Glutathione Levels Discriminate between Oxidative Stress and Transforming Growth Factor-β Signaling in Activated Rat Hepatic Stellate Cells*

(Received for publication, March 9, 1999, and in revised form, August 25, 1999)

Pieter J. De Bleser‡, Guoxiong Xu‡, Krista Rombouts‡, Vera Rogiers§, and Albert Geerts‡¶¶
From the ‡Laboratory for Cell Biology and Histology and §Laboratory for Toxicology, Free University Brussels, B-1090 Brussels, Belgium

Reactive oxygen species are implicated in the pathogenesis of several diseases, including Alzheimer’s disease, multiple sclerosis, human immunodeficiency virus, and liver fibrosis. With respect to liver fibrosis, we have investigated differences in antioxidant enzymes expression in stellate cells (SCs) and parenchymal cells from normal and CCl₄-treated rat livers. We observed an increase in the expression of catalase in activated SCs. Treatment with transforming growth factor-β (TGF-β) increased the production of H₂O₂. Treatment of the cultures decreased TGF-β expression. Addition of H₂O₂ resulted in increased TGF-β production. 3-Amino-1,2,4-triazole abolished the capacity of SCs to remove H₂O₂. A paradoxical increase in capacity was observed when the cells were pretreated with diethyl maleate. Treatment with 3-amino-1,2,4-triazole increased TGF-β production. A paradoxical decrease of TGF-β production was observed with diethyl maleate. Treatment of the cells with N-acetylcysteine resulted in increased TGF-β production. TGF-β decreased the capacity of the SCs to remove H₂O₂. An increase in the capacity to remove H₂O₂ was observed when TGF-β was removed by neutralizing antibodies. In conclusion, our results suggest: 1) a link between cellular GSH levels and TGF-β production and 2) that cellular GSH levels discriminate whether H₂O₂ is the result of oxidative stress or as second messenger in the TGF-β signal transduction pathway.

Increasing experimental evidence suggests that reactive oxygen species (ROS) such as H₂O₂, O₂⁻, and OH⁻, are implicated in the development and progress of several diseases, including Alzheimer’s disease (1), multiple sclerosis (2), human immunodeficiency virus (3), and liver fibrosis (4–6). In normal conditions, low amounts of ROS are produced as by-products of the aerobic respiration. In high doses, ROS are noxious to the cell leading to impaired metabolic functions, growth inhibition, and ultimately cell death (7). Cells therefore employ several antioxidant enzyme systems to maintain low levels of ROS.

With respect to liver fibrosis, the stellate cell is considered to be the main effector cell of liver fibrogenesis. In normal conditions, stellate cells produce controlled amounts of collagens type III and IV (8). In fibrogenesis, stellate cells proliferate and undergo transition into myofibroblast-like cells, secreting large amounts of extracellular matrix components. This process is driven to a large extent by the autocrine and paracrine secretion of TGF-β (9). TGF-β has been shown to induce the production of H₂O₂ in several cell types such as lung fibroblasts (10), hepatocytes (11), osteoblastic cells (12), and aortic endothelial cells (13). Recently ROS and, in particular H₂O₂, have been proposed to act as signaling mediators for a variety of growth factors such as tumor necrosis factor, interleukin 1 (14), and TGF-β (12). Both nuclear factor (NF)-κB and activator protein-1 were shown to be regulated by the intracellular redox state (15, 16). In activated stellate cells oxidative stress results in NF-κB activation (17).

In the present study, we investigated 1) the links between antioxidant enzymes expression and the capacity to remove extracellular H₂O₂ and 2) the roles of glutathione (GSH), TGF-β, and H₂O₂ in the control of the autocrine TGF-β loop in activated stellate cells. Our results suggest a model in which cellular GSH levels discriminate whether H₂O₂ is considered as oxidative stress or as second messenger in the TGF-β signal transduction pathway.

MATERIALS AND METHODS

Isolation and Purification of the Cells—Male adult Wistar rats (350–400 g) were used in all experiments. The rats were treated according to the guidelines of the Council of International Organizations of Medical Sciences for the care and use of laboratory animals in research. All procedures were performed with the animals under ether anesthesia. For the isolation of cells from CCl₄-treated rat livers, rats received 6 injections of CCl₄ intraperitoneally. CCl₄ was dissolved in an equal volume of paraffin oil. The first injection consisted of 150 μl of CCl₄/100 g of body weight. Subsequent injections consisted of 100 μl of CCl₄/100 g of body weight, intraperitoneally (2 injections weekly at equal intervals). Rats were sacrificed 72 h after the last injection. Parenchymal cells were isolated and purified by collagenase digestion, 1 × g sedimentation, and low speed centrifugation according to Rogiers et al. (18). Stellate cells were isolated by collagenase/pronase digestion. Stellate cells were purified by centrifugation through 13% and 11% (w/v) sucrose solutions. These procedures were performed with the animals under ether anesthesia.

RNA Extraction and Northern Hybridization Analysis—Total RNA was extracted as described by Chomczynski and Sacchi (20). Purity and concentration were determined by measuring light absorbance at 260 and 280 nm. For Northern hybridization analysis, 20 μg of RNA obtained from the isolated cells was electrophoresed in 1% agarose/2% paraformaldehyde gels. The gels were then stained with acridine orange (15 mg/liter in 0.01 mol/liter sodium phosphate buffer pH 6.5) for 3 min. After destaining, gels were viewed and photographed on a UV transilluminator. RNA was transferred to Hybond N filters (Amersham

* This work was supported by Fonds voor Geneeskundig Wetenschappelijk Onderzoek Grants 30.078.90, 9.0011.95, G.0044.96, and 1.5.618.98 and by Onderzoeksraad Vrije Universiteit Brussel Grant 1903220550. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence and reprint requests should be addressed: Laboratory for Cell Biology and Histology, Free University Brussels, Laarbeeklaan 103, B-1090 Brussels-Jette, Belgium. Tel.: 32-2-477-44-07; Fax: 32-2-477-44-12; E-mail: bert@cyto.vub.ac.be.

§The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; SC, stellate cell; TGF-β, transforming growth factor-β; ELISA, enzyme-linked immunosorbent assay; kb, kilobase pair(s); NF, nuclear factor; DAB, diaminobenzidine; DCFDA, 2′,7′-dichlorofluorescein diacetate; PBS, phosphate-buffered saline; NAC, N-acetylcysteine; AMT, 3-amino-1,2,4-triazole; DEM, diethyl maleate; GSHPs, glutathione peroxidase.
Pharmacia Biotech, Little Chalfont, United Kingdom) by capillary blotting. RNA was cross-linked to the filter by UV illumination (1200 microwatts/cm²) using a Stratalinker (Stratagene, La Jolla, CA). Filters were prehybridized for 2 h and subsequently hybridized overnight with cDNA probes at 42 °C. After hybridization, filters were washed at 65 °C to a stringency of 0.1x SSC, 0.1% SDS, 70 °C to pre-flashed Hyperfilm-MP (Amersham Pharmacia Biotech) using intensifying screens. Antisense RNA probes complementary to rat copper/zinc-containing superoxide dismutase (Cu/Zn-SOD), manganese-containing superoxide dismutase (Mn-SOD), glutathione peroxidase (GSHPx), and catalase mRNA sequences were synthesized by in vitro transcription from linearized plasmid templates using [32P]uridine triphosphate ([32P]UTP, 800 Ci/mmol, ICN, Aesse, Belgium) and the RNA labeling kit (Amersham Pharmacia Biotech). 18S ribosomal cDNA was labeled with 32P-deoxyctydine triphosphate ([32P]dCTP, 3000 Ci/mmol, Amersham Pharmacia Biotech) using a megaprime extension kit (Amersham Pharmacia Biotech). As a control for equal loading, correct transfer, and integrity of the samples, the blots were later probed for the presence of 18S ribosomal RNA.

cDNA Probes—The cDNAs containing rat catalase, GSHPx, Cu/Zn-SOD, and Mn-SOD sequences were kindly provided by Dr. J. L. Tilly (Johns Hopkins University, Baltimore, MD) (21). The 5.6-kb 18S ribosomal cDNA probe was a kind gift from Dr. R. V. Gunatka (University of Missouri, Columbia, MO).

Hydrogen Peroxide Removal by Stellate Cells—In these experiments hydrogen peroxide levels were measured by the method of Jiang et al. (22). Xylenol orange (100 μM), ammonium ferrous sulfate (250 μM), and ascorbit (100 μM) were dissolved in sulfuric acid (25 μM). Fifty μl of sample was combined with 50 μl of substrate in a 96-well microplate and incubated at room temperature for 45 min. Absorbance was read at 570 nm. A standard curve was generated by including in the assay a 2-fold serial dilution of a known amount of hydrogen peroxide. For the measurement of removal rate, 300 μl of GBSS, containing 100 μM of H₂O₂, was added to each well of a 24-well plate, containing the cultured stellate cells. At 5-min time intervals, 50-μl portions of the medium were withdrawn and subjected to H₂O₂ determination, as described above.

Measurement of Glutathione—Hepatic stellate cell glutathione was measured using the GSH-400 colorimetric assay for glutathione (Oxis International, Portland, ME).

Determination of Hydrogen Peroxide Levels—The method of White et al. (23) was modified. The latter was based on the oxidation of o-phenylenediamine by horseradish peroxidase in the presence of hydrogen peroxide. o-Phenylenediamine (16 μM) and horseradish peroxidase (1 unit/ml of Tris-HCl buffer, pH 8.0) were immediately prior to use. For the assay, 50 μl of sample was combined with 75 μl of substrate in a 96-well microplate and incubated for 30 min at 37 °C. Subsequently the reaction was quenched with sulfuric acid (3 N), and the absorbance was measured at 490 nm, using 690 nm as reference wavelength. Hydrogen peroxide levels in freshly conditioned media were measured 90 min after the addition of 0, 2.5, 5, 10, and 20 ng/ml TGF-β1. The control consisted of media, conditioned in the presence of excess catalase. Results were presented relative to untreated cultures, taken as 100%.

Determination of Intracellular Peroxides—Intracellular peroxides levels were assessed using 2′,7′-dichlorofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR). Cells treated with 0, 2.5, 5, 10, and 20 ng/ml TGF-β for 60 min at 37 °C were incubated with 5 μM DCFDA. Micrographs were taken with the same exposure time (8 s) for all conditions.

TGF-β Assay—The TGF-β bioassay was essentially the one described by Tada et al. (24). In short, CCL 64 cells (106 cells/well) were cultured in 96-well flat-bottom microtiter plates in the presence of conditioned medium for 48 h. Inhibition of cell proliferation was measured using the colorimetric bromodeoxyuridine cell proliferation ELISA (Roche Molecular Biochemicals, Brussels, Belgium).

Fibronectin ELISA—Fibronectin concentrations in conditioned media of stellate cell cultures were measured using a direct ELISA. Ninety-six-well ELISA plates (Nunc, Denmark) were coated with samples diluted in coating buffer (1.59 mg/ml Na₂CO₃, 2.93 mg/ml NaHCO₃, 0.2 mg/ml triton X-100, pH 9.8) and incubated at 4 °C for 1 h. After washing, rabbit anti-fibrinogen antibody (1:1000) was added and incubated for 1.5 h at room temperature. After washing, anti-rabbit immunoglobulin G (dilution, 1:10000) (Sigma) was added and incubated for 1.5 h at room temperature. The plates were washed and incubated with alkaline phosphatase-labeled, affinity-purified goat anti-rabbit immunoglobulin G (dilution, 1:8000) (Sigma). After washing, p-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer containing 0.1 mg/ml MgCl₂·6H₂O, pH 9.8) was added and the plates were incubated at room temperature up to 60 min. The optical densities were read at 405 nm using a microplate reader (Bio-Rad, 3550 UV).

Immunocytochemistry—At the indicated time points, the medium was removed from stellate cells cultured on coverslips. Cells were washed with PBS and fixed for 10 min with acetone at −20 °C. Subsequently, the coverslips were preincubated with PBS containing 1% (w/v) BSA (Sigma) for 15 min and washed three times for 5 min each with PBS. Each coverslip was incubated with the primary antibody (diluted in PBS, containing 0.3% Triton X-100). A 1:1000 diluted mouse monoclonal antibody clone 1A4 (Sigma) raised against α-smooth muscle actin synthetic peptide was used. Incubation with the primary antibody for 18 h at 4 °C in a humid atmosphere was followed by washing three times for 5 min each with PBS and subsequent application of horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech), dilution 1:100, for 90 min at room temperature. After rinsing three times in PBS, peroxidase was visualized by diaminobenzidine (DAB) (Fluka, Buchs, Switzerland) and H₂O₂. Twenty mg of DAB was dissolved in 50 ml of Tris-HCl (10 mM) (pH 7.4). The solution was filtered, kept in the dark, and shortly before use, 1 ml of 1% CoCl₂·6H₂O and 0.8 ml of 1% Ni(NH₄)₂(SO₄)₃ were added under constant stirring. Finally, 15 μl of a 30% H₂O₂ solution was added. Addition of Co²⁺ and Ni²⁺ ions strongly enhanced the reaction product. Sections were incubated in the substrate solution for 10-12 min. Nuclei were counterstained with hematoxylin. Controls consisted of substitution of the primary antibody by normal mouse IgG and omission of the primary antibody. They were invariably negative.

Statistics—Data were expressed as mean values ± S.D. Statistical analysis of the observed differences was performed using one-way blocked analysis of variance, and values were considered significant at p < 0.05.

RESULTS

Northern Hybridization Analysis—First, we examined by Northern hybridization analysis the mRNA steady state levels of catalase, GSHPx, Cu/Zn-SOD, and Mn-SOD in freshly isolated stellate and parenchymal cells from both normal and CCl₄-treated rat livers and in stellate cells kept in culture for 3, 6, and 12 days. The results were shown in Figs. 1 and 2. Whereas strong signals for catalase mRNA were detected in freshly isolated stellate cells from fibrotic animals and in culture-activated stellate cells, freshly isolated stellate cells of normal livers did not express catalase mRNA. Using ribosomal RNAs as size standards, the catalase cRNA recognized a mRNA species of approximately 2.7 kb. No differences in steady state catalase mRNA levels were found when total RNA of parenchymal cells derived from normal livers was compared with that of fibrotic rat livers (Fig. 2).

GSHPx cRNA recognized a mRNA of approximately 1.2 kb. In stellate cells, isolated from fibrotic animals, the levels of GSHPx expression tended to be slightly increased as compared with the levels observed in stellate cells from normal animals. In parenchymal cells derived from fibrotic animals, the level of GSHPx expression was decreased as compared with normal parenchymal cells. With time in culture, the levels of GSHPx mRNA expression tended to decrease.

The cRNA for Cu/Zn-SOD detected a transcript with the size of approximately 0.9 kb. We found no differences in the levels of transcription in the stellate cells derived from fibrotic animals compared with freshly isolated stellate cells from normal livers. The level of transcription in parenchymal cells derived from fibrotic animals was lower than in normal parenchymal cells. Again, there was a slight tendency for decrease in the level of transcription with the time the stellate cells were cultured.

The cRNA for Mn-SOD detected multiple bands in our blots. The minor bands corresponded to transcript sizes of approximately 0.9 kb. We found no differences in the levels of transcription in the stellate cells derived from fibrotic animals as compared with freshly isolated stellate cells from normal livers. In parenchymal cells derived from fibrotic animals, the level of GSHPx expression was decreased as compared with normal parenchymal cells. With time in culture, the levels of GSHPx mRNA expression tended to decrease.

The cRNA for Cu/Zn-SOD detected a transcript with the size of approximately 1.2 kb. In stellate cells, isolated from fibrotic animals, the levels of GSHPx expression tended to be slightly increased as compared with the levels observed in stellate cells from normal animals. In parenchymal cells derived from fibrotic animals, the level of GSHPx expression was decreased as compared with normal parenchymal cells. With time in culture, the levels of GSHPx mRNA expression tended to decrease.
Mn-SOD mRNA was transiently expressed in cultured stellate cells. No expression was observed in freshly isolated stellate cells. Strongest expression was observed at day 3 in culture and decreased at days 6 and 12.

Considering the results obtained with the freshly isolated cells, the major observation was the induction of expression of catalase in stellate cells from fibrotic animals. This was also reflected in culture-activated stellate cells. From the third day in culture onwards, we observed strong signals for catalase mRNA. This observation suggested that activated stellate cells were better equipped than quiescent stellate cells to remove efficiently H$_2$O$_2$.

Removal of Extracellular H$_2$O$_2$ by Cultured Stellate Cells—Using a xylene orange assay, the kinetics of H$_2$O$_2$ removal was determined in cultures of stellate cells, kept in culture for 3 days, and in cultures of activated stellate cells, kept in culture for 9 days. The latter cells showed clear morphological signs of activation. Cells were challenged to 100 $\mu$M H$_2$O$_2$, and every 5 min the remaining H$_2$O$_2$ concentration was measured. The results were shown in Fig. 3. Whereas the rate of H$_2$O$_2$ removal by activated stellate cells was first order with an half-life of approximately 7 min, the same number of 3-day-old stellate cells was not able to remove efficiently added H$_2$O$_2$.

As most cells had two antioxidant enzymes able to remove H$_2$O$_2$, catalase and GSHPx, we tried to identify which of the two enzymes was responsible for H$_2$O$_2$ breakdown using specific inhibitors. These were, respectively, 3-amino-1,2,4-triazole (AMT) and diethyl maleate (DEM) (Fig. 4). When the cells were challenged with 100 $\mu$M H$_2$O$_2$ (Fig. 4A), pre-treatment with 8 mM AMT abolished almost completely the capacity of activated stellate cells to remove H$_2$O$_2$. A paradoxical, opposite effect was obtained when the cells were pre-treated with 1 mM DEM. This indicated that the capacity to remove the added H$_2$O$_2$ was due to catalase rather than GSHPx. As GSHPx is more effective at low H$_2$O$_2$ concentrations, the cells were also challenged with 10 $\mu$M H$_2$O$_2$ (Fig. 4B). At this concentration, DEM-treated cells removed H$_2$O$_2$ less efficiently than controls.

Fibrogenesis is driven by the autocrine expression of TGF-β by activated stellate cells. TGF-β has been shown to induce the...
production of H_2O_2 in several cell types such as lung fibroblasts (10), hepatocytes (11), osteoblastic cells (12), and aortic endothelial cells (13). If the autocrine TGF-β loop in stellate cells were mediated by H_2O_2, then increased expression of catalase could be a physiological adaptation to the high levels of H_2O_2 generated in the cells following exposure to TGF-β. In the following experiments, we investigated this hypothesis.

**Effect of TGF-β on H_2O_2 Production by Stellate Cells**—Both in quiescent (Fig. 5A) and activated (Fig. 5B) stellate cells, the levels of hydrogen peroxide increased in a dose-dependent way with the amount of TGF-β added. At a concentration of 20 ng/ml TGF-β, a 300% increase of hydrogen peroxide concentration was observed.

To confirm these results, the intracellular oxidized states were determined using the highly sensitive fluorescent assay with DCFDA. Quiescent stellate cells were treated with 0, 2.5, 5, and 10 ng/ml TGF-β for 60 min at 37 °C. DCFDA (5 μM) was added, and micrographs were taken with an epifluorescence photo-microscope, using the same exposure time for all samples. Increasing concentrations of TGF-β resulted in increased fluorescence (Fig. 5C).

**Effect of H_2O_2 or Menadione on TGF-β Production by Stellate Cells**—Conditioned media were prepared from 8-day-old, activated stellate cell cultures treated with 0, 10, 20, and 40 μM H_2O_2 for 16 h. Next, the media were assayed for TGF-β activity using a bioassay. The results were shown in Fig. 6A. Untreated stellate cells produced 75 pg/ml TGF-β. With increasing concentrations of H_2O_2, TGF-β synthesis reached a concentration of 261 pg/ml at 40 μM H_2O_2, corresponding to an approximately 3-fold increase as compared with the untreated cells. As the added H_2O_2 is rapidly removed by the cells (Fig. 3), this is rather a model for acute oxidative stress. To investigate what happens under conditions of chronic oxidative stress, we studied the effect of treating the cells with 0, 12.5, 25, and 50 μM menadione. While untreated cells produced 324 pg/ml TGF-β, addition of menadione resulted in a dramatic decrease of TGF-β production (Fig. 6B).

**Effect of Modulation of Cellular H_2O_2 Levels on the Secretion of TGF-β**—We investigated the effect of extracellular catalase (Fig. 7A), AMT (Fig. 7B), and DEM (Fig. 7C) on the production of TGF-β by 8-day-old, activated stellate cells in culture. At this time point, the autocrine TGF-β loop in stellate cells was well established (25). Untreated stellate cells synthesized 189 pg/ml active TGF-β per 24 h. Stellate cells conditioned in the presence of 100 units/ml catalase synthesized 37 pg/ml active TGF-β per 24 h, corresponding to a 5-fold decrease in the level of active TGF-β (Fig. 6A). Stellate cells conditioned in the presence of 4 mM AMT per 24 h synthesized 318 pg/ml active TGF-β (70% increase). At a concentration of 8 mM AMT, stellate cells produced 524 pg/ml active TGF-β (277% increase) per 24 h (Fig. 7B). Treatment of stellate cells with DEM resulted paradoxically in a decrease of TGF-β production. While control cells synthesized 970 pg/ml active TGF-β, treatment with 0.25 mM DEM resulted in a production of 60 pg/ml TGF-β, corresponding to a 16-fold decrease in active TGF-β production (Fig. 7C). TGF-β has been shown to stimulate both fibronectin synthesis and transdifferentiation of stellate cells into myofibroblast-like cells (9). If H_2O_2 acts as a mediator in the TGF-β signal transduction pathway, than removal of H_2O_2 by exogenous catalase should attenuate both the effect of TGF-β on fibronectin synthesis and stellate cell activation. These hypotheses were tested in the next experiments.

**Effect of Catalase on Fibronectin Production by Cultured Stellate Cells**—Conditioned media were prepared from stellate cell cultures treated with different concentrations of catalase. These media were assayed for fibronectin using a specific
ELISA. The results were shown in Fig. 8. Untreated stellate cell cultures synthesized 51 ng/ml fibronectin. With increasing concentrations of catalase, the synthesis of fibronectin decreased in a dose-dependent way, reaching a concentration of 5 ng/ml at 1000 units/ml catalase.

Effect of Catalase on Stellate Cell Activation—Freshly isolated stellate cells were cultured on coverslips for 3, 6, and 9 days. One group was left untreated (control), while the other half was treated with catalase (500 units/ml) from day 2 onward. In the untreated group (left panel), staining for α-smooth muscle actin became strong from day 6 onward and was localized in filaments throughout the cytoplasm. When the stellate cells were treated with catalase (right panel), the cells remained smaller, staining was much less pronounced and was localized perinuclear. Nuclei were counterstained with hematoxylin (original magnification, ×133).

Effect of TGF-β on H₂O₂ Removal by Activated Stellate Cells—Two paradoxical events emerged from the DEM treatment of stellate cells; first, these cells removed H₂O₂ more efficiently than control cells and, second, the production of TGF-β in these cells was dramatically decreased. This latter observation suggested a link between cellular GSH levels and the autocrine TGF-β loop. As the increased breakdown of H₂O₂ by DEM-treated cells could be due to the removal of TGF-β from the system, we also investigated the effects of 1) adding excess TGF-β to the cells and 2) removal of TGF-β from the system by using neutralizing antibodies. The results were shown in Fig. 10. Treatment of the cells with neutralizing anti-TGF-β antibodies (50 μg/ml) resulted in a strong increase in the capacity of the cells to remove H₂O₂, reminiscent of the effect observed with DEM (Fig. 10A). The opposite effect was observed when the cells were treated with excess TGF-β (5 ng/ml). This resulted in a decrease in the capacity of the cells to remove H₂O₂, mimicking the effect of AMT (Fig. 10B).

Effect of N-Acetylcysteine (NAC) on TGF-β Production—As
Furthermore, activated, but not quiescent, stellate cells were treated with excess TGF-β(5 ng/ml). This resulted in a decrease in the capacity of the cells to remove H₂O₂, mimicking the effect of AMT.

The above data suggested the existence of a link between cellular GSH levels and TGF-β production, we also investigated the effects of treating the cells with various concentrations of NAC on TGF-β production (Fig. 11). NAC was used to provide extra cysteine for de novo glutathione synthesis (26). While untreated cells produced 21 pg/ml TGF-β, treatment with 2.5, 5, or 10 mM NAC increased TGF-β production with 33–40%. Treatment with 20 mM NAC increased TGF-β production approximately 3-fold. Under these conditions, the cells produced 59 pg/ml TGF-β.

**DISCUSSION**

TGF-β is generally considered to be the main mediator of fibrogenesis. TGF-β induces its own expression in stellate cells thereby creating a self-perpetuating circle of events (9). Recently ROS and, in particular, H₂O₂ have been proposed to act as signaling mediators for tumor necrosis factor, interleukin 1 (14), and TGF-β (12). This hypothesis was based on the observations that these growth factors were able to induce the production of H₂O₂ in several cell types (10–13) and that both NF-κB and activator protein-1 were regulated by the intracellular redox state (15, 16). A direct consequence of this model is that cells must be able to discriminate between H₂O₂ produced by oxidative stress and H₂O₂ acting as second messenger in signal transduction pathways. In the present paper, we propose a model that discriminates between these possibilities.

First, we investigated the differences in anti-oxidant enzyme expression in parenchymal and stellate cells from both normal and fibrotic rat livers and culture-activated stellate cells. We found that the expression of catalase mRNA was strongly induced in activated stellate cells both in vitro and in vivo. Furthermore, activated, but not quiescent, stellate cells were able to remove efficiently exogenously added H₂O₂. As catalase removes specifically H₂O₂, this suggested a special role for H₂O₂ in the activation of stellate cells. Our current hypothesis concerning the relationship between TGF-β and oxidative stress is that the autocrine TGF-β loop in stellate cells is mediated by endogenously produced H₂O₂. In support of this hypothesis, we showed that TGF-β₁ increased the levels of H₂O₂ in the medium of cultured hepatic stellate cells. This increase was abolished by adding catalase, indicating that H₂O₂ was the major cause of the increased oxidized state. TGF-β₁ also increased the intracellular peroxide levels of the stellate cell, as demonstrated by DCFDA and fluorescence microscopy. The role of H₂O₂ as a mediator in the TGF-β signal transduction pathway was studied by investigating the effect of modulating the cellular H₂O₂ levels on both the production of TGF-β and the synthesis of fibronectin. Treatment of culture-activated stellate cells with catalase resulted in a dose-dependent decrease of both TGF-β production and fibronectin synthesis. In parallel, the spontaneous differentiation into the myofibroblast-like phenotype was retarded, as evidenced by staining for α-smooth muscle actin, a stellate cell activation marker. This result was in support of the finding by Lee et al. (17), in which the antioxidant, d-a-tocopherol, blocked the activation of stellate cells cultured on plastic, as assessed by α-smooth muscle actin immunohistochemistry. When, conversely, the stellate cells were treated with a pulse of H₂O₂, an increase in the production of TGF-β was observed. This observation is in support of the data by García-Trevijano et al. (27), who found that H₂O₂ treatment of stellate cells resulted in the up-regulation of α1(I) procollagen mRNA. This gene is known to be regulated at the transcriptional level by TGF-β (28).

Decreased TGF-β production by DEM-treated stellate cells suggested a link between cellular GSH content and the extent of the autocrine TGF-β loop. This hypothesis was verified by treating stellate cells with increasing concentrations of NAC, a
precursor of GSH (26), which resulted in increased production of TGF-β. A similar observation was reported by Shan et al. (26), who found that treatment of mesangial cells with NAC resulted in a 40% increase of TGF-β mRNA expression. Kidney mesangial cells and hepatic stellate cells are both of mesenchymal origin and share many characteristics including extracellular matrix production and vitamin A storage (29).

Considering that 1) the cellular GSH content is linked to the extent of the autocrine TGF-β loop, 2) DEM-treated cells remove H2O2 more efficiently than control cells, 3) removal of TGF-β from the system with neutralizing antibodies results in a strong increase in the efficiency of H2O2 break-down, and (4) addition of excess TGF-β to the system results in the opposite effect, we postulate that cellular GSH may play a role in discriminating between exogenous oxidative stress and H2O2 generated in the TGF-β signaling pathway. This model is summarized in Fig. 12.

Exogenous oxidative stress (Fig. 12, left panel) is essentially a diffusion process, i.e. the initial H2O2 concentrations that enter the cell will be low. As it is generally accepted that GSHPs is more effective at low H2O2 concentration (30), this will result in the consumption of GSH necessary for its detoxification. Since our experiments demonstrated that the cellular GSH content was linked to the autocrine TGF-β loop, this will result in abrogation of TGF-β production. All H2O2 will be considered as oxidative stress and will be efficiently removed. The same happens when TGF-β is removed from the system with neutralizing antibodies (Fig. 10A). When, however, H2O2 is generated intracellularly in the TGF-β signaling pathway (Fig. 12, right panel), its concentration may locally reach high concentrations, making GSHPs less effective for its detoxification. In addition, it was shown by Thannickal et al. (13) that TGF-β-induced H2O2 production in bovine pulmonary artery endothelial cells occurs at a site inaccessible to detoxification by GSH. In this case, the cellular GSH concentrations will remain high and the autocrine TGF-β loop will not be interrupted. In this event, H2O2 will be considered as mediator in the signaling transduction pathway. Moreover, addition of excess TGF-β further decreases the capacity of the cells to remove H2O2 (Fig. 10B), resulting in higher intracellular H2O2 concentrations.

The fact that treatment of stellate cells with a single initial dose of H2O2 resulted in an increase in TGF-β production is not in contradiction with our model. In this experimental approach, the added H2O2 is rapidly removed by the stellate cells. This will not lead to depletions of the cellular glutathione stores, and the cells may consequently consider this H2O2 as a mediator in the signal transduction pathway. The opposite was observed when chronic oxidative stress was applied by treating the cells with menadione, a redox cycler. In this case, TGF-β production was strongly decreased, as expected when glutathione becomes depleted.

In conclusion, 1) activation of stellate cells results in a strong increase of catalase expression; 2) activated but not quiescent stellate cells are able to remove efficiently added H2O2; 3) this capacity is due to catalase, rather than GSH peroxidase; 4) TGF-β stimulates stellate cells to produce H2O2; 5) H2O2 levels, TGF-β activity, and differentiation are closely linked, suggesting a role for H2O2 as a mediator in the TGF-β signal transduction cascade in stellate cells; 6) increased catalase expression may provide a way for the stellate cells to control cellular H2O2 levels and indirectly the autocrine TGF-β loop; 7) there is a link between cellular GSH levels and TGF-β production; and 8) cellular GSH levels discriminate whether H2O2 is considered as oxidative stress or as second messenger in the TGF-β signaling pathway.

Acknowledgments—We are grateful to J.-M. Lazou and Kit Van den Berg (Laboratory for Cell Biology and Histology, Free University Brussel, Brussels, Belgium) for their expert technical assistance.

REFERENCES

1. Reynolds, W. F., Rhees, J., Maciejewski, D., Paladino, T., Sieburg, H., Maki, R. A., and Masliah, E. (1999) Exp. Neurol. 155, 31–41
2. Vladimirova, O., O’Connor, J., Cahill, A., Alder, H., Butunoi, C., and Kalman, B. (1998) Multi. Sci. 4, 413–418
3. Pitarka, D. L., Mullan, E. M., Bilek, M. L., Stevens, P., and Allen, R. C. (1998) J. Lab. Clin. Med. 132, 284–283
4. Pietrangelo, A. (1996) Semin. Liver Dis. 16, 13–30
5. Thannickal, V. J. (1993) Alcohol 16, 465–467
6. Thannickal, V. J., and Fanburg, B. L. (1995) J. Gastroenterol. Hepatol. 10, Suppl. 1, S50–S55
7. Sanchez, A., Alvarez, A. M., Benito, M., and Fabregat, J. (1996) J. Biol. Chem. 271, 7416–7422
8. Geerts, A., De Blieser, P., Hautekeete, M. L., Niki, T., and Wisse, E. (1994) in The Liver: Biology and Pathobiology (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachtier, D. A., and Shafritz, D. A., eds) pp. 819–838, Raven Press, Ltd., New York
9. Bachem, M. G., Meyer, D., Melchior, R., Sell, K. M., and Grossner, A. M. (1992) J. Clin. Invest. 89, 19–27
10. Thannickal, V. J., and Fanburg, B. L. (1995) J. Biol. Chem. 270, 30334–30338
11. Koyanagi, Y., Fuji, J., Suzuki, K., Kawata, S., Matsuzawa, Y., and Taniguchi, N. (1994) J. Biol. Chem. 269, 15488–15492
12. Ohba, M., Shihanuma, M., Kuroki, T., and Nose, K. (1994) J. Cell Biol. 126, 1079–1088
13. Thannickal, V. J., Hasson, P. M., White, A. C., and Fanburg, B. L. (1993) Ann. J. Physiol. 265, L622–L626
14. Meier, B., Redeke, H. H., Selle, S., Younes, M., Sies, H., Resch, K., and Habenermel, G. G. (1989) Biochem. J. 263, 539–545
15. Sun, Y., and Oberley, L. W. (1996) Free Radical Biol. Med. 21, 335–348
16. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) Science 249, 1157–1161
17. Lee, K. S., Buck, M., Hougkum, K., and Choi, K. J. (1995) J. Clin. Invest. 96, 2461–2468
18. Rogiers, V., Paeme, G., Vercruysse, A., and Bouwens, L. (1984) in Pharmacological, Morphological and Physiological Aspects of Liver Aging (Van Bezoyen, C., ed) pp. 121–126, Elsevier, Rijswijk, The Netherlands
19. De Blieser, P., Geerts, A., Van Eyken, P., Vrĳsen, R., Lazo, J. M., Desmet, V., and Wisse, E. (1993) in Cells of the Hepatic Sinusoid (Wisse, E., Kronek, D., and McCuskey, R., eds) pp. 218–221, Kupffer Cell Foundation, Rijswijk, The Netherlands
20. Chomskyński, P., and Saczki, N. (1997) Anal. Biochem. 162, 156–159
21. Tilly, J. L. and Tilly, K. I. (1995) Endocrinology 136, 242–252
22. Jiang, Z. Y., Woolard, A. C., and Welf, S. P. (1990) FEBS Lett. 268, 69–71
23. White, S. R., Kulp, G. V., Spaethe, S. M., Van Alstyne, E., and Leff, A. R. (1991) J. Immunol. Methods 144, 257–263
24. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., and Tsukamoto, K. (1986) J. Clin. Investigat. 87, 3130–3135
25. Gressner, A. M., and Bachem, M. G. (1990) Semin. Liver Dis. 10, 30–46
26. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., and Tsukamoto, K. (1986) J. Endocrinol. 136, 242–252
27. Garcia-Trevijano, E. R., Iraburu, M. J., Fontana, L., Dominguez-Rosales, J. A., Auster, A., Covarrubias-Pinedo, A., and Rojkind, M. (1999) J. Gastroenterol. Hepatol. 14, 690–707
28. Garcia-Trevijano, E. R., Iraburu, M. J., Fontana, L., Dominguez-Rosales, J. A., Auster, A., Covarrubias-Pinedo, A., and Rojkind, M. (1999) J. Hepatology 29, 960–970
29. Greenwel, P., Inagaki, Y., Hu, W., Walsh, M., and Ramirez, F. (1997) J. Biol. Chem. 272, 19738–19745
30. Bauer, P., and Wake, K. (1996) Endocrinology 136, 242–252
31. Makino, N., Mochizuki, Y., Bannai, S., and Sugita, Y. (1994) J. Biol. Chem. 269, 1020–1025