Original Article

Inhibitory effects of clinical reagents having anti-oxidative activity on transforming growth factor- β 1-induced expression of α -smooth muscle actin in human fetal lung fibroblasts

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ABSTRACT — Excessive production of transforming growth factor- β 1 (TGF β 1) plays an important role in lung fibrosis, in which the differentiation of fibroblasts into myofibroblasts is a key process. Increased formation of reactive oxygen species (ROS) induced by TGF β 1 is a common pathological feature of fibrosis. In the present study, probucol and lovastatin, which are therapeutics used for hyperlipidemia and proposed to act as anti-oxidants, were examined in terms of their effect on TGF β 1-induced formation of ROS and expression of α -smooth muscle actin (α SMA), a myofibroblast marker, in human fetal lung fibroblasts (HFL1 cells). The effects of anti-oxidative enzymes and reagents including *N*-acetyl-L-cysteine, α -tocopherol, and lecithinized-superoxide dismutase (SOD) on TGF β 1-induced expression of α SMA were also examined. Treatment with probucol (30 μ M) and lovastatin (1 μ M and 3 μ M), in addition to lecithinized-SOD, significantly inhibited the TGF β 1-induced formation of ROS and α SMA. Other anti-oxidants including *N*-acetyl-L-cysteine had marginal effects on α SMA expression under the conditions. Probucol and lovastatin, established therapeutics, may be worth trying in patients with both lung fibrosis and hypercholesterolemia.

Key words: Fibrosis, TGF β 1, Probucol, Lovastatin, HFL1 cells

INTRODUCTION

Transforming growth factor- β 1 (TGF β 1) regulates a wide variety of cellular functions in normal development and tissue homeostasis, and is involved in pathogenesis. It is reported that dys-regulated expression of TGF β 1 plays an important role in the pathogenesis of aberrant airway development in a number of chronic lung diseases including idiopathic pulmonary fibrosis (IPF, Hu *et al.*, 2003; Gauldie *et al.*, 2006; Liu and Gaston Pravia, 2010). The appearance of fibroblast foci is one pathological hallmark of IPF, and a greater profusion of fibroblast foci is associated with a worse prognosis (King *et al.*, 2001; Hu *et al.*, 2003; Gauldie *et al.*, 2006). Myofibroblasts, showing a phenotype intermediate between that of fibroblasts

and smooth muscle cells, are the key effector for fibrogenesis and characterized by the expression of α -smooth muscle actin (α SMA). In addition, treatment of patients with drugs such as methotrexate and bleomycin has been known to cause pulmonary fibrotic responses including expression of α SMA (Keane *et al.*, 2005; Ohbayashi *et al.*, 2010).

TGF β 1 is a potent fibrogenic cytokine and mediates the conversion of dermal fibroblasts into myofibroblasts. Since TGF β 1-mediated fibrosis is regulated by oxidative stress and/or cellular redox state (Kinnula and Crapo, 2003; Kinnula *et al.*, 2005; Liu and Gaston Pravia, 2010), redox modulatory therapy may be useful for treatment. Anti-oxidants such as GSH and *N*-acetyl-L-cysteine (NAC) have been tried clinically for the treatment of

lung fibrotic diseases, but the efficacy of these reagents remains to be comprehensively evaluated (Kinnula *et al.*, 2005; Liu and Gaston Pravia, 2010). In the present study, we examined the effects of probucol and lovastatin, which are lipid-lowering agents with anti-oxidative properties, on TGF β 1-induced expression of α SMA in human fetal lung fibroblasts (HFL1 cells). Also, we examined the effects of anti-oxidative enzymes including lecithinized-superoxide dismutase (SOD) and anti-oxidants such as NAC and glutathione ethyl ester on TGF β 1-induced expression of α SMA under same conditions. Anti-oxidant activities of probucol and lovastatin were examined by measuring the level of intracellular reactive oxygen species (ROS) in HFL1 cells.

MATERIALS AND METHODS

Cell culture

HFL1 cells (lung, diploid, human, passage 14) were purchased from American Type Culture Collection (Lot No. 4619685, Manassas, VA, USA). The HFL1 cells (1×10^5 /well) were cultured in DMEM supplemented with 5% fetal bovine serum (Hyclone Co., Logan, UT, USA). The cells at 60~70% confluence were incubated with serum-free medium containing 0.1% albumin for 24 hr, and then stimulated for 48 hr with 10 ng/ml TGF β 1 in 5% serum-containing medium. Addition of the anti-oxidative enzymes and reagents (20 μ l solution) was repeated twice in the cells in the same plate (5 ml of medium): the first (20 μ l) and second (20 μ l) treatments were carried out 2 hr before and 24 hr after the TGF β 1 treatment, respectively, and the total concentration achieved by the two applications is shown. Cytotoxicity was determined by counting cell number or by leakage of lactate dehydrogenase (Kurosawa *et al.*, 2009).

Reagents

Human TGF β 1 was purchased from PeproTech (#100-21C, Rocky Hill, CT, USA). Lovastatin and probucol were from Cayman (Ann Arbor, MI, USA) and Otsuka (Tokushima, Japan), respectively, and formula of the two reagents were shown in Fig. 1. Catalase (#3556, from human erythrocytes), SOD from *E. coli* (S5639, Mn-SOD), SOD from bovine brain erythrocytes (#S2515, CuZn-SOD), NAC, ($\pm$)- α -tocopherol, and glutathione ethyl ester were from Sigma (St. Louis, MO, USA). Lecithinized-SOD (phosphatidylcholine (PC)-bound with human CuZn-SOD, 3,000 U/mg) was a gift from LTT BioPharm (Tokyo, Japan). The concentrations of reagents and enzymes used in this study were the same as those in previous studies using cultured cells: lovastatin (Watts *et*

al., 2006; Ghavami *et al.*, 2010), probucol (Tsukamoto *et al.*, 2000; Du *et al.*, 2010), anti-oxidative enzymes including catalase and SOD (Tsukamoto *et al.*, 2000; Qi *et al.*, 2009), lecithinized-SOD (Igarashi *et al.*, 1994), and anti-oxidants such as NAC and glutathione ethyl ester (Sugiura *et al.*, 2009; Ono *et al.*, 2009; Felton *et al.*, 2009).

Immunoblotting of α SMA

Treated cells were harvested and homogenized in cell lysis buffer as described with minor modifications (Orido *et al.*, 2010). Bands immunoreactive to anti- α SMA antibody (Clone 1A4, Sigma) and anti- β -tubulin antibody (TUB2.1, Sigma) were visualized using a chemiluminescent reagent (Amersham, Buckinghamshire, UK). The effects of reagents were evaluated against the net increase of TGF β 1-induced expression of α SMA

Measurement of intracellular ROS

The level of intracellular ROS was measured as a function of 2',7'-dichloro-fluorescein (DCF) fluorescence as described previously (Kurosawa *et al.*, 2009) with minor modifications. Briefly, TGF β 1-treated HFL1 cells were incubated with 5 μ M non-fluorescent 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA, Sigma) in the dark, and DCF fluorescence was observed under a laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany: 488 nm excitation and evoked emission filtered with a 520-nm filter). DCFH is sensitive to oxidation by peroxynitrite, H₂O₂, and hydroxyl radical, while it is not

Fig. 1. Formula of probucol and lovastatin.

suitable for the measurement of nitric oxide and superoxide anion in biological systems (Myhre *et al.*, 2003). The images were obtained in a fluorescent detector with the indicated PMT voltages. The images with greater PMT voltages showed a lower level of intracellular ROS. An increase in ROS is shown as a pseudo-color in Fig. 4.

Data presentation

Data are the mean $\pm$ S.D. for the indicated number of independent experiments. The statistical significance of differences between two groups was assessed using the two-tailed Student's *t*-test. Multiple comparisons against a single control group were made by a one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered significant.

RESULTS

Effects of anti-oxidative enzymes on TGF β 1-induced expression of α SMA

Stimulation of HFL1 cells with 10 ng/ml TGF β 1 significantly enhanced α SMA expression at 48 hr: the value was $224 \pm 35\%$ ($n = 7$, $P < 0.05$) compared with the control without TGF β 1 (Fig. 2A). The response was marginal at 24 hr. Next, we examined the effects of anti-oxidative enzymes and reagents on the response to TGF β 1. In preliminary experiments, a single application of the test compounds such as lecithinized-SOD and probucol did not have a significant effect on the TGF β 1-induced response. Thus, taking account of the possible degradation and/or time-dependent invalidity of the tested compounds, the application was repeated twice at an interval of 24 hr in the subsequent experiments. Treatment with 100 μ g/ml (300 U/ml) and 500 μ g/ml (1,500 U/ml) lecithinized-SOD (Fig. 2A) and 1,000 U/ml catalase (Fig. 2B) reduced the expression of α SMA induced by TGF β 1. The decrease caused by these anti-oxidative enzymes was slight (approximately 20~30% inhibition), but significant (Fig. 2C). In two preliminary experiments, treatment with both lecithinized-SOD and catalase (500 μ g/ml and 1,000 U/ml, respectively) additionally inhibited the TGF β 1-evoked response: the values were 45% and 52% of the control (100%) without the enzymes. The effects of 500 μ g/ml lecithinized-SOD and 1,000 U/ml catalase on α SMA expression without TGF β 1 were variable: $74.6 \pm 25.8\%$ (% of control, $n = 5$) and $84.2 \pm 12.4\%$ ($n = 5$), respectively. Although treatment with lecithinized-SOD solution produced small white particles, probably lecithin vesicles, in the medium after approximately 10 hr, the reagent did not show cytotoxicity, and lecithin vesicles without SOD showed neither an inhibitory effect

on α SMA level nor cytotoxicity (data not shown). The effects of other anti-oxidative enzymes, CuZn-SOD and Mn-SOD, at 300 μ g/ml on α SMA expression with TGF β 1 were marginal: the values were $72.6 \pm 28.1\%$ and $84.8 \pm 17.3\%$, respectively ($n = 3$). Also, the enzymes had no

Fig. 2. Effects of anti-oxidative enzymes on expression of α SMA in HFL1 cells. HFL1 cells were pretreated with 50 μ g/ml and 250 μ g/ml of lecithinized-SOD (PC-SOD, Panel A) and catalase (500 U/ml, Panel B) for 2 hr, and then stimulated with 10 ng/ml of TGF β 1 in medium containing 5% serum. These same concentrations of enzymes were repeatedly added to the same dishes 24 hr after TGF β 1 treatment, and α SMA levels were determined 48 hr after the stimulation. Total concentrations of the enzymes following two applications are shown. Levels of α SMA and β -tubulin are shown in the upper and lower panels, respectively. Pictures are from typical representative experiments, and quantitative data are shown in Panel C. The data were calculated as the ratio of α SMA to β -tubulin and normalized as a percentage of the TGF β 1-induced response. Data are the mean $\pm$ S.D. for the indicated number of independent experiments. ^a $P < 0.05$, significantly different from the control.

effect on α SMA expression without TGF β 1: approximately $90 \pm 15\%$ ($n = 4$).

Effects of anti-oxidative reagents on TGF β 1-induced expression of α SMA

Under our conditions, treatment with 3 mM NAC had variable effects on the TGF β 1-induced expression of α SMA after 48 hr: half inhibition in two experiments and no inhibition in the other three. Treatment with 6 mM glutathione ethyl ester did not inhibit the TGF β 1-evoked response: the value was $97.4 \pm 6.5\%$ ($n = 3$), the same as the control value without the anti-oxidant. The level of α SMA without TGF β 1 was not modified by these anti-oxidants. Treatment with 60 μ M α -tocopherol did not affect α SMA expression: the value without TGF β 1 was $102 \pm 6\%$ (% of control, $n = 4$) and the TGF β 1-induced response was $94.6 \pm 11.3\%$ ($n = 5$) in the presence of α -tocopherol.

Next, we investigated the effects of probucol and lovastatin, which are used clinically and proposed to have an anti-oxidative effect (Liao and Laufs, 2005; Alexeeff *et al.*, 2007; Yamashita and Matsuzawa, 2009), on α SMA expression. Treatment with 10 μ M and 30 μ M probucol significantly inhibited the TGF β 1-induced expression of α SMA (Fig. 3). Probucol did not modify the expression of α SMA without TGF β 1. Treatment with 1 μ M and 3 μ M lovastatin significantly inhibited the TGF β 1-induced expression of α SMA. In the absence of TGF β 1, treatment with 1 μ M lovastatin significantly decreased the basal level of α SMA ($71.3 \pm 5.8\%$, $n = 3$, $P < 0.05$), although 3 μ M lovastatin had no effect. Treatment with statins for 48 hr is reported to cause cell death in human lung mesenchymal cells (Ghavami *et al.*, 2010), but lovastatin at the concentrations tested was not cytotoxic (data not shown).

Effects of probucol, lovastatin, and lecithinized-SOD on the TGF β 1-induced increase in ROS

First, we confirmed that the treatment with 5 μ M H_2O_2 for 90 min increased ROS levels in HFL1 cells (Fig. 4A). Treatment with 10 ng/ml TGF β 1 for 16 hr increased ROS levels (Fig. 4B), and the response was approximately 8-fold of that in the control cells (Fig. 5). The TGF β 1-induced response was marginal at 2 hr and 4 hr, but marked from 6 hr after stimulation (data not shown). Treatment of HFL1 cells with 500 μ g/ml lecithinized-SOD, 30 μ M probucol, and 3 μ M lovastatin significantly inhibited the TGF β 1-induced increase of ROS levels 16 hr after the stimulation (Figs. 4B and 5). The reagents including lecithinized-SOD did not change the basal ROS levels in control cells without TGF β 1 (data not shown). The treatment with the reagents including probucol and

lovastatin showed partial and/or limited inhibitory effects on the H_2O_2 -induced response probably because of a large amount of ROS in H_2O_2 -treated cells (PMT voltage, 380 in a detector).

DISCUSSION

Inhibition of TGF β 1-induced expression of α SMA by probucol and lovastatin

Oxidative stress has been shown to regulate fibrosis, specifically TGF β 1-induced fibrosis, in various cells including lung fibroblasts and lung epithelial cells (Sugiura *et al.*, 2009; Felton *et al.*, 2009; Liu and Gaston Pravia, 2010). Oxidative stress (i.e. ROS) includes superoxide radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals. Since DCFH is sensitive to H_2O_2 and hydroxyl radical, but not superoxide radicals, in biological systems (Myhre *et al.*, 2003), TGF β 1 appeared

Fig. 3. Effects of probucol and lovastatin on expression of α SMA in HFL1 cells. HFL1 cells were stimulated with 10 ng/ml TGF β 1 in medium containing 5% serum in the presence of probucol and lovastatin at the indicated concentrations. The reagents were added by two applications, and the total concentration is shown. Pictures in Panel A are from a typical representative experiment, and quantitative data are shown in Panel B. ^a $P < 0.05$, significantly different from the control.

Probucol and lovastatin on fibrosis

Fig. 4. Effects of lecithinized-SOD, probucol, and lovastatin on ROS levels. In Panel A, HFL1 cells were stimulated with vehicle (None) or 5 μ M H_2O_2 for 90 min. In Panel B, the cells were pre-treated with 500 μ g/ml lecithinized-SOD (PC-SOD), 30 μ M probucol, and 3 μ M lovastatin for 2 hr, and then stimulated with 10 ng/ml TGF β 1 for 16 hr. Then, the cells underwent a DCF fluorescence assay for the determination of ROS levels. The images were obtained at an enhanced intensity in a fluorescent detector with the indicated PMT voltages. The images obtained with greater PMT voltages showed a lower level of intracellular ROS. An increase in ROS is shown as a pseudo-color, and two pictures are from two different fields in the same dish. Quantitative data are shown in Fig. 5.

to increase H_2O_2 and hydroxyl radical as ROS in HFL1 cells under our conditions. Probucol is a diphenolic compound with anti-oxidant activity that reduces arteriosclerosis and restenosis in coronary arteries (Yamashita and Matsuzawa, 2009). In this study, treatment with probucol inhibited the TGF β 1-induced formation of ROS at 16 hr (Fig. 4B) and expression of α SMA at 48 hr (Fig. 3) after

TGF β 1 stimulation. Statins including lovastatin are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, and also block the isoprenylation and resulting activation of small GTP-binding proteins such as the Rho family. Statins attenuate oxidative stress in cells through inhibition of Rac1, a member of the Rho family, and regulation of ROS formation by NADPH oxidase (Liao and

Fig. 5. Inhibition of TGFβ1-induced formation of ROS by lecithinized-SOD (PC-SOD), probucol, and lovastatin. The response is expressed as a percentage of ROS levels in the control cells. Data are the mean $\pm$ S.D. for the indicated number of independent experiments. ^a $P < 0.05$ and ^b $P < 0.05$, significantly different from the control and from the value obtained with TGFβ1, respectively.

Laufs, 2005). Treatment with lovastatin at 3 μ M inhibited the TGFβ1-induced formation of ROS and expression of α SMA. We showed for the first time that probucol and lovastatin, which are established compounds in clinical use with good safety profiles, inhibited the TGFβ1-induced formation of ROS and resulting expression of α SMA in HFL1 cells. Oxidative stress can induce TGFβ gene expression, and a synthesized latent form of TGFβ should be activated by ROS for binding to its receptors (Gauldie *et al.*, 2006; Liu and Gaston Pravia, 2010). It was found that lung fibroblasts expressed higher levels of TGFβ1 and TGFβ2 mRNA in response to exogenous TGFβ1, suggesting a positive autocrine feedback mechanism (DiCamillo *et al.*, 2006; Blaauwboer *et al.*, 2011). Treatment of mice with lovastatin (p.o., 10 mg/kg) attenuated subchronic radiation effects including increased expression of TGFβ in lung cells (Ostrau *et al.*, 2009). Thus, reagents having anti-oxidative activity may inhibit the TGFβ1-induced expression of α SMA via multiple mechanisms. In the present study, lovastatin at 1 μ M had a greater inhibitory effect on the TGFβ1-induced expression of α SMA than did 30 μ M probucol. Probulcol is proposed to scavenge ROS by the two phenol moieties of the reagent (Yamashita and Matsuzawa, 2009), and lovastatin

appears to inhibit formation of ROS by enzymatic inhibition. This character of lovastatin may explain its greater inhibition of α SMA expression.

On the basis of findings that the concentration of GSH is decreased in the lung-lining fluid of patients with fibrotic disease, GSH and NAC have been used clinically for the treatment of various lung fibrotic diseases, although the efficacy of these reagents remains to be comprehensively evaluated (Kinnula *et al.*, 2005; Sadowska *et al.*, 2006; Liu and Gaston Pravia, 2010). It is reported that glutathione redox status regulated TGFβ1-induced fibrogenic effects including expression of α SMA in lung cells including HFL1 cells (Sugiura *et al.*, 2009; Ono *et al.*, 2009; Felton *et al.*, 2009). Under our conditions, however, treatment with neither NAC nor glutathione ethyl ester affected α SMA levels. This discrepancy may be explained by the following factors. Oxidative/redox conditions including GSH metabolism and ROS formation are dependent on various cellular conditions including cell clones, passages, and serum. In fact, the expression and/or endogenous level of α SMA in HFL1 cells differs among reports: a substantial level was found in both this study and previous reports (Ichikawa *et al.*, 2008; Ono *et al.*, 2009), but a trace amount of the protein was reported elsewhere (Sugiura *et al.*, 2009). Felton *et al.* (2009) reported that repeated application of 5 mM NAC (5 times, at every 24 hr for 5 days) decreased TGFβ1-induced expression of α SMA in alveolar epithelial cells, and the single application had no effect. Thus, application of the anti-oxidants at tested concentrations (2 times) in the present study may be not enough to regulate glutathione redox status in HFL1 cells, and additional experiments on the application of NAC (dose and time) are in progress in our laboratory. Also, multiple pools and/or sub-cellular compartments of ROS, which differed in sensitivity to water-soluble anti-oxidants and to lipid-soluble anti-oxidants, are proposed (Moldovan and Moldovan, 2004). The effects of lipid soluble anti-oxidants and statins on TGFβ1-induced α SMA expression should be solved.

Inhibition of α SMA expression by lecithinized-SOD

SODs are the only enzymatic system decomposing superoxide radicals to H_2O_2 and are hypothesized to play a significant role against oxidative stress, specifically in lung (Kinnula *et al.*, 2005; Gao *et al.*, 2008). Treatment with lecithinized-SOD (also shown as PC-SOD) significantly inhibited TGFβ1-induced expression of α SMA (Fig. 2). Although addition of lecithinized-SOD can increase H_2O_2 formation from superoxide radicals in TGFβ1-stimulated cells, endogenous catalase may effec-

tively reduce H_2O_2 level in cells. Liposome encapsulation of anti-oxidative enzymes is postulated to provide a longer half-life and better penetration of target cells (Igarashi *et al.*, 1994; Kinnula and Crapo, 2003). Lecithinized-SOD appeared to have characteristics similar to liposome-encapsulated SOD, and treatment with the reagent protected against pulmonary fibrosis in mice (Tamagawa *et al.*, 2000; Tanaka *et al.*, 2010) and in a patient with amyopathic dermatomyositis (Kawashima *et al.*, 2010). Although particles probably due to lecithin formed in the culture medium, they were not cytotoxic. The usefulness of lecithinized-SOD should be examined in animal models and patients with lung fibrotic diseases.

In summary, the present study demonstrated that probucol and lovastatin, which are established compounds in clinical use with safety profiles, inhibited the TGF β 1-induced formation of ROS and resulting expression of α SMA in HFL1 cells. Lecithinized-SOD also decreased the TGF β 1-induced expression of α SMA. Recent studies show probucol and statins acting as anti-oxidants to be useful for the prevention and/or treatment of various diseases including declines in lung function (Franks and Gardner, 2006; Watts *et al.*, 2006; Alexeeff *et al.*, 2007; Tanous *et al.*, 2008; Yamashita and Matsuzawa, 2009). Probucol and lovastatin may be worth testing as therapeutic agents for patients with both lung fibrosis and hypercholesterolemia.

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