

Glutathione restores collagen degradation in TGF- β -treated fibroblasts by blocking plasminogen activator inhibitor-1 expression and activating plasminogen

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Vayalil, Praveen K., Mitchell Oلمان, Joanne E. Murphy-Ullrich, Edward M. Postlethwait, and Rui-Ming Liu. Glutathione restores collagen degradation in TGF- β -treated fibroblasts by blocking plasminogen activator inhibitor-1 expression and activating plasminogen. *Am J Physiol Lung Cell Mol Physiol* 289: L937–L945, 2005; doi:10.1152/ajplung.00150.2005.—Transforming growth factor (TGF)- β plays an important role in tissue fibrogenesis. We previously demonstrated that reduced glutathione (GSH) supplementation blocked collagen accumulation induced by TGF- β in NIH-3T3 cells. In the present study, we show that supplementation of GSH restores the collagen degradation rate in TGF- β -treated NIH-3T3 cells. Restoration of collagen degradation by GSH is associated with a reduction of type I plasminogen activator inhibitor (PAI)-1 expression/activity as well as recovery of the activities of cell/extracellular matrix-associated tissue-type plasminogen activator and plasmin. Furthermore, we find that NIH-3T3 cells constitutively express plasminogen mRNA and possess plasmin activity. Blockade of cell surface binding of plasminogen/plasminogen activation with tranexamic acid (TXA) or inhibition of plasmin activity with aprotinin significantly reduces the basal level of collagen degradation both in the presence or absence of exogenous plasminogen. Most importantly, addition of TXA or active PAI-1 almost completely eliminates the restorative effects of GSH on collagen degradation in TGF- β treated cells. Together, our results suggest that the major mechanism by which GSH restores collagen degradation in TGF- β -treated cells is through blocking PAI-1 expression, leading to increased PA/plasmin activity and consequent proteolytic degradation of collagens. This study provides mechanistic evidence for GSH's putative therapeutic effect in the treatment of fibrotic disorders.

transforming growth factor- β ; plasminogen activation

PROGRESSIVE FIBROSIS IN VITAL ORGANS, such as the lung, kidney, and heart, is a major cause of morbidity and mortality (7). Despite the high frequency and severity of these fibrotic diseases, there are few, if any, effective therapies because the pathophysiological processes leading to fibrosis remain poorly defined. Fibrosis is a complex tissue response characterized by increased production and deposition of extracellular matrix (ECM), of which collagens are a major component (7, 67). Various cytokines, chemokines, and growth factors have been shown to play important roles in the development of fibrosis; however, transforming growth factor (TGF)- β is considered to be the most potent and ubiquitous profibrogenic mediator (8, 20, 23, 46, 71). Despite intensive investigations, the complex

molecular mechanisms underlying TGF- β -stimulated collagen accumulation are still not completely defined.

In vitro, TGF- β 1 lowers the intracellular concentration of reduced glutathione (GSH), the most abundant intracellular free thiol and an important antioxidant, in a variety of cell types (2, 40, 52, 61, 62). A decreased GSH content has also been demonstrated in animals with experimentally induced fibrosis (10, 54) and in the lung lining fluid of patients with fibrotic lung diseases such as idiopathic pulmonary fibrosis and cystic fibrosis (9, 11, 59). Furthermore, aerosolized administration of *N*-acetylcysteine (NAC), a precursor of GSH, attenuates bleomycin-induced lung fibrosis in mice (25). Although NAC may function as a reductant directly modulating signaling pathways or other biological processes, these observations suggest that GSH depletion may contribute to the development of fibrosis. We previously demonstrated that TGF- β decreased cellular GSH content in mouse embryonic fibroblasts (NIH-3T3) (40). Restoration of intracellular GSH concentration with exogenous GSH, NAC, or GSH ester reduced TGF- β -stimulated collagen accumulation, suggesting that GSH depletion is critical for TGF- β -stimulated collagen accumulation (40).

In the present study, we clearly demonstrate that GSH supplementation blocks TGF- β -induced collagen accumulation by restoring collagen degradation through the inhibition of protease inhibitor plasminogen activator inhibitor (PAI)-1 expression/activity and subsequent activation of plasminogen.

MATERIALS AND METHODS

Cell culture and treatment. NIH-3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown routinely on 10-cm culture dishes in DMEM (GIBCO) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μ g/ml streptomycin [complete medium (CM)] at 37°C in a humidified 95% air/5% CO₂ atmosphere. The cells were treated under the same conditions as described below for all experiments unless specifically indicated: cells were seeded at a density of 2×10^5 cells/ml and cultured in CM for 24 h and then serum starved for 24 h in the medium containing 0.1% FBS. After serum starvation, the cells were treated with TGF- β (R&D Systems, Minneapolis, MN) and/or other compounds in serum-free medium for various periods of time as indicated.

Northern blot analyses of mRNA content. Northern blot analysis of steady-state mRNA content was performed as described previously (39). Briefly, RNA (20 μ g) was electrophoresed through a 1.2%

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agarose-1.1% formaldehyde gel, transferred onto nylon membranes, which were hybridized at 60°C with [³²P]dCTP (ICN Biomedical Research Products, Costa Mesa, CA)-labeled PAI-1, procollagen α2(I), and 18S cDNAs sequentially after stripping off the probe. The membranes were scanned and radioactivity was quantified with InstantImager (Packard Instruments).

Preparation of cell lysates and Western blot analysis. The cells were lysed with a cocktail containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1% phosphatase inhibitor (Sigma Chemical, St. Louis, MO). The cell lysates were centrifuged at 14,000 g for 30 min at 4°C, and the supernatant was used for Western analyses as described previously (39). Autoradiographic bands were semiquantitated via densitometric analysis (QuantityOne image analysis software; Bio-Rad, Hercules, CA).

Determination of collagen degradation. We assessed collagen degradation by measuring the time-dependent reduction of collagen-bound radioactivity after pulse-chase labeling the cells with [³H]proline (19, 24, 57). In brief, the cells were seeded on 12-well plates at a density of 2×10^5 cells/ml in CM. After 24 h, the medium was replaced, and the cells were incubated in serum-free medium for 6 h, followed by CM containing 1 μCi/ml [³H]proline (ICN Biomedical Research Products) and 25 μg/ml ascorbic acid for 18 h. Subsequently, the cells were washed three times with prewarmed medium containing 2 mM cold proline (16) and treated with TGF-β (1 ng/ml) or solvent (the chase medium) in the presence or absence of GSH (1, 2.5, or 5 mM), plasminogen (5 μg/ml; Calbiochem, San Diego, CA), tranexamic acid (TXA, 10 mM), aprotinin (5 μg/ml), or mouse active PAI-1 (20 ng/ml, Molecular Innovations) in the chase medium for various periods of time as indicated. In preliminary studies, we examined collagen degradation in the medium and ECM/cell separately. The results showed that >95% of the collagen degradation was associated with the ECM/cell portion. Therefore, total collagen degradation, including the degradation in media and ECM/cells, was analyzed together. In brief, media and the cells/ECM lysates were collected together at each time interval after treatments and heated at 95°C for 10 min to inactivate endogenous proteases. We determined newly synthesized collagens by measuring the amount of collagenase-sensitive radioactivity (collagen) as described previously (19, 51) with slight modification. Briefly, proteins were precipitated with ice-cold TCA (final concentration 5%) in the presence of bovine serum albumin (100 μg). TCA precipitates were then resuspended in collagenase digestion buffer (50 mM Tris pH 7.5, 5 mM CaCl₂, 2.5 mM N-ethylmorpholine), and collagens were digested with highly purified collagenase at 37°C for 4 h. Undigested proteins were then precipitated with TCA, and the radioactivity in the supernatant was determined via liquid scintillation counting. Total collagen degraded at $time_x$ was calculated by the following

$$\% \text{ collagen degraded} = \frac{(\text{cpm at } time_0 - \text{cpm at } time_x)}{\text{cpm at } time_0} \times 100$$

where $time_0$ represents the time just before TGF-β or solvent was added and $time_x$ represents the time when samples were collected after TGF-β or solvent treatment.

Zymographic analysis of tissue type plasminogen activator activity. After treatment, the media were collected and concentrated by ultrafiltration, and cells/ECM were collected with 1% Triton X-100 in PBS buffer. The activities of tissue- and urokinase-type plasminogen activator (t-PA and u-PA) were determined by zymographic analysis (29). Briefly, equal amounts of proteins were loaded onto 12% polyacrylamide gel containing 2 mg/ml casein in the presence of 5 μg/ml plasminogen. After electrophoresis, we initiated the enzyme reaction by incubating the gel in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 h, and we developed the lytic areas by staining the gel with a solution containing 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie blue G250. The gels were destained in the same solution

without dye and scanned with Bio-Rad Fluor-s MultiImaging system. The photo-negative images of the gels were presented in the figures and assessed semiquantitatively with QuantityOne image-analyzing software. To avoid the possible interference of matrix metalloproteinases (MMPs), EDTA (2 mM) was included in the glycine-NaOH buffer during the incubation period. Gels without plasminogen were also run simultaneously to ensure that the lytic areas were plasminogen activators. The bands were identified based on both molecular weight and the loss of lytic activity upon inclusion of the plasmin inhibitor aprotinin (2 μg/ml).

Determination of plasmin activity in the medium. Plasmin activity was measured according to the manufacturer's protocol using a specific chromogenic substrate Tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate (Chromozym PL from Roche Applied Sciences), which is cleaved by plasmin into a residual peptide and 4-nitroaniline (73). The assay was carried out at 25°C in a 0.5-ml reaction mixture containing final concentrations of 33 mM Tris (pH 8.2), 6.4 mM NaCl, and 0.5 mM Chromozym PL dissolved in 100 mM glycine-0.2% vol/vol Tween 20 buffer. The absorbance of 4-nitroaniline produced was monitored at 405 nm. We calculated plasmin activity using the extinction coefficient for 4-nitroaniline ($\epsilon_{405 \text{ nm}} = 1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). To ensure specificity, the results were confirmed by inclusion of plasmin inhibitor aprotinin (2 μg/ml), which almost completely inhibited plasmin activity.

RT-PCR. Total RNA was treated with RNase-free DNase I (Promega, Madison, WI) at 37°C for 30 min and reverse transcribed into cDNAs with oligo (dT) 12–18 as primer. We performed PCR with a DNA thermal cycler (Biometa) using the following primers for murine plasminogen (74): forward primer 5'-AAC TAC TGC CGG AAC CCA GAT-3' (corresponding to murine plasminogen cDNA 988–1009 bp) and reverse primer 5'-GGT CTT CGA ATG CCT GTG TGG A-3' (complementary to 1239–1260 bp). The PCR conditions were: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 45 s for 35 cycles, and finally at 72°C for 5 min. GAPDH was amplified at the same time to show the equal amplification conditions among samples. PCR was also conducted with DNase I-digested RNAs without reverse transcription to exclude the possibility of DNA contamination in RT-PCR reactions. The amplified cDNA fragment (273 bp) were subjected to 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Reverse zymographic analysis of PAI-1 activity. Reverse zymography was performed as described previously (26) with minor modifications. Samples were applied to mini 12% SDS-PAGE gels containing purified human plasminogen, casein, and conditioned medium (CM) from MDA-MB-231 cells (used as a source for PA's). After electrophoresis at 4°C, SDS was washed out with 25% Triton X-100, and the gel was incubated at 37°C for 18 h in 0.1 M glycine (pH 8.3) containing EDTA (2 mM) to block the activity of MMPs in the CM. Consequently, the PA in CM of MDA-MB-231 cells converted the plasminogen throughout the gels into plasmin, which in turn degraded the casein in a diffuse pattern. By this method, PAI-1 activity yields dark bands on the lightly stained background, due to an inhibition of casein degradation. Two parallel control gels were treated similarly, but they did not contain either casein or plasminogen. The former served as a control to assure that the dark bands in the casein-containing gels did not result from just staining of sample proteins and the latter to demonstrate the degradation of casein in the gel is due to the generation of plasmin. Band intensity was determined with the Bio-Rad's Fluor-s MultiImaging system and QuantityOne image-analyzing software.

ELISA analysis of PAI-1 antigen. PAI-1 antigen was measured with a commercially available ELISA kit (Molecular Innovations, Southfield, MI) that detects active and inactive PAI-1, as well as PAI-1/PA complexes as described before (49, 53). CM (100 μl) was incubated in 96-well plates coated with capture antibody followed by addition of rabbit anti-murine PAI-1 polyclonal IgG (1:2,000). The bound polyclonal antibody was subsequently reacted for 30 min with secondary

antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:10,000). 3,3',5,5'-Tetramethylbenzidine was used as the substrate for color development, and the absorbance was measured at 450 nm. The concentrations of PAI-1 in the samples were determined by comparison to known amounts of human PAI-1 (0–5 ng/ml).

Statistical analysis. Data were presented as means \pm SE and were evaluated by one-way analysis of variance. Statistical significance was determined post hoc by Tukey's test wherein $P < 0.05$ was considered significant.

RESULTS

Effects of GSH supplementation on TGF- β -induced procollagen $\alpha 2(I)$ mRNA and protein expression. We reported previously that TGF- β increased procollagen $\alpha 2(I)$, one of the three procollagen chains that compose collagen I, but not procollagen $\alpha 1(III)$, mRNA in NIH-3T3 cells (40). We also reported that GSH supplementation blocked collagen protein accumulation induced by TGF- β in these same cells. In this study, we found that neither 5 mM GSH nor 4 mM GSH ester, which were shown in our previous study to restore intracellular GSH concentration in TGF- β -treated NIH-3T3 cells (40), had significant effects on TGF- β -induced procollagen $\alpha 2(I)$ mRNA expression (Fig. 1); however, GSH dramatically reduced TGF- β -stimulated accumulation of procollagen $\alpha 2(I)$ protein by 75% (Fig. 2). These data suggest that GSH supplementation abrogates TGF- β -mediated collagen accumulation through posttranscriptional mechanisms.

Effects of TGF- β and GSH supplementation on collagen degradation. As GSH supplementation blocks TGF- β -mediated collagen protein accumulation through a posttranscriptional mechanism, we further examined whether it alters collagen degradation in TGF- β -treated NIH-3T3 cells. The results showed that TGF- β (1 ng/ml) inhibited collagen degradation (8 and 24 h), whereas these inhibitory effects were reversed by GSH supplementation (Fig. 3). For example, by 24 h, 35% of newly synthesized collagen was degraded in untreated cells,

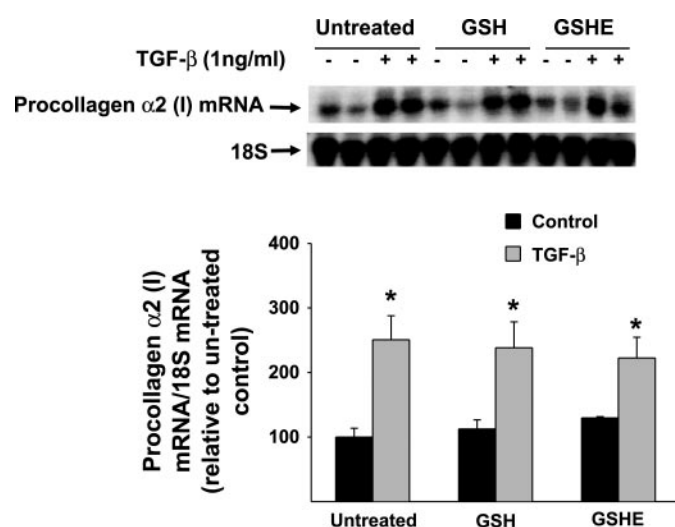


Fig. 1. GSH or GSH ester (GSHE) had no effect on transforming growth factor (TGF)- β -stimulated procollagen $\alpha 2(I)$ mRNA expression in NIH-3T3 cells. The cells were pretreated with 5 mM GSH or 4 mM GSHE for 30 min and then treated with TGF- β (1 ng/ml) in the presence or absence of GSH/GSHE for 24 h. Procollagen $\alpha 2(I)$ mRNA was analyzed by Northern hybridization and normalized by 18S mRNA as described in MATERIALS AND METHODS. * $P < 0.05$ compared with respective untreated control ($n = 4$).

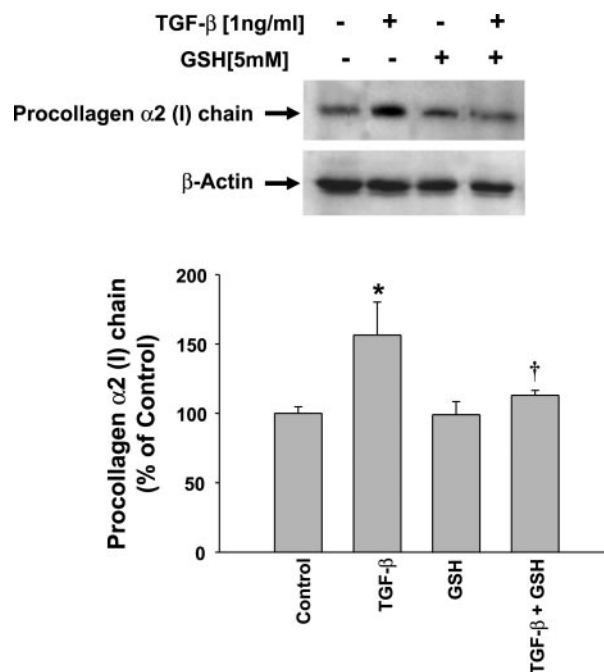


Fig. 2. GSH abrogated the increase in procollagen $\alpha 2(I)$ protein in TGF- β -treated NIH-3T3 cells. Experimental conditions were the same as described in Fig. 1. The cell lysates were prepared, separated on SDS-PAGE, and blotted on to a membrane, which was probed with a specific antibody to procollagen $\alpha 2(I)$ as described in MATERIALS AND METHODS. β -Actin was used to normalize the amount of protein loading. * $P < 0.05$ compared with untreated control ($n = 4$); † $P < 0.05$ compared with TGF- β alone-treated cells ($n = 4$).

whereas only 13% was degraded in TGF- β -treated cells. GSH supplementation (5 mM) restored the collagen degradation rate in TGF- β -treated cells to 29%. Although lower concentrations of GSH (1 and 2.5 mM) partially restored the collagen degra-

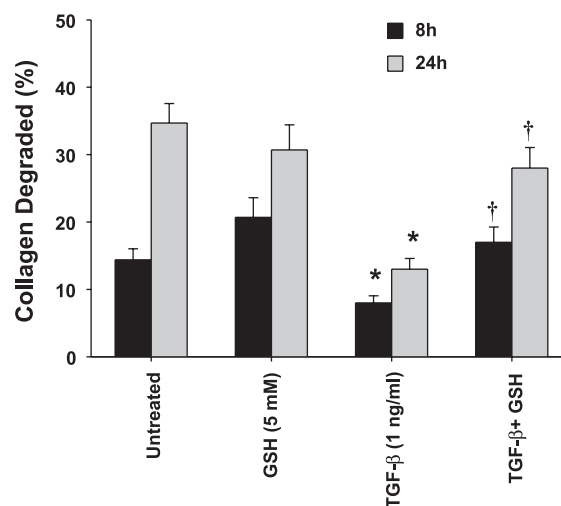
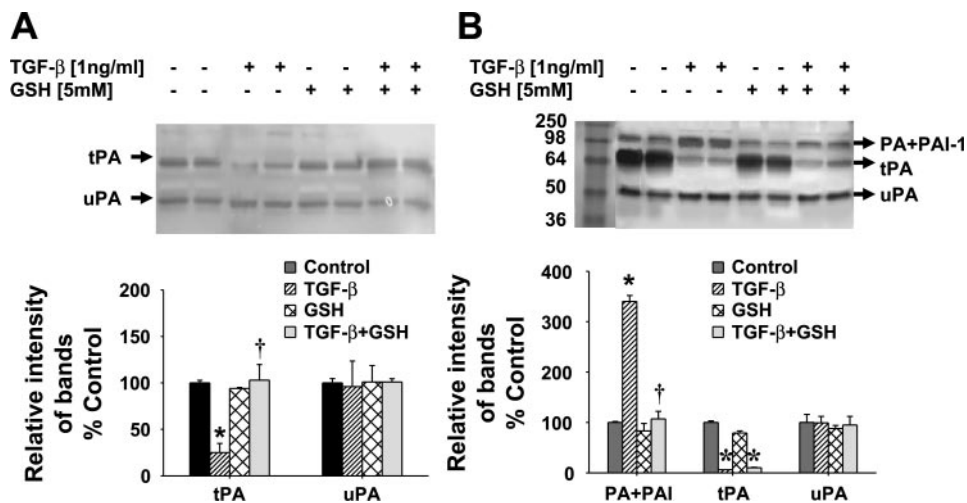


Fig. 3. GSH restored collagen degradation rate in TGF- β -treated NIH-3T3 cells. After being labeled with [3 H]proline for 18 h, NIH-3T3 cells were washed with chase medium and treated with 1 ng/ml TGF- β in the presence or absence of GSH in chase medium for various periods of time as indicated. Total collagen degradation was determined as described in MATERIALS AND METHODS. The values are means \pm SE of 3 separate experiments performed in triplicate. * $P < 0.05$ compared with untreated control at correspondent time point; † $P < 0.05$ compared with TGF- β alone-treated group at correspondent time point ($n = 6-9$).

Fig. 4. GSH restored the cell-associated tissue-type plasminogen activator (t-PA) activity and reduced plasminogen activator-plasminogen activator inhibitor (PA/PAI) complex levels in the conditioned medium in TGF- β -treated NIH-3T3 cells. The cells were pretreated with 5 mM GSH for 30 min and then treated with 1 ng/ml TGF- β in the presence or absence of GSH for 24 h. t-PA and urokinase-type plasminogen activator (u-PA) activities in the cell/extracellular matrix-associated portion (A) and in the medium (B) were determined by zymography as described in MATERIALS AND METHODS. The pictures represent the inverted images of the zymography gel, and the dark bands represent lytic zone. * $P < 0.05$ compared with its respective controls; † $P < 0.05$ compared with TGF- β alone-treated group ($n = 4$)



dation in TGF- β -treated cells, the effect was not statistically significant (data not shown). GSH up to 5 mM had no effect on the basal level collagen degradation. These data indicate that GSH supplementation reduces net collagen accumulation in TGF- β -treated cells, at least in part, by restoring collagen degradation inhibited by TGF- β .

Effects of TGF- β and GSH supplementation on plasminogen activators and plasmin activities. The plasminogen/plasmin system has been implicated in the process of collagen degradation in vivo through multiple yet not completely defined mechanisms (12, 21, 27, 45, 65). To further elucidate the mechanism underlying the restoration of collagen degradation by GSH in TGF- β -treated cells we examined the effects of GSH on cell-/ECM-associated and soluble (in the conditioned medium) t-PA and u-PA activities in TGF- β -treated cells. Direct zymography analysis of cell/ECM revealed two major lytic zones (the dark bands on the photo-negative images of the gels) at 63 and 48 kDa, which correspond to the known molecular masses of t-PA and u-PA (Fig. 4A). TGF- β inhibited the activities of cell-/ECM-associated and soluble t-PA, but not u-PA. Similar effects of TGF- β were also observed in time-course experiments (data not shown). Importantly, although GSH (5 mM) alone did not affect cell-/ECM-associated or soluble levels of t-PA or u-PA, it selectively reversed the inhibition of TGF- β on the cell-/ECM-associated t-PA activity (Fig. 4A). Moreover, analysis of CM showed an additional lytic band at 110 kDa, consistent with the known molecular mass of PA/PAI-1 complex (Fig. 4B). TGF- β increased the levels of PA/PAI-1 complex in the conditioned medium, whereas co-treatment with GSH eliminated such increase. These data suggest that GSH restores the activity of cell/ECM associated t-PA probably by inhibiting TGF- β -induced PAI-1 expression/activity. Consistent with the finding that GSH restored t-PA activity, we found that GSH supplementation reversed the inhibition of plasmin activity by TGF- β (Fig. 5A).

Plasma-circulating plasminogen is presumed to be liver derived. Whether fibroblasts also express plasminogen has not been previously characterized. To clarify where the plasminogen come from in our cell system, we performed RT-PCR analyses to determine whether NIH-3T3 cells also express plasminogen. Electrophoresis analysis of RT-PCR products revealed a clear band at 273 bp, which corresponds to the

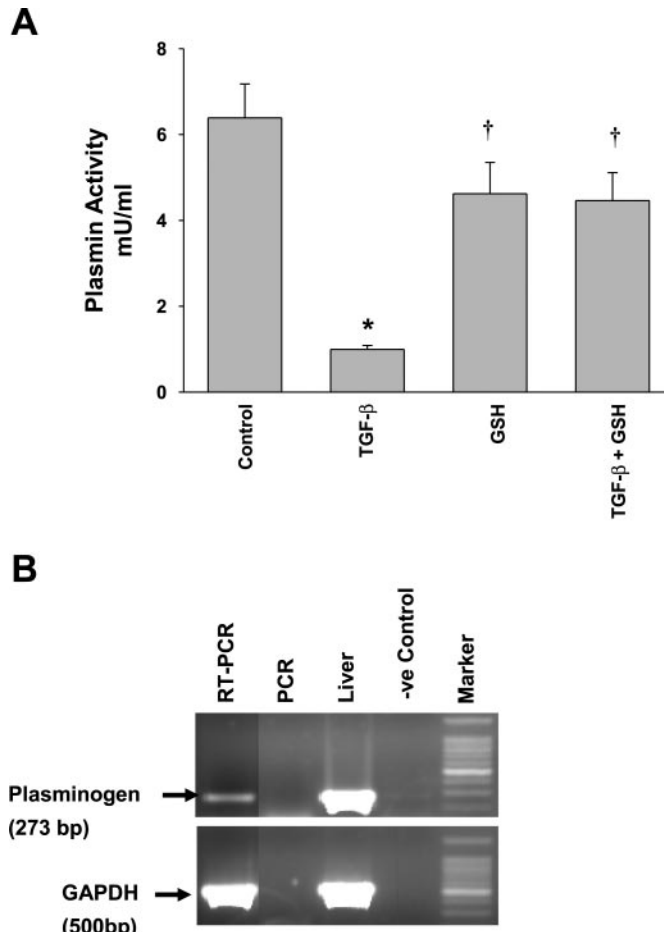


Fig. 5. NIH-3T3 cells constitutively express plasminogen mRNA and GSH reversed TGF- β -mediated inhibition of plasmin activity in NIH-3T3 cells. A: plasmin activity in the conditioned media. Media were collected after treatment, and plasmin activity was determined with a chromogenic substrate Tosyl-glycyl-prolyl-lysine-4-nitrilide-acetate as described in MATERIALS AND METHODS. B: RT-PCR showing the expression of plasminogen in NIH-3T3 cells. RNAs were isolated from untreated NIH-3T3 cells (lanes 1 and 2) or mouse liver (lane 3, positive control). Equal amounts of RNA were used for RT-PCR reactions (lane 1) or direct PCR reactions (lane 2) with the primers specific for murine plasminogen and GAPDH. Lane 4, negative control (no template). * $P < 0.05$ compared with untreated control; † $P < 0.05$ compared with TGF- β alone-treated group ($P < 0.05$, $n = 3-6$).

RT-PCR fragment of plasminogen amplified by the primers (Fig. 5B, lanes 1 and 3). No detectable PCR product was found when the same amount of RNA from NIH-3T3 cells was directly used as the template for PCR (lane 2), indicating that the band in lane 1 is not due to DNA contamination. These data indicate that, in addition to hepatocytes, fibroblast-like cells (NIH-3T3 cells) also express plasminogen under basal conditions.

Effect of plasminogen/plasmin inhibitors on collagen degradation in TGF- β - and GSH-treated cells. To further verify the involvement of the PA/plasmin system in collagen degradation in our cell model, we examined collagen degradation in the presence or absence of the plasminogen receptor binding/plasminogen activation inhibitor, TXA, and the plasmin active-site inhibitor, aprotinin, in the presence or absence of exogenously added plasminogen. The results showed that addition of exogenous plasminogen (5 μ g/ml) enhanced collagen degradation (Fig. 6). Both TXA and aprotinin partially inhibited collagen degradation in the presence and absence of exogenously added plasminogen (Fig. 6, top). These data confirm the importance of PA/plasmin system in the degradation of collagens in NIH-3T3 cells. Most importantly, TXA abrogated the restorative effect of GSH on collagen degradation in TGF- β -treated cells in the presence or absence of exogenously added plasminogen (Fig. 6, bottom). These data provide direct evidence that GSH supplementation restores the collagen degradation in TGF- β -treated cells by, at least in part, restoring the activity of plasminogen activator/plasmin cascade.

Effects of GSH supplementation on TGF- β -induced expression of type I PAI. PAI-1 plays an important role in controlling ECM turnover in part by inhibiting the activity of plasminogen activators/plasmin cascade (46, 48, 64). Increased PAI-1 activity is a hallmark of tissue and organ fibrosis and TGF- β is a strong inducer of PAI-1 (41). Therefore, we examined the effects of TGF- β and/or GSH on PAI-1 expression and activity by Northern hybridization, Western blotting, ELISA, and reverse zymography. TGF- β increased the steady-state level of PAI-1 mRNA in a time-dependent manner in NIH-3T3 cells (Fig. 7A). Although neither GSH nor GSH ester had a significant effect on the basal level of PAI-1 mRNA expression, both reduced TGF- β -stimulated PAI-1 mRNA expression (48 and 57%, respectively; Fig. 7B).

Consistent with the above results, TGF- β substantially increased the total PAI-1 protein content (Fig. 8A) and activity (Fig. 8B) in the CM as well as intracellular PAI-1 protein content (Fig. 8C) by 24 h. GSH ester and/or GSH dramatically reduced such stimulatory effects of TGF- β on PAI-1 protein content and activity, whereas neither GSH nor GSH ester treatment induced a significant impact on basal PAI-1 protein levels/activity.

Effects of exogenous active PAI-1 on the collagen degradation. To determine whether GSH restores collagen degradation by inhibiting PAI-1 expression, we tested the effect of exogenous active PAI-1 (20 ng/ml) almost completely eliminated the stimulatory effect of GSH on the collagen degradation in TGF- β -treated cells (Fig. 9). These data suggest that inhibition of PAI-1 expression is the primary mechanism by which GSH restores the collagen degradation in TGF- β -treated cells.

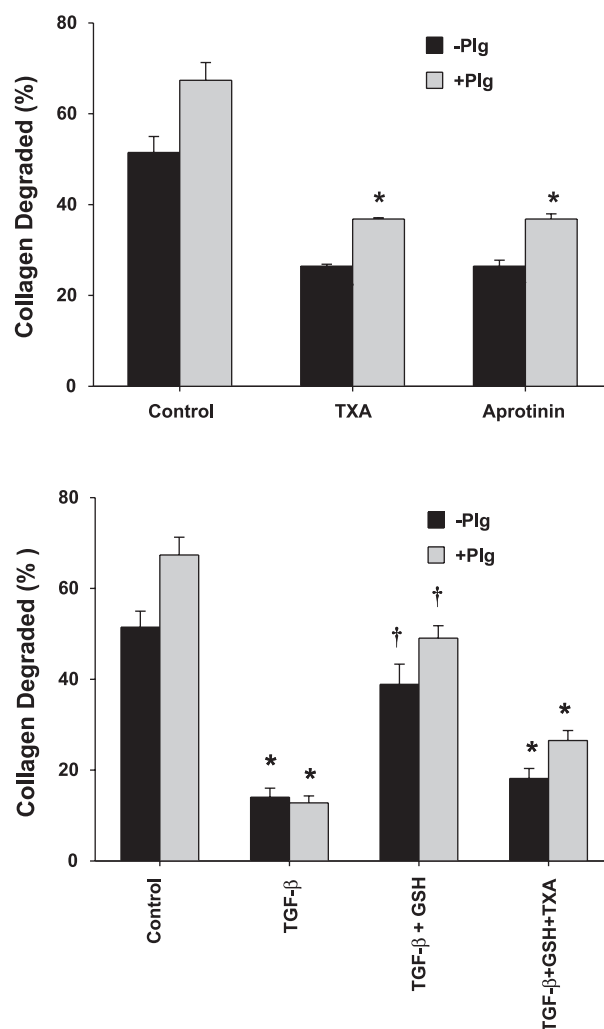
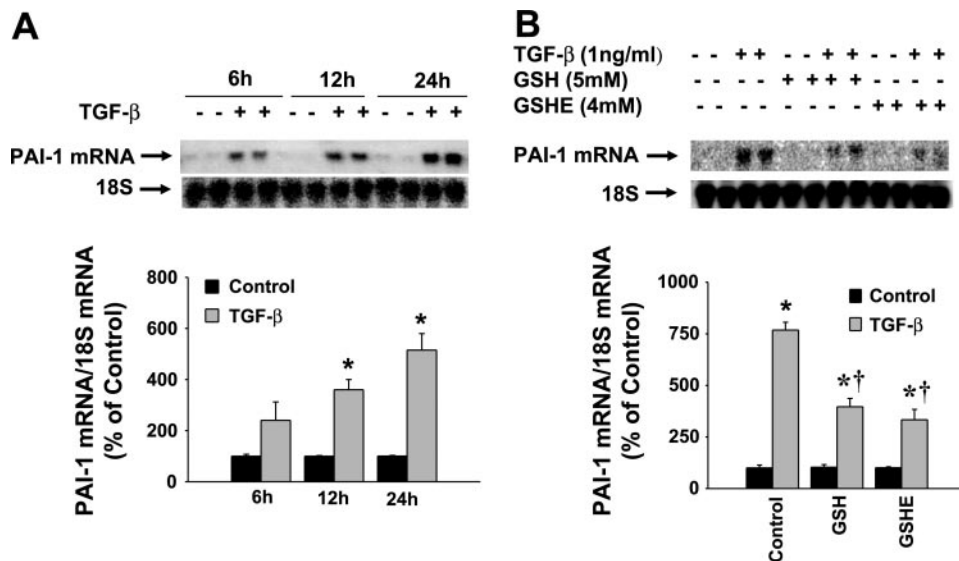


Fig. 6. Plasminogen activation/plasmin inhibitors reduced collagen degradation and abrogated the stimulatory effect of GSH on collagen degradation in TGF- β -treated NIH-3T3 cells. *Top*: cells were pulse-labeled with [3 H]proline for 18 h and then treated with 1 ng/ml TGF- β in the presence or absence of 10 mM tranexamic acid (TXA) or 5 μ g/ml aprotinin with (+) or without (-) exogenous plasminogen (PLG). *Bottom*: cells were pulse-labeled with [3 H]proline for 18 h and then treated with 1 ng/ml TGF- β with or without 5 mM GSH and/or 10 mM TXA in the presence or absence of exogenous PLG. Collagen degradation was determined as described in MATERIALS AND METHODS. *Significantly different from corresponding PLG \pm , untreated control group; †significantly different from corresponding PLG \pm , TGF- β -treated group ($P < 0.05$, $n = 6-11$).

DISCUSSION

In previous studies, we demonstrated that TGF- β concomitantly decreases cellular GSH pools while increasing collagen accumulation in murine embryonic fibroblasts (40). Addition of exogenous NAC, GSH, or GSH ester limited the net GSH decline and blocked collagen accumulation, suggesting that GSH depletion may play a critical role in TGF- β 's fibrogenic effects (40). Our current results suggest that GSH supplementation blocks TGF- β -induced collagen accumulation, at least in part, through enhancing collagen degradation. Inhibition of PAI-1 expression, coupled with restoration of t-PA and plasmin activities, accounts for the augmented collagen degradation by GSH. These conclusions are based on the following results: 1) GSH blocked TGF- β -induced increase in procolla-

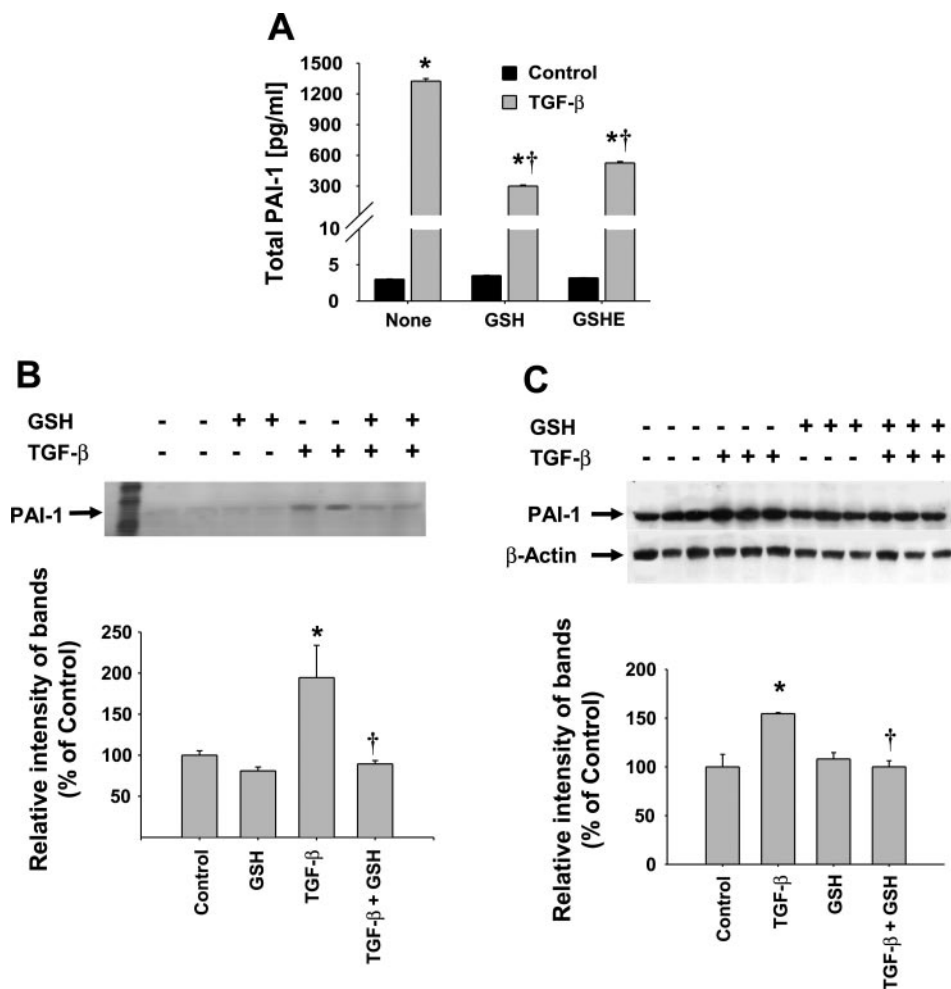
Fig. 7. GSH inhibited TGF- β -induced PAI-1 mRNA expression in NIH-3T3 cells. *A*: time-dependent induction of PAI-1 by TGF- β . The cells were treated with 1 ng/ml TGF- β for different periods of time as indicated. *B*: effect of GSH or GSHE on TGF- β -induced PAI-1 expression. The cells were treated with 5 mM GSH or 4 mM GSHE for 30 min and then with 1 ng/ml TGF- β in the presence or absence of GSH/GSHE for 24 h. After treatment, RNA was isolated, and Northern hybridization analyses of PAI-1 mRNA were conducted as described in MATERIALS AND METHODS. * $P < 0.05$ compared with untreated control; † $P < 0.05$ compared with TGF- β alone-treated group.



gen $\alpha 2(I)$ protein chain levels (Fig. 2); 2) TGF- β suppressed collagen degradation, whereas GSH reversed such effect (Fig. 3); 3) TGF- β induced PAI-1 expression and activity, which was accompanied by suppression of t-PA and plasmin activities, whereas GSH supplementation inhibited TGF- β -induced

PAI-1 expression/activity and restored both t-PA and plasmin activities (Figs. 4, 5, 7, and 8); 4) plasminogen activation/plasmin inhibitors, TXA and aprotinin, suppress collagen degradation in the presence or absence of exogenously added plasminogen, and TXA abolished the stimulatory effect of

Fig. 8. GSH reduced TGF- β -stimulated increases in PAI-1 protein content and activity in NIH-3T3 cells. NIH-3T3 cells were treated as described in Fig. 1. *A*: total amount of PAI-1 antigen in the conditioned medium was determined by ELISA; *B*: PAI-1 activity in the conditioned medium by reverse zymography; *C*: cellular levels of PAI-1 by Western blot analyses as described in MATERIALS AND METHODS. *Significantly different from untreated control; †significantly different from TGF- β alone-treated group ($P < 0.05$, $n = 4$).



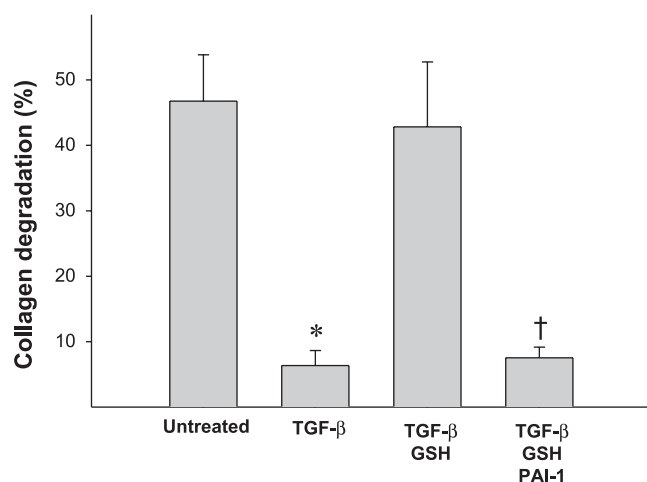


Fig. 9. Addition of exogenous PAI-1 blocked the restorative effect of GSH on the collagen degradation in TGF- β -treated fibroblasts. After being labeled with [3 H]proline for 18 h, NIH-3T3 cells were washed with chase medium and treated with 1 ng/ml TGF- β in the presence or absence of GSH and/or PAI-1 (20 ng/ml) in chase medium for 24 h. Total collagen degradation was determined as described in MATERIALS AND METHODS. The values are means \pm SE of 2 separate experiments performed in triplicate. *Significantly different from the untreated control ($P < 0.05$); †significantly different from TGF- β + GSH treatment ($P < 0.05$).

GSH on collagen degradation in TGF- β -treated cells (Fig. 6); and 5) addition of active PAI-1 completely eliminated the stimulatory effect of GSH on the collagen degradation in TGF- β -treated cells (Fig. 9).

Degradation of newly synthesized collagen can occur in both intracellular and extracellular compartments. Before secretion, 15–30% of newly synthesized collagens are degraded intracellularly (6, 42, 55, 56). Extracellular collagen degradation has been suggested to be mediated mainly by two mechanisms: receptor-mediated phagocytosis and subsequent intracellular degradation and extracellular/cell surface associated protease-mediated degradation (3, 17, 38, 45). As we measured the total collagen degradation, we were unable to discriminate in which compartment collagen degradation was inhibited by TGF- β and reversed by GSH supplementation. However, we speculate that both TGF- β and GSH affect mainly the extracellular collagen degradation. This speculation is based on the following results: 1) t-PA, plasmin and PAI-1 function mainly in the extracellular compartment, and GSH supplementation elevated the activities of t-PA and plasmin and inhibited PAI-1 expression (45); and 2) addition of exogenous active PAI-1 protein almost completely eliminated the stimulatory effect of GSH on the collagen degradation in TGF- β -treated cells.

Both in vitro and in vivo studies suggest that the plasminogen activator/plasmin system plays an important role in extracellular matrix degradation (12, 15, 21, 27, 45, 48, 65). Plasmin activates pro-MMPs including interstitial collagenases, which in turn degrade collagens and other extracellular matrix proteins (12, 18, 43, 45). In addition to activating MMPs, plasmin can also directly degrade fibrin, denatured collagens, as well as components of the basement membrane and extracellular matrix including type IV collagen (3, 4, 15, 20, 68). In this study, we showed that the collagen degradation was significantly inhibited by plasminogen activation inhibitor TXA or plasmin inhibitor aprotinin in the presence or absence of exogenous

plasminogen. These data further support the role of the plasminogen system in collagen degradation. Plasma-circulating plasminogen is presumed to be liver derived; however, reports also demonstrate regulatable plasminogen gene expression in extrahepatic cells/tissues (5, 69, 74). Interestingly, we found in this study that NIH-3T3 fibroblasts also express plasminogen (Fig. 5). Although carry-over plasminogen from serum may contribute in part to the plasmin activity detected in the medium, these data suggest that fibroblasts are another potential source of plasminogen/plasmin.

In addition to plasminogen system, the activities of MMPs, the major group of proteases involved in the regulation of ECM turnover (12, 18, 43, 45), are also regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). Studies have shown that TGF- β induces TIMPs and inhibits the activities of MMPs (3, 50, 63, 70). Whether TIMPs are also involved in the regulation of the collagen degradation in our cell model and whether TGF- β and/or GSH also affect the expression/activity of TIMPs need to be explored further.

Under physiological conditions, cell-associated plasmin activity is regulated at multiple levels including plasminogen/plasminogen activator protein concentration, plasminogen/plasminogen activator receptor number, as well as the activities of plasminogen activator inhibitors (mainly PAI-1) (26, 45, 72). Our data demonstrate that exogenous GSH or GSH ester inhibits TGF- β -stimulated PAI-1 expression at both the mRNA and protein levels. GSH also abrogated formation of the PAI-1 complex and reversed the inhibitory effects of TGF- β on cell-/ECM-associated t-PA and plasmin activities. These results suggest that GSH enhances plasmin activity in TGF- β -treated cells probably by blocking induction of PAI-1 protein and thereby eliminating the binding/inhibition of PAI-1 to ECM/cell-associated t-PA. The key finding that addition of PAI-1 almost completely abrogated the restorative effect of GSH on collagen degradation in TGF- β -treated fibroblasts further supports the notion that GSH reverses TGF- β -mediated inhibition of collagen degradation mainly by blocking the induction of PAI-1.

The mechanisms whereby intracellular GSH concentrations modulate TGF- β -induced PAI-1 gene expression remain unclear. It is interesting to note that GSH alone did not affect basal collagen degradation, PAI-1 expression, or the activities of t-PA/u-PA/plasmin. Most importantly, GSH has no effect on TGF- β -stimulated procollagen $\alpha 2(I)$ or TGF- β mRNA (data not shown) expression. These data suggest that GSH blocks PAI-1 expression by altering specific signaling pathways rather than interacting directly with TGF- β /TGF- β receptors.

There is increasing evidence that reactive oxygen species (ROS) participate in the signaling cascades that mediate TGF- β 's effects (22, 28, 32, 34). Our previous study demonstrated that TGF- β is capable of producing ROS in NIH-3T3 cells. Limiting ROS production by inhibiting NADPH oxidase completely eliminates TGF- β -induced collagen accumulation (40), suggesting that ROS mediate TGF- β 's effects in these fibroblasts. Although it remains unclear whether ROS are involved in TGF- β -induced PAI-1 gene expression in NIH-3T3 cells, ROS have been linked to the induction of PAI-1 expression by several stimuli in other cell types (1, 13, 33, 60, 66, 75). Interestingly, binding sites for activation protein 1 (AP-1), a redox-sensitive transcription factor, have been identified in the PAI-1 promoter (14, 30, 35, 36, 44, 47). Therefore, modulation

of AP-1 binding to DNA may represent one potential mechanism by which ROS modulates TGF- β -induced PAI-1 gene expression. GSH is the most abundant intracellular free thiol and an important antioxidant. GSH can directly scavenge ROS or reduce hydrogen peroxide and organic peroxides through glutathione peroxidase-catalyzed reactions. GSH can also regulate gene expression by modifying the redox status of protein cysteine residues, including transcription factors, through glutaredoxin-catalyzed reactions (31, 37, 58). Thus GSH may inhibit TGF- β -induced PAI-1 expression by scavenging ROS and/or reducing oxidized protein cysteine residues induced by TGF- β . Nevertheless, further studies are required to fully characterize the underlying mechanism whereby GSH blocks the induction of PAI-1 by TGF- β .

As an extension to our previous work (40), we used murine embryonic fibroblasts to unravel the mechanism of antifibrogenic effects of GSH. It should be emphasized that although these fibroblast-like cells have been widely used to study fibroblast biology, they are derived from mouse embryo. Thus further experimentation with primary fibroblasts will be necessary to extrapolate these findings to human diseases.

In summary, we have shown that GSH supplementation restores collagen degradation in TGF- β -treated cells, likely by inhibiting PAI-1 expression, which constrains the loss of plasmin activity and subsequently facilitates collagen degradation. The results from this study shed new light on the mechanism of TGF- β 's fibrogenic actions and provide a mechanistic understanding regarding the potential therapeutic value of GSH in the treatment of fibrotic disorders.

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REFERENCES

- Adair JC, Knoefel JE, and Morgan N. Controlled trial of N-acetylcysteine for patients with probable Alzheimer's disease. *Neurology* 57: 1515-1517, 2001.
- Arsalane K, Dubois CM, Muanza T, Begin R, Boudreau F, Asselin C, and Cantin AM. Transforming growth factor-beta1 is a potent inhibitor of glutathione synthesis in the lung epithelial cell line A549: transcriptional effect on the GSH rate-limiting enzyme gamma-glutamylcysteine synthetase. *Am J Respir Cell Mol Biol* 17: 599-607, 1997.
- Arthur MJ. Degradation of matrix proteins in liver fibrosis. *Pathol Res Pract* 190: 825-833, 1994.
- Arthur MJ. Fibrosis and altered matrix degradation. *Digestion* 59: 376-380, 1998.
- Bae YH, Hwang JY, Kim YH, and Koh JY. Anti-oxidative neuroprotection by estrogens in mouse cortical cultures. *J Korean Med Sci* 15: 327-336, 2000.
- Bienkowski RS. Collagen degradation in human lung fibroblasts: extent of degradation, role of lysosomal proteases, and evaluation of an alternate hypothesis. *J Cell Physiol* 121: 152-158, 1984.
- Border WA and Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331: 1286-1292, 1994.
- Branton MH and Kopp JB. TGF-beta and fibrosis. *Microbes Infect* 1: 1349-1365, 1999.
- Brown DM, Beswick PH, Bell KS, and Donaldson K. Depletion of glutathione and ascorbate in lung lining fluid by respirable fibres. *Ann Occup Hyg* 44: 101-108, 2000.
- Cabre M, Camps J, Paternain JL, Ferre N, and Joven J. Time-course of changes in hepatic lipid peroxidation and glutathione metabolism in rats with carbon tetrachloride-induced cirrhosis. *Clin Exp Pharmacol Physiol* 27: 694-699, 2000.
- Cantin AM, Hubbard RC, and Crystal RG. Glutathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 139: 370-372, 1989.
- Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, and Collen D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 17: 439-444, 1997.
- Ceriello A, Curcio F, dello Russo P, Pegoraro I, Stel G, Amstad P, and Cerutti P. The defence against free radicals protects endothelial cells from hyperglycaemia-induced plasminogen activator inhibitor 1 over-production. *Blood Coagul Fibrinolysis* 6: 133-137, 1995.
- Chen YQ, Sloan-Lancaster J, Berg DT, Richardson MA, Grinnell B, and Tseng-Crank J. Differential mechanisms of plasminogen activator inhibitor-1 gene activation by transforming growth factor-beta and tumor necrosis factor-alpha in endothelial cells. *Thromb Haemost* 86: 1563-1572, 2001.
- Chuang-Tsai S, Sisson TH, Hattori N, Tsai CG, Subbotina NM, Hanson KE, and Simon RH. Reduction in fibrotic tissue formation in mice genetically deficient in plasminogen activator inhibitor-1. *Am J Pathol* 163: 445-452, 2003.
- Clark JG, Dedon TF, Wayner EA, and Carter WG. Effects of interferon-gamma on expression of cell surface receptors for collagen and deposition of newly synthesized collagen by cultured human lung fibroblasts. *J Clin Invest* 83: 1505-1511, 1989.
- Davidson FF, Lister MD, and Dennis EA. Binding and inhibition studies on lipocortins using phosphatidylcholine vesicles and phospholipase A2 from snake venom, pancreas, and a macrophage-like cell line. *J Biol Chem* 265: 5602-5609, 1990.
- Davis GE, Pintar Allen KA, Salazar R, and Maxwell SA. Matrix metalloproteinase-1 and -9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices. *J Cell Sci* 114: 917-930, 2001.
- Diegelmann RF, Bryson GR, Flood LC, and Graham MF. A microassay to quantitate collagen synthesis by cells in culture. *Anal Biochem* 186: 296-300, 1990.
- Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol* 15: 290-301, 2000.
- Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, and Simon RH. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 97: 232-237, 1996.
- Garcia-Trevijano ER, Iraburu MJ, Fontana L, Dominguez-Rosales JA, Auster A, Covarrubias-Pinedo A, and Rojkind M. Transforming growth factor beta1 induces the expression of alpha1(I) procollagen mRNA by a hydrogen peroxide-C/EBPbeta-dependent mechanism in rat hepatic stellate cells. *Hepatology* 29: 960-970, 1999.
- Gressner AM, Weiskirchen R, Breitkopf K, and Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 7: d793-d807, 2002.
- Gunn JM, Clark MG, Knowles SE, Hoppgood MF, and Ballard FJ. Reduced rates of proteolysis in transformed cells. *Nature* 266: 58-60, 1977.
- Hagiwara SI, Ishii Y, and Kitamura S. Aerosolized administration of N-acetylcysteine attenuates lung fibrosis induced by bleomycin in mice. *Am J Respir Crit Care Med* 162: 225-231, 2000.
- Hajjar KA and Acharya SS. Annexin II and regulation of cell surface fibrinolysis. *Ann NY Acad Sci* 902: 265-271, 2000.
- Hattori N, Sisson TH, Xu Y, and Simon RH. Upregulation of fibrinolysis by adenovirus-mediated transfer of urokinase-type plasminogen activator genes to lung cells in vitro and in vivo. *Hum Gene Ther* 10: 215-222, 1999.
- Herrera B, Alvarez AM, Sanchez A, Fernandez M, Roncero C, Benito M, and Fabregat I. Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transforming growth factor (beta) in fetal hepatocytes. *FASEB J* 15: 741-751, 2001.
- Heussen C and Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 102: 196-202, 1980.
- Hirashima Y, Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, and Terao T. Transforming growth factor-beta1 produced by ovarian cancer cell line HRA stimulates attachment and invasion through an up-regulation of plasminogen activator inhibitor type-1 in human peritoneal mesothelial cells. *J Biol Chem* 278: 26793-26802, 2003.

31. **Holmgren A.** Thioredoxin and glutaredoxin systems. *J Biol Chem* 264: 13963–13966, 1989.
32. **Hong Y, Peng H, La Fata V, and Liao J.** Hydrogen peroxide-mediated transcriptional induction of macrophage colony-stimulating factor by TGF- β 1. *J Immunol* 159: 2418–2423, 1997.
33. **Jiang Z, Seo JY, Ha H, Lee EA, Kim YS, Han DC, Uh ST, Park CS, and Lee HB.** Reactive oxygen species mediate TGF- β 1-induced plasminogen activator inhibitor-1 upregulation in mesangial cells. *Biochem Biophys Res Commun* 309: 961–966, 2003.
34. **Junn E, Lee KN, Ju HR, Han SH, Im JY, Kang HS, Lee TH, Bae YS, Ha KS, Lee ZW, Rhee SG, and Choi I.** Requirement of hydrogen peroxide generation in TGF- β 1 signal transduction in human lung fibroblast cells: involvement of hydrogen peroxide and Ca^{2+} in TGF- β 1-induced IL-6 expression. *J Immunol* 165: 2190–2197, 2000.
35. **Keeton MR, Curriden SA, van Zonneveld AJ, and Loskutoff DJ.** Identification of regulatory sequences in the type I plasminogen activator inhibitor gene responsive to transforming growth factor β . *J Biol Chem* 266: 23048–23052, 1991.
36. **Kutz SM, Hordines J, McKeown-Longo PJ, and Higgins PJ.** TGF- β 1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. *J Cell Sci* 114: 3905–3914, 2001.
37. **Landino LM, Moynihan KL, Todd JV, and Kennett KL.** Modulation of the redox state of tubulin by the glutathione/glutaredoxin reductase system. *Biochem Biophys Res Commun* 314: 555–560, 2004.
38. **Laurent GJ.** Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. *Am J Physiol Cell Physiol* 252: C1–C9, 1987.
39. **Liu RM, Borok Z, and Forman HJ.** 4-Hydroxy-2-nonenal increases gamma-glutamylcysteine synthetase gene expression in alveolar epithelial cells. *Am J Respir Cell Mol Biol* 24: 499–505, 2001.
40. **Liu RM, Liu Y, Forman HJ, Olman M, and Tarpey MM.** Glutathione regulates transforming growth factor- β -stimulated collagen production in fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 286: L121–L128, 2004.
41. **Loskutoff DJ and Quigley JP.** PAI-1, fibrosis, and the elusive provisional fibrin matrix. *J Clin Invest* 106: 1441–1443, 2000.
42. **Mattana J, Margiloff L, and Chaplia L.** Oxidation of extracellular matrix modulates susceptibility to degradation by the mesangial matrix metalloproteinase-2. *Free Radic Biol Med* 27: 315–321, 1999.
43. **Monea S, Lehti K, Keski-Oja J, and Mignatti P.** Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism. *J Cell Physiol* 192: 160–170, 2002.
44. **Mucsi I, Skorecki KL, and Goldberg HJ.** Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor- β 1 on gene expression. *J Biol Chem* 271: 16567–16572, 1996.
45. **Netzel-Arnett S, Mitola DJ, Yamada SS, Chrysovergis K, Holmbeck K, Birkedal-Hansen H, and Bugge TH.** Collagen dissolution by keratinocytes requires cell surface plasminogen activation and matrix metalloproteinase activity. *J Biol Chem* 277: 45154–45161, 2002.
46. **Olman MA.** Mechanisms of fibroproliferation in acute lung injury. In: *Acute Respiratory Distress Syndrome*, edited by Matthay MA and Lenfant C. New York: Dekker, 2003, p. 313–354.
47. **Olman MA, Hagood JS, Simmons WL, Fuller GM, Vinson C, and White KE.** Fibrin fragment induction of plasminogen activator inhibitor transcription is mediated by activator protein-1 through a highly conserved element. *Blood* 94: 2029–2038, 1999.
48. **Olman MA, Mackman N, Gladson CL, Moser KM, and Loskutoff DJ.** Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. *J Clin Invest* 96: 1621–1630, 1995.
49. **Olman MA, Marsh JJ, Lang IM, Moser KM, Binder BR, and Schleef RR.** Endogenous fibrinolytic system in chronic large-vessel thromboembolic pulmonary hypertension. *Circulation* 86: 1241–1248, 1992.
50. **Overall CM, Wrana JL, and Sodek J.** Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor- β . *J Biol Chem* 264: 1860–1869, 1989.
51. **Peterkofsky B and Diegelmann R.** Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry* 10: 988–994, 1971.
52. **Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Kotliansky VE, Matthay MA, and Sheppard D.** TGF- β 1 is a critical mediator of acute lung injury. *J Clin Invest* 107: 1537–1544, 2001.
53. **Prabhakaran P, Ware LB, White KE, Cross MT, Matthay MA, and Olman MA.** Elevated levels of plasminogen activator inhibitor-1 in pulmonary edema fluid are associated with mortality in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 285: L20–L28, 2003.
54. **Purucker E, Wernze W, and Krandik G.** Glutathione in plasma, liver, and kidney in the development of CCl₄-induced cirrhosis of the rat. *Res Exp Med (Berl)* 195: 193–199, 1995.
55. **Rennard SI, Stier LE, and Crystal RG.** Intracellular degradation of newly synthesized collagen. *J Invest Dermatol* 79, Suppl 1: 77s–82s, 1982.
56. **Ripley CR, Fant J, and Bienkowski RS.** Brefeldin A inhibits degradation as well as production and secretion of collagen in human lung fibroblasts. *J Biol Chem* 268: 3677–3682, 1993.
57. **Rodemann HP and Bayreuther K.** Abnormal collagen metabolism in cultured skin fibroblasts from patients with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 81: 5130–5134, 1984.
58. **Rouhier N, Gelhaye E, and Jacquot JP.** Glutaredoxin-dependent peroxidation of protein-protein interaction and catalytic mechanism. *J Biol Chem* 277: 13609–13614, 2002.
59. **Roum JH, Buhl R, McElvany NG, Borok Z, and Crystal RG.** Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol* 75: 2419–2424, 1993.
60. **Sachsenmaier C, Radler-Pohl A, and Zinck R.** Involvement of growth factor receptors in the mammalian UVC response. *Cell* 78: 963–972, 1994.
61. **Sanchez A, Alvarez AM, Benito M, and Fabregat I.** Apoptosis induced by transforming growth factor- β in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J Biol Chem* 271: 7416–7422, 1996.
62. **Sanchez A, Alvarez AM, Benito M, and Fabregat I.** Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor β in fetal rat hepatocytes in primary culture. *Hepatology* 26: 935–943, 1997.
63. **Seeland U, Haeuseler C, Hinrichs R, Rosenkranz S, Pfitzner T, Scharfetter-Kochanek K, and Bohm M.** Myocardial fibrosis in transforming growth factor- β 1 [TGF- β 1] transgenic mice is associated with inhibition of interstitial collagenase. *Eur J Clin Invest* 32: 295–303, 2002.
64. **Seiffert D, Mimuro J, Schleef RR, and Loskutoff DJ.** Interactions between type I plasminogen activator inhibitor, extracellular matrix and vitronectin. *Cell Differ Dev* 32: 287–292, 1990.
65. **Sisson TH, Hanson KE, Subbotina N, Patwardhan A, Hattori N, and Simon RH.** Inducible lung-specific urokinase expression reduces fibrosis and mortality after lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 283: L1023–L1032, 2002.
66. **Swiatkowska M, Szmraj J, Al-Nedawi KN, and Pawlowska Z.** Reactive oxygen species upregulate expression of PAI-1 in endothelial cells. *Cell Mol Biol Lett* 7: 1065–1071, 2002.
67. **Trojanowska M, LeRoy EC, Eckes B, and Krieg T.** Pathogenesis of fibrosis: type I collagen and the skin. *J Mol Med* 76: 266–274, 1998.
68. **Tuan TL, Wu H, Huang EY, Chong SS, Laug W, Messadi D, Kelly P, and Le A.** Increased plasminogen activator inhibitor-1 in keloid fibroblasts may account for their elevated collagen accumulation in fibrin gel cultures. *Am J Pathol* 162: 1579–1589, 2003.
69. **Twining SS, Wilson PM, and Ngamkitidechakul C.** Extrahepatic synthesis of plasminogen in the human cornea is up-regulated by interleukin-1 α and -1 β . *Biochem J* 339: 705–712, 1999.
70. **Uria JA, Jimenez MG, Balbin M, Freije JM, and Lopez-Otin C.** Differential effects of transforming growth factor- β on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol Chem* 273: 9769–9777, 1998.
71. **Wells RG.** Fibrogenesis. V. TGF- β signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 279: G845–G850, 2000.
72. **Werner F, Razaq TM, and Ellis V.** Tissue plasminogen activator binds to human vascular smooth muscle cells by a novel mechanism. Evidence for a reciprocal linkage between inhibition of catalytic activity and cellular binding. *J Biol Chem* 274: 21555–21561, 1999.
73. **Wohl R, Summaria L, and Robbins K.** Kinetics of activation of human plasminogen by different activator species at pH 7.4 and 37 degrees C. *J Biol Chem* 255: 2005–2013, 1980.
74. **Zhang L, Seiffert D, Fowler BJ, Jenkins GR, Thinnis TC, Loskutoff DJ, Parmer RJ, and Miles LA.** Plasminogen has a broad extrahepatic distribution. *Thromb Haemost* 87: 493–501, 2002.
75. **Zhao W, Spitz DR, Oberley LW, and Robbins ME.** Redox modulation of the pro-fibrogenic mediator plasminogen activator inhibitor-1 following ionizing radiation. *Cancer Res* 61: 5537–5543, 2001.