis as described under Materials and Methods. Data are presented as a percentage change relative to control cells arbitrarily set at 100%. Three independent experiments were similar.

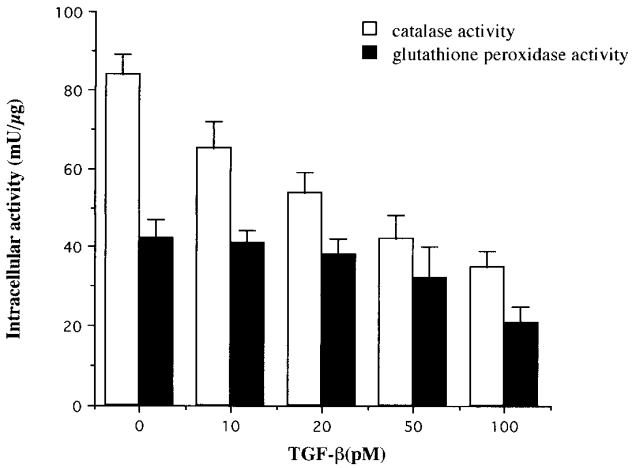

FIG. 4. The effect of activity of catalase and glutathione peroxidase. Cells were grown in 10-cm diameter culture dishes to confluence. The proteins were extracted when confluent SMC was stimulated by the indicated concentrations of TGF-β for 24hr. All measurements of activity of catalase and glutathione peroxidase were obtained from triplicate experiments in duplicate and are expressed as mean±S.D. Units are defined as follows: catalase, micromoles of H_2O_2 consumed per minute; glutathione peroxidase, nanomoles of NADPH oxidized per minute.

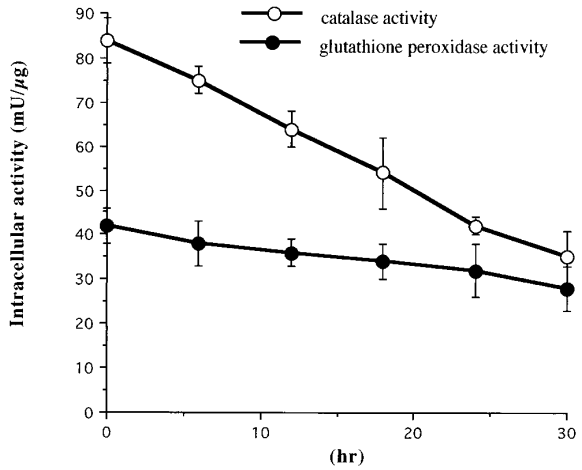

FIG. 5. Time cource of activity of catalase and glutathione peroxidase of SMC treated by TGF-β. Cells were grown in 10-cm diameter culture dishes to confluence. The proteins were extracted when confluent SMC was stimulated by TGF-β(50pM) for indicated times. All measurements of activity of catalase and glutathione peroxidase were obtained from independent triplicate experiments in duplicate and are expressed as mean±S.D. Units are defined as described in the legend to Fig. 4.

that TGF-β can inhibit (16) or stimulate growth of cultured VSMC (17). As shown in Fig.1, our results demonstrated that addition of 50pM of TGF-β alone were did not significantly increase the incorporation of [^{3}H] thymidine into DNA in the course of 30hr. The effects on the incorporation of [^{3}H] thymidine into DNA were not observed at concentrations of 10-100pM (data not shown). By contrast, the addition of PDGF-BB(1nM) alone was moderately able to increase the incorporation of [^{3}H] thymidine into DNA. The increase was approximately 3-fold above that observed for the control. Furthermore, when TGF-β and PDGF were added together, the increase in [^{3}H] thymidine incorporation into DNA was approximately 15-fold above that observed for TGF-β alone. These results indicate that although TGF-β is not a major mitogen in rat VSMC, it dramatically enhances the mitogenic response of VSMC to PDGF.

Antioxidants Inhibit the Enhancement of [^{3}H] Thymidine Incorporation into DNA by TGF-β and PDGF-BB Together

Recently, active oxygen species were reported to stimulate VSMC growth (10). To determine the involvement of ROS in the enhancement of [^{3}H] thymidine incorporation into DNA by TGF-β and PDGF-BB together, we cultured VSMCs with TGF-β, PDGF-BB and antioxidants together. Fig.2 shows that catalase, an enzyme that hydrolyses hydrogen peroxide, and N-acetyl-L-cysteine (NAC), glutathione precursor / radical scavenger, prevented the enhancement of [^{3}H] thymidine incorporation into DNA by TGF-β and PDGF-BB, whereas superoxide dismutase (SOD) had no significant effect. Aminotriazole (ATZ), catalase inhibitor, increased the enhancement of [^{3}H] thymidine incorporation into DNA by TGF-β and PDGF-BB. The nitric oxide synthase inhibitor nitroarginine (0.1-1mM) did not significantly affect the enhancement of DNA synthesis. Catalase, NAC, SOD, ATZ, and nitroarginine alone have no effect on [^{3}H] thymidine incorporation into DNA compared to control. These results suggest

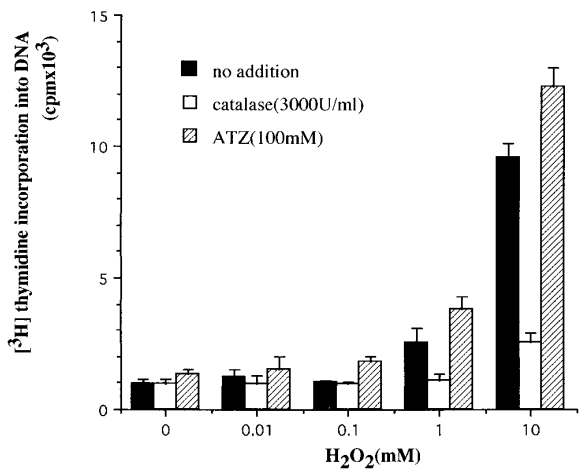

FIG. 6. The effect of H_2O_2 on the DNA synthesis of SMC. DNA synthesis was determined in H_2O_2-stimulated VSMC treated without or with aminotriazole (100mM) or catalase (3000U/ml). The data represent the mean±S.D. of three independent experiments in duplicate.

that ROS derived from H_2O_2 is involved in the enhancement of [^{3}H] thymidine incorporation into DNA by TGF-β and PDGF-BB.

The Content of H_2O_2 of VSMCs Treated by TGF-β and PDGF-BB

For ROS to fulfill the role of signaling intermediates for PDGF-BB and TGF-β, they must be able to induce the production of ROS. We measured the relative concentrations of H_2O_2 in VSMC using DCFH-DA and fluorescene-activated cell sorting (FACS). DCFH-DA is oxidized to membrane-impermeable, fluolecent DCFH-DA in the presence of H_2O_2 and possibly derived from it. As shown in Fig.3, fluorescence intensity induced by TGF-β and PDGF together increased in a way that was dependent upon TGF-β concentrations (0-100pM), but the increase in fluolescene intensity was not observed by TGF-β alone compared to control. Thus, the addition of both TGF-β and PDGF-BB increased the generation of ROS in VSMCs.

The Effect of Catalase Activity and Glutathione Peroxidase Activity by TGF-β

As shown in Fig.3, the content of H_2O_2 was almost unchanged by TGF-β alone compared to control. Furthermore, it was reported that PDGF generates H_2O_2 in VSMCs (11). One possibility is that TGF-β impairs the H_2O_2 catabolising system. Therefore, we measured the activities of two enzymes which can catabolise H_2O_2, catalase and glutathione peroxidase, after addition of TGF-β for 24hr. Catalase is found in many types of cells and scavenges hydrogen peroxide as its sole substrate. Glutathione peroxidase is an antioxidative enzyme that scavenges various peroxides. Three isozymes, cellular glutathione peroxidase, extracellular glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase, are known (18), and each contains a seleno-cysteine in its catalytic center. Cellular glutathione peroxidase, the most characteristic form, can react with hydrogen peroxide and organic peroxides. Fig.4 shows that TGF-β decreased the activity of catalase and glutathione peroxidase in a dose-dependent manner (0-100pM). Further, we investigated the time course of the change in activity of catalase and glutathione peroxidase to clarify the mechanism of inactivation of these enzymes by TGF-β. Fig.5 shows the slow decrease in the activities of these enzymes.

H_2O_2 Enhances [^3H] Thymidine Incorporation into DNA in VSMCs

If H_2O_2 is the signaling molecule that mediates TGF-β- and PDGF- induced DNA synthesis, then an increase in intracellular concentrations of H_2O_2 mimics the effects of TGF-β and PDGF on the enhancement of DNA synthesis. We investigated whether direct addition of H_2O_2 enhances mitogenic signals in the cells (Fig.6). The direct addition of H_2O_2 enhanced [^{3}H] thymidine incorporation into DNA in the cells in a dose-dependent manner. This enhancement was significantly increased with aminotriazole (100mM), a catalase inhibitor, and was blunted with catalase (3000U/ml). These results suggest that H_2O_2 is involved in mitogenic signals in VSMCs.

In summary, we described in this study that TGF-β enhances the response by PDGF-BB and that this phenomenon at least partly resulted from the impairment of ROS catabolism system caused by the inactivation of catalase and glutathione peroxidase by TGF-β. However, further study is needed to clear the underlying mechanism of inactivation of the enzymes.

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