

# Modulation of Transforming Growth Factor- $\beta$ 1 Antiproliferative Effects on Endothelial Cells by Cysteine, Cystine, and *N*-Acetylcysteine

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## Abstract

Early passaged bovine pulmonary artery endothelial cells exposed to 0.1–2.0 ng/ml transforming growth factor-beta 1 (TGF- $\beta$ 1) showed concentration-dependent growth inhibition, as assessed by [ $^3$ H]thymidine labeling and cell counts, over a 96-h interval. Most of the inhibition of [ $^3$ H]thymidine labeling measured at 96 h persisted when the medium was replaced with TGF- $\beta$ 1-free medium after 24 h, but the inhibition of labeling was prevented by the presence of anti-TGF- $\beta$ 1 antibody in the replacement medium. Additions of 2 mM cysteine, 1 mM cystine, or 2 mM *N*-acetylcysteine at the time of the initial addition of TGF- $\beta$ 1 blocked the inhibitory effect of TGF- $\beta$ 1 on [ $^3$ H]thymidine labeling when this was assessed after 72–96 h, but not at earlier times. Prevention of the inhibitory effect on cellular proliferation produced by cysteine, cystine and *N*-acetylcysteine was associated with elevation of cellular glutathione that was present at 48–96 h. There was no evidence for direct inactivation of TGF- $\beta$ 1 by the thiol-amino acids. Conditioned medium from TGF- $\beta$ 1-treated endothelial cells inhibited proliferation of mink lung carcinoma (CCL64) cells, supporting a previously reported concept of autocrine production of TGF- $\beta$ 1 by the endothelial cells. The inhibitory action of the conditioned medium was partially prevented when 1 mM cysteine was added during conditioning. Thus, TGF- $\beta$ 1 treatment of endothelial cells appears to set off autocrine production by these cells of TGF- $\beta$ 1 that perpetuates the inhibition of cellular proliferation. Replenishment of cellular glutathione with thiol-amino acids counteracts the growth-inhibitory effect of TGF- $\beta$ 1 through a currently undefined mechanism. (*J. Clin. Invest.* 1992. 90:1649–1656.) Key words: CCL64 cells • cell proliferation • glutathione • thiol-amino acids • [ $^3$ H]thymidine labeling

## Introduction

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>1</sup> is a 25-kD dimeric protein that belongs to a family of five closely related peptides.

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1. Abbreviations used in this paper: TdR, thymidine; TGF, transforming growth factor.

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It has numerous effects on growth and differentiation of many cell types (1, 2). Initially TGF- $\beta$ 's were found to stimulate the anchorage independent growth of fibroblasts in soft agar. Later studies revealed that they also inhibit the proliferation of a number of other cells in culture, including endothelial cells (3–5). TGF- $\beta$ 's also stimulate collagen synthesis (6–8), suppress T and B lymphocyte activity (9, 10), promote chemotaxis for monocytes and macrophages (11), and act as mitogens for osteoblasts (12). Recently TGF- $\beta$ 1 was shown to suppress human immunodeficiency virus replication in cells of the monocyte/macrophage lineage (13). In general, mammalian cells possess receptors for TGF- $\beta$ 's, and the peptides are thought to regulate cell function via paracrine and autocrine systems (1, 2).

Experiments performed in this laboratory have shown that TGF- $\beta$ 1 effects on endothelial cells are O<sub>2</sub> dependent and blocked by antioxidants. We have referred to this as a "pro-oxidant" effect of TGF- $\beta$ 1 (14). Furthermore, TGF- $\beta$ 1 lowers cellular glutathione over the same time period as it produces the pro-oxidant effect (15). To further investigate the modulation of the TGF- $\beta$ 1 effect on endothelial cells, we have carried out experiments that evaluate its reversibility. In this report we show that TGF- $\beta$ 1 produces both early (within 24 h) and protracted (several days) effects on endothelial cells manifested by inhibition of radioactive thymidine (TdR) uptake and cellular proliferation. When cysteine, cystine, or *N*-acetylcysteine is applied with TGF- $\beta$ 1, the late effect on TdR uptake, but not the early one, is significantly attenuated. The attenuation of inhibition of TdR uptake with these agents is associated with elevation of total cellular glutathione. Studies utilizing anti-TGF- $\beta$ 1 antibody in endothelial cell cultures and conditioned medium of endothelial cells on mink lung carcinoma cells (CCL64) support a concept that TGF- $\beta$ 1 up-regulates its own production in an autocrine manner and that this is likely to play a role in the prolonged growth inhibitory effect of TGF- $\beta$ 1. Furthermore, cellular glutathione availability appears to regulate the growth inhibitory process produced by TGF- $\beta$ 1, and we propose that this effect may occur through regulation of the autocrine production of TGF- $\beta$ 1.

## Methods

**Reagents.** Porcine platelet TGF- $\beta$ 1 and chicken anti-TGF- $\beta$ 1 antibody were obtained from R&D Systems, Minneapolis, MN. The TGF- $\beta$ 1 was reconstituted in 4 mM HCl according to instructions of the supplier.

**Cell culture.** Third- to sixth-passaged bovine pulmonary artery endothelial cells were procured as previously described (16). Cells were grown at 37°C in RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone Laborato-

ries, Logan, UT) and antibiotics (100 U/ml penicillin G potassium, 100  $\mu$ g/ml streptomycin sulfate, and 1.25  $\mu$ g/ml amphotericin B) and plated at a density of  $\sim 1\text{--}4 \times 10^4$  cells per 35-mm culture dish. Additions of TGF- $\beta$ 1 were made 48 h after plating when cell density was  $\sim 2\text{--}8 \times 10^4$  cells per dish. For experiments using low seeding density and prolonged incubation periods, 4 ml of media was used without refeeding; for all other experiments, 2 ml of culture media was used and cells were refed at 48-h intervals. In experiments where unbound TGF- $\beta$ 1 removal from medium was carried out, the 2–4 ml of medium was carefully aspirated and the cellular monolayer was washed once with an equal volume of medium before reapplying new incubation medium without TGF- $\beta$ 1.

**Harvesting of cells and counting.** Cell monolayers were thoroughly washed twice with phosphate buffered saline (PBS) at 37°C and incubated with PBS containing 0.1% trypsin and 1 mM EDTA for 2 min at 37°C. The released cells were resuspended by pipetting and diluted in Isoton (Coulter Electronics, Hialeah, FL) for counting in a model ZM counter (Coulter). Protein measurement was done utilizing procedure of Lowry et al. (17).

**Quantitation of [ $^3$ H]TdR uptake by endothelial cells.** [ $^3$ H]TdR uptake was determined as previously described (18). [Methyl- $^3$ H]TdR purchased from Dupont/New England Nuclear, Boston, MA, with a specific activity of 49.5 Ci/mmol was diluted in phosphate-buffered saline (PBS) and was added to the 35-mm culture dishes to quantitate the rate of [ $^3$ H]TdR uptake. After incubating for desired periods at 37°C, the medium containing the radioactive nucleoside was carefully aspirated off and the dishes were then washed twice with 5 ml of ice-cold PBS. 2 ml of 0.01 N NaOH was then added to the dishes and they were left at 4°C for 15–20 min to lyse the cells. The content of the dishes was transferred carefully to test tubes that were vigorously mixed by vortexing. 0.5 ml of this solution was counted in Ecolite (ICN Chemicals, Costa Mesa, CA) in a scintillation counter.

**Measurement of cellular glutathione.** Culture dishes were rinsed three times with warm PBS and incubated for 3 min with 1.0 ml trypsin-EDTA at 37°C. The cells were rapidly suspended by pipetting and chilled immediately on ice. An aliquot of 0.2 ml of the cellular suspension was removed and diluted for counting in a model ZM Coulter counter. The remaining 0.8 ml of suspension was treated with 0.1 ml 10% perchloric acid to precipitate cell proteins, sonicated, and centrifuged at 2,600 g for 20 min. The supernatant obtained from centrifugation was immediately frozen at  $-20^\circ\text{C}$  for subsequent assays of cellular glutathione. To assay for total cellular glutathione, the previously frozen perchloric acid-treated supernatants were thawed and sonicated. The pH was adjusted to 7.0 with 0.3 M potassium hydroxide-3-(*N*-morpholino) propanesulfonic acid (pH 13.6) to neutralize the perchloric acid, and the sonicate was centrifuged and assayed for total glutathione by the Tietze method as described by Akerboom and Sies (19).

Briefly, the sum of the oxidized (GSSG) and reduced (GSH) forms of glutathione were determined using a kinetic assay in which GSH or GSSG and glutathione reductase reduce 5,5'-dithiobis (2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoate. The formation of 5-thio-2-nitrobenzoate was assessed spectrophotometrically at 412 nm. Each assay was individually calibrated with standard glutathione and the concentration of each sample was adjusted by dilution to ensure that the reaction rate was on the linear portion of the standard curve.

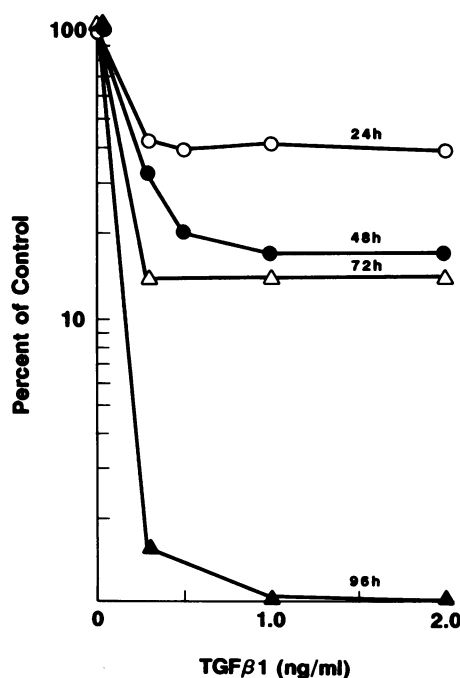
**Inhibition of mink lung carcinoma cell proliferation by endothelial cell conditioned medium.** Endothelial cells were treated with 0.1–2.0 ng/ml TGF- $\beta$ 1 for 24 h. After this, the media containing TGF- $\beta$ 1 were removed and the culture dishes were rinsed once with TGF- $\beta$ 1-free medium. The cells were then reincubated in TGF- $\beta$ 1-free medium for an additional 96 h. In some of the cultures, 1 mM cysteine was present during the final 96-h incubation period. Conditioned medium thus obtained was applied directly to mink lung carcinoma line CCL64 obtained from the American Type Culture Collection, Rockville, MD, and cell proliferation was measured 48 h later by determination of cell number by Coulter counter analysis.

**Experimental procedures.** All experiments were done at least in duplicate and representative experiments are shown. For some experiments multiple samples ( $n = 4$ ) were tested and standard deviations were calculated.

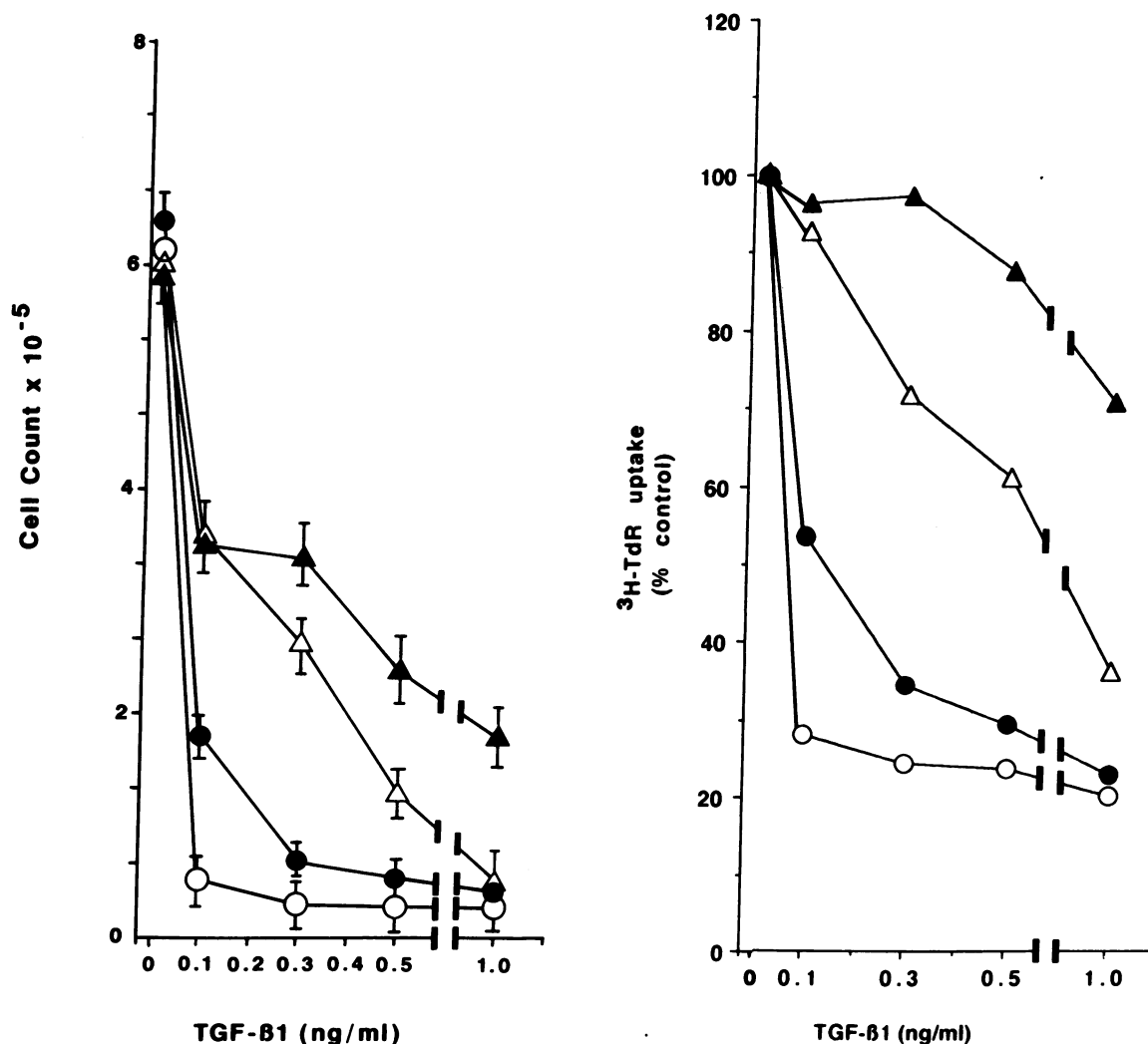
## Results

**Effect of TGF- $\beta$ 1 on [ $^3$ H]TdR uptake by bovine pulmonary artery endothelial cells.** We previously found that TGF- $\beta$ 1 at concentrations of 0.1–2.0 ng/ml inhibits uptake of radioactive thymidine by bovine pulmonary artery endothelial cells when it is measured at 48 h of incubation (14). We present more detailed observations of this effect in Fig. 1. At 24 h 0.3 ng/ml TGF- $\beta$ 1 resulted in 42% inhibition of [ $^3$ H]TdR uptake. This inhibitory effect increased with incubation time, and at 96 h inhibition was almost complete at concentrations from 0.3 to 2.0 ng TGF- $\beta$ 1/ml.

**Reversibility of the TGF- $\beta$ 1 effect on [ $^3$ H]TdR uptake by endothelial cells.** To evaluate whether the effect of TGF- $\beta$ 1 on endothelial cell proliferation was reversible, TGF- $\beta$ 1 was added to the endothelial cell cultures and then removed from some dishes by replacement of medium following cell monolayer washes 24 h later, whereas it was allowed to remain on others for the full duration of the experiment. Some replacement media at 24 h contained anti-TGF- $\beta$ 1 antiserum. Incubations were continued with the new media for another 96 h at which time cell counts were measured. As seen in Fig. 2 (left), TGF-



**Figure 1.** Effect of TGF- $\beta$ 1 treatment on the rate of [ $^3$ H]TdR uptake by pulmonary artery endothelial cells. Cells were plated 48 h before addition of TGF- $\beta$ 1 in fresh media. [ $^3$ H]TdR uptake for 1 h was carried out as described in Methods after incubation at the following times after exposure to TGF- $\beta$ 1: 24 h ( $\circ$ ); 48 h ( $\bullet$ ); 72 h ( $\Delta$ ); and 96 h ( $\blacktriangle$ ). Radioactive uptake by TGF- $\beta$ 1-treated cells is expressed as percentage of controls and is plotted on a logarithmic scale.

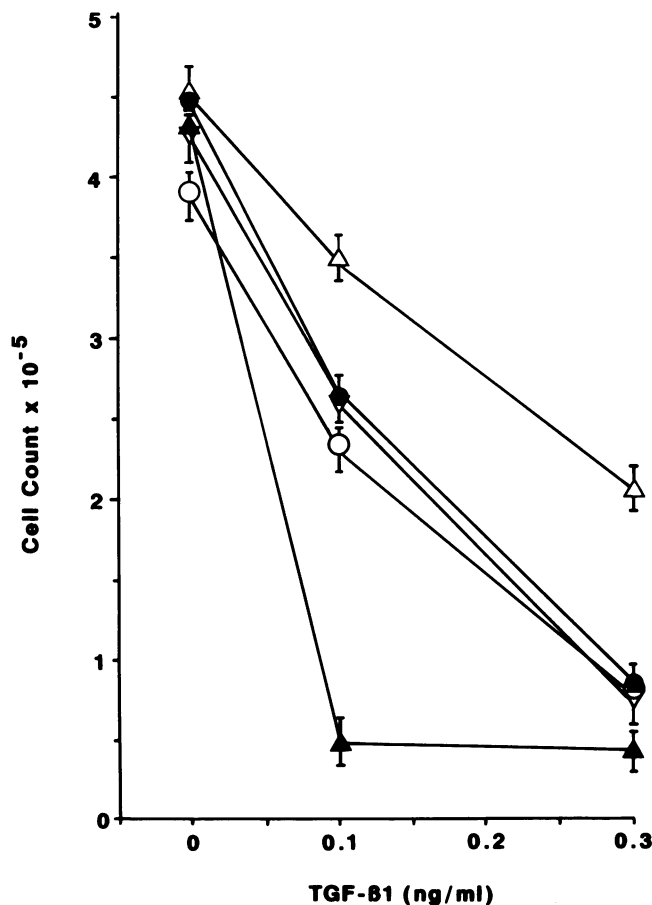


**Figure 2.** *Left:* effect of TGF- $\beta$ 1 removal at 24 h and anti-TGF- $\beta$ 1 antibody on inhibition of endothelial cell multiplication by TGF- $\beta$ 1. 2  $\mu$ g/ml anti-TGF- $\beta$ 1 antibody was used. Cell number was determined at 120 h. (○) TGF- $\beta$ 1 continuously present; (●) TGF- $\beta$ 1-free medium removed at 24 h of incubation and TGF- $\beta$ 1-free medium added; (Δ) TGF- $\beta$ 1 continuously present and anti-TGF- $\beta$ 1 added at 24 h; (▲) TGF- $\beta$ 1 removed at 24 h and medium containing anti-TGF- $\beta$ 1 added at 24 h. Means and standard deviations are shown. *Right:* effect of TGF- $\beta$ 1 removal at 2 h and anti-TGF- $\beta$ 1 antibody on inhibition of [ $^3$ H]TdR uptake by TGF- $\beta$ 1. The same protocol as that at left was carried out except TGF- $\beta$ 1 was removed and replacements were made at 2 h and [ $^3$ H]TdR uptake was measured for 1 h after a total incubation of 48 h. (○) TGF- $\beta$ 1 continuously present; (●) TGF- $\beta$ 1-containing medium removed at 2 h of incubation and TGF- $\beta$ 1-free medium added; (Δ) TGF- $\beta$ 1 continuously present and anti-TGF- $\beta$ 1 added at 2 h; (▲) TGF- $\beta$ 1 removed at 2 h and medium containing anti-TGF- $\beta$ 1 added at 2 h. 2.0 ng/ml chicken anti-TGF- $\beta$ 1 antibody was used.

$\beta$ 1 at concentrations as low as 0.1 ng/ml prevented cellular proliferation when left in contact with the cells for 120 h. A slightly less pronounced effect was observed when media were replaced with TGF- $\beta$ 1-free media at 24 h. Addition of anti-TGF- $\beta$ 1 antibody to medium at 24 h partially reversed the TGF- $\beta$ 1 effect, and more complete reversal was observed when media were exchanged at 24 h to TGF- $\beta$ 1-free media that contained anti-TGF- $\beta$ 1 antibody. A similar effect was observed with [ $^3$ H]TdR uptake measured during a 48–49-h interval when incubations were carried out for 49 h and changes in media were made at 2 h (Fig. 2, right). Removal of TGF- $\beta$ 1-containing medium at 2 h produced a similar inhibitory effect on cell proliferation as removal at 6 or 24 h when cell counts

were done at 120 h (Fig. 3). However, there was a partial reversibility of the TGF- $\beta$ 1 effect if the medium containing TGF- $\beta$ 1 was removed at 30 min. This observation suggests that TGF- $\beta$ 1 binding to cell receptors occurred rapidly and was nearly complete by 2 h.

*Attenuation of prolonged effects of TGF- $\beta$ 1 on endothelial cell [ $^3$ H]TdR uptake by cysteine, N-acetylcysteine, and cystine.* After the demonstration that the TGF- $\beta$ 1 effect on the endothelial cells was reversible and with an awareness that the cellular glutathione level is important in the modulation of the TGF- $\beta$ 1 effect (15), we considered the possibility that thiol-amino acids might prevent the TGF- $\beta$ 1 effects on endothelial cells. As shown in Fig. 4, a and b, when TGF- $\beta$ 1 treatment was carried



**Figure 3.** Reversal of TGF- $\beta$ 1 effect on cellular proliferation by replacement of medium containing TGF- $\beta$ 1 at various times after addition to cultures. Experiments were done similarly to those in Fig. 2 (left). Replacement culture medium contained no TGF- $\beta$ 1. Cell numbers were determined at 120 hrs incubation. For zero points TGF- $\beta$ 1 was never introduced. ( $\Delta$ ) medium containing TGF- $\beta$ 1 not changed. Medium containing TGF- $\beta$ 1 replaced after: ( $\Delta$ ) 30 min; ( $\bullet$ ) 2 h; ( $\circ$ ) 6 h; and ( $\nabla$ ) 24 h. Means and standard deviations are shown.

out in the presence or absence of 2 mM cysteine or its analogue, 2 mM *N*-acetylcysteine, the rates of [ $^3$ H]TdR uptake were similarly suppressed for the first 72 h (data obtained at 24 and 48 h also showing no effect of the thiol-amino acid are not shown). However, at 96 h the rates of [ $^3$ H]TdR uptake were higher in cultures treated with combined TGF- $\beta$ 1 and cysteine or *N*-acetylcysteine as compared with TGF- $\beta$ 1 alone (Fig. 4, *c* and *d*), indicating an attenuation of the TGF- $\beta$ 1 effect after  $\sim$  72 h of contact with the thiol-amino acid.

The effect of TGF- $\beta$ 1 was somewhat reduced by removing it from culture media at 24 h after initiation of experiments (Fig. 2), and we wished to determine whether the recovery process might be augmented by the addition of cysteine. These experiments were carried out similarly to those shown in Fig. 2 (right), except initial media were replaced with media containing 2 mM cysteine after 24 h in some samples, and proliferation was assessed by [ $^3$ H]TdR incorporation at 120–121 h. As seen in Fig. 5, addition of cysteine even after 24 h attenuated

the TGF- $\beta$ 1 effect on the rate of [ $^3$ H]TdR uptake at 120 h. These data support the likelihood that cysteine does not react directly with TGF- $\beta$ 1 to inactivate it.

In aerobic solution cysteine is largely converted to cystine within 24 h (20). Because our experiments were carried out for a prolonged time interval, we determined whether cystine might also attenuate the TGF- $\beta$ 1 effects. Cultures were treated with TGF- $\beta$ 1 in the presence or absence of cystine (0.2–1.0 mM) and the rate of [ $^3$ H]TdR uptake was determined at 120 h of incubation. As shown in Fig. 6, cystine also attenuated the effect of TGF- $\beta$ 1 on the rate of [ $^3$ H]TdR uptake by the endothelial cells.

In that TGF- $\beta$ 1 is a homodimeric protein joined by -S-S-bridge(s), we considered the possibility that the effects of TGF- $\beta$ 1 may be attenuated by cysteine through direct inactivation of TGF- $\beta$ 1 through the reduction of -S-S- bridges in the biologically active molecule. However, preincubation of TGF- $\beta$ 1 at concentrations up to 1.0 ng/ml with cysteine at concentrations up to 2.0 mM for 2 h failed to alter the inhibitory effect of TGF- $\beta$ 1 on [ $^3$ H]TdR uptake by endothelial cells at 48 h of incubation (Fig. 7). Longer preincubation (up to 48 h) also failed to influence the growth inhibitory effect of TGF- $\beta$ 1 (data not shown).

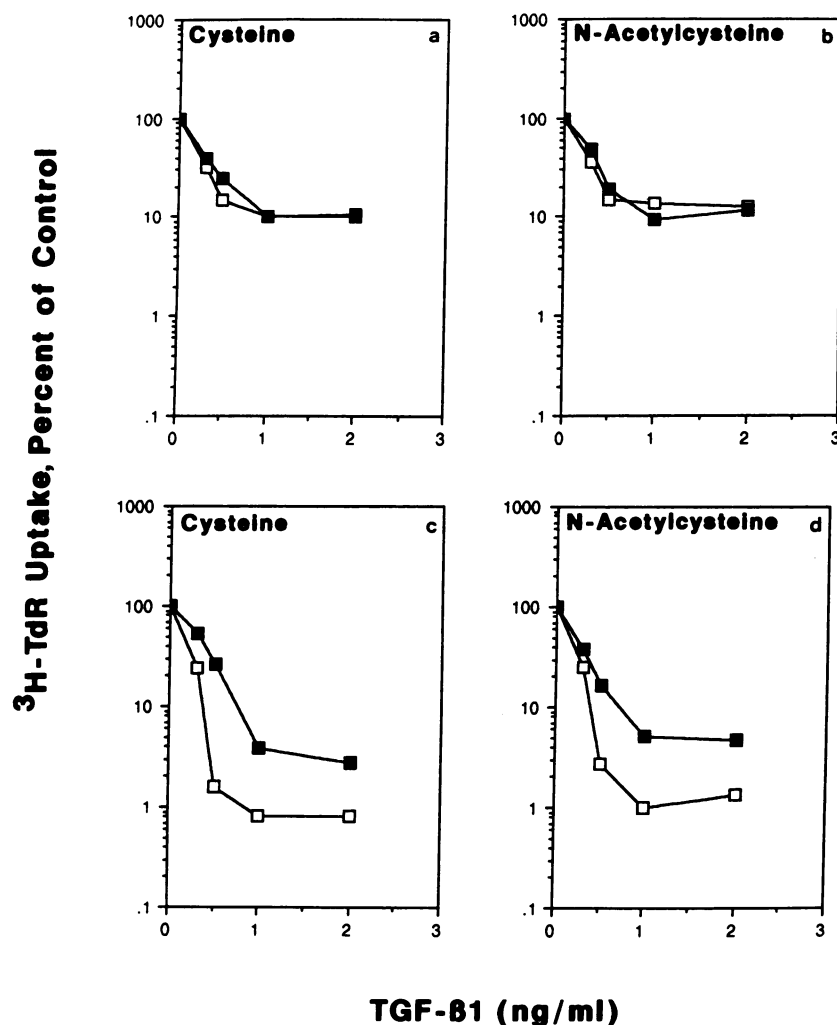
*Influence of cysteine, N-acetylcysteine, and cystine on cellular glutathione in the presence of TGF- $\beta$ 1.* Endothelial cells were seeded at low density and grown as described in Methods. Cells were then exposed to 1 ng/ml TGF- $\beta$ 1 with and without cysteine (2 mM), cystine (1 mM), or *N*-acetylcysteine (2 mM) in 4 ml of medium for 96 h and cell counts and glutathione levels were measured. TGF- $\beta$ 1 alone reduced both cell counts and cellular glutathione (Fig. 8). The addition of cysteine, cystine, and *N*-acetylcysteine to the medium of the TGF- $\beta$ 1-exposed cells reversed total cellular glutathione level and cell counts toward or to control levels.

*Growth-inhibitory effects of conditioned media from TGF- $\beta$ 1-treated endothelial cells on mink lung carcinoma cells.* CCL64 cells showed pronounced growth inhibition in the presence of 0.1–1.0 ng/ml TGF- $\beta$ 1. We utilized this cell line to investigate the possible autocrine production of TGF- $\beta$ 1 by endothelial cells treated with TGF- $\beta$ 1 for 24 h. As shown in a representative experiment in Table I, conditioned medium from cell cultures pretreated with TGF- $\beta$ 1 produced inhibition of CCL64 cell proliferation. The presence of cysteine during the conditioning period greatly reduced the inhibition of CCL64 growth by the conditioned medium.

## Discussion

Although TGF- $\beta$ 1 has previously been demonstrated to inhibit the proliferation of endothelial cells in culture (4), details regarding the mechanism of inhibition and its reversibility have not been adequately explored. We have proposed that inhibition of proliferation by TGF- $\beta$ 1 may be produced by a “pro-oxidant” effect of this cytokine (14) and that the pro-oxidant effect may be potentiated by the lowering of cellular glutathione by TGF- $\beta$ 1 (15).

In the current experiments, TGF- $\beta$ 1 was found to have a prolonged and progressive inhibitory effect on [ $^3$ H]TdR uptake by bovine pulmonary artery endothelial cells in culture



**Figure 4.** Influence of cysteine and *N*-acetylcysteine on [ $^3\text{H}$ ]TdR uptake inhibition by TGF- $\beta$ 1. (□) no cysteine or *N*-acetylcysteine; (■) + 2 mM cysteine (*a* and *c*), or 2 mM *N*-acetylcysteine (*b* and *d*). [ $^3\text{H}$ ]TdR uptake was carried out at 72–73 h for (*a* and *b*) and at 96–97 h for (*c* and *d*). Other details are in Methods.

even when it was removed from the medium after 24 h. Furthermore, removal at 2 h produced the same inhibitory effect as removal at 24 h, suggesting that TGF- $\beta$ 1 rapidly binds to its receptor and sets into motion a progressive and irreversible inhibition of cellular proliferation. The rapid binding of TGF- $\beta$ 1 to its receptor is consistent with other reports (21). Experiments showing that (*a*) an antibody to TGF- $\beta$ 1 reverses the inhibitory effect of TGF- $\beta$ 1 when added 24 h after the initial introduction of TGF- $\beta$ 1 concurrently with removal of TGF- $\beta$ 1 from the medium, and (*b*) conditioned medium from TGF- $\beta$ 1-treated cells contains an antiproliferative factor for mink lung carcinoma cells suggest that autocrine production of TGF- $\beta$ 1 occurs, thus perpetuating inhibition of cellular proliferation. In this regard, our interpretations are similar to those forwarded by other authors who have observed stimulation of cell growth by anti-TGF- $\beta$ 1 antibody and attributed this effect to autocrine production of TGF- $\beta$ 1 (22, 23). It is possible that the antibody is influencing intracellular TGF- $\beta$ 1 that is still available in the cell for liganding after 24 h; however, we think this mechanism is less likely, particularly in view of our data with conditioned medium and the current understanding of TGF- $\beta$ 1 receptor interaction. It has been shown that bound

TGF- $\beta$ 1 is internalized and degraded by lysosomal enzymes within 4 h at 37°C (21).

Inhibition of proliferation of the mink lung carcinoma cell has been used conventionally as a bioassay for the measurement of TGF- $\beta$ 1 (1, 2). In the current experiments, we have made use of this assay to determine if an antiproliferative substance might be released into the media of TGF- $\beta$ 1-treated endothelial cells. TGF- $\beta$ 1 treatment does, indeed, stimulate the production of such an inhibitory substance, and, consistent with a mechanism of regulation of its production by thiol-amino acid availability, exogenous cysteine attenuates the inhibition. Although we cannot be sure that the inhibitory substance is TGF- $\beta$ 1, our experiments with antibody to TGF- $\beta$ 1 support this contention.

Cysteine, cystine, and *N*-acetylcysteine all attenuated both the TGF- $\beta$ 1 antiproliferative and glutathione-depleting effects on endothelial cells, but this was not observed for the first 48–72 h of incubation. Thus, the blocking effect of these agents on inhibition of cellular proliferation is delayed. The lack of a quantitative correlation between the ability of the thiol amino acid to elevate cellular glutathione and its ability to prevent reduction of the cell count at 96 h may reflect the limited time

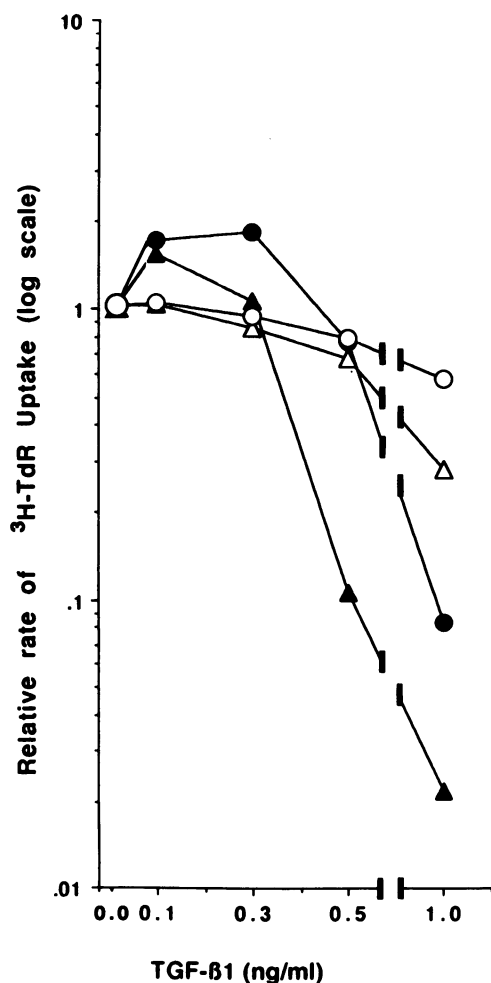


Figure 5. Effect of delayed addition of 2 mM cysteine to TGF- $\beta$ 1 treated cells. TGF- $\beta$ 1 was added to endothelial cells at 48 h after plating and 24 h later medium was changed to allow TGF- $\beta$ 1 removal and/or 2 mM cysteine addition. In control experiments TGF- $\beta$ 1 was left in the medium for the duration of the incubation. Total duration of incubation was 121 h and [ $^3$ H]TdR incorporation was carried out during the 120–121-h interval. (▲) TGF- $\beta$ 1 for entire incubation; (Δ) TGF- $\beta$ 1 removed at 24 h when medium changed; (●) TGF- $\beta$ 1 for duration of incubation plus cysteine added at 24 h; (○) TGF- $\beta$ 1 removed and cysteine added at 24 h. The rates of [ $^3$ H]TdR uptake are plotted relative to the rate with no TGF- $\beta$ 1.

point of our measurement. Maintenance or elevation of cellular glutathione by these thiol-amino acids or the *N*-acetylcysteine analogue might be anticipated since they provide substrate for intracellular synthesis of glutathione (24). Cysteine is internalized by sodium-dependent pathways (A or ASC carrier system) (25, 26) and cystine by the sodium-independent  $X_c^-$  system. *N*-acetylcysteine probably forms cysteine through a mixed disulfide reaction and cysteine is subsequently internalized through the A or ASC carrier system (27). Since our experiments were prolonged, any cysteine initially added to medium is probably totally oxidized to cystine within 24 h (28).

Consideration was given to the possibility that the thiol amino acids might inactivate TGF- $\beta$ 1 directly, perhaps by the formation of mixed disulfides or the oxidation of thiol groups on the molecule. This is unlikely because (a) the disulfide

Table I. Effect of Pulmonary Artery Endothelial Cell Conditioned Medium on CCL64 Mink Lung Carcinoma Cell Growth

Treatment	CCL64 cell number per 35-mm dish at 48 h $\times 10^{-5}$
A. Medium conditioned for 120 h with endothelial cells	4.44 $\pm$ 0.37
B. As in A except 2.0 ng/ml TGF- $\beta$ 1 present during 0–24 h	2.69 $\pm$ 0.17
C. As in A except 1 mM cysteine present during 24–120 h	4.5 $\pm$ 0.031
D. As in B except 1 mM cysteine present during 24–120 h	3.42 $\pm$ 0.23*

CCL64 cells were plated 48 h before the addition of conditioned medium and cells were incubated with conditioned medium for 48 h. Before the addition of conditioned medium, (i.e., at  $t = 0$  h), CCL64 cell count on replicate samples was  $0.78 \pm 0.11 \times 10^5$  per 35-mm dish ( $n = 4$ ).

Quadruplicate samples were done for each time point and the means and standard deviations are shown.

\*  $P < 0.05$  compared to B, Student's  $t$  test.

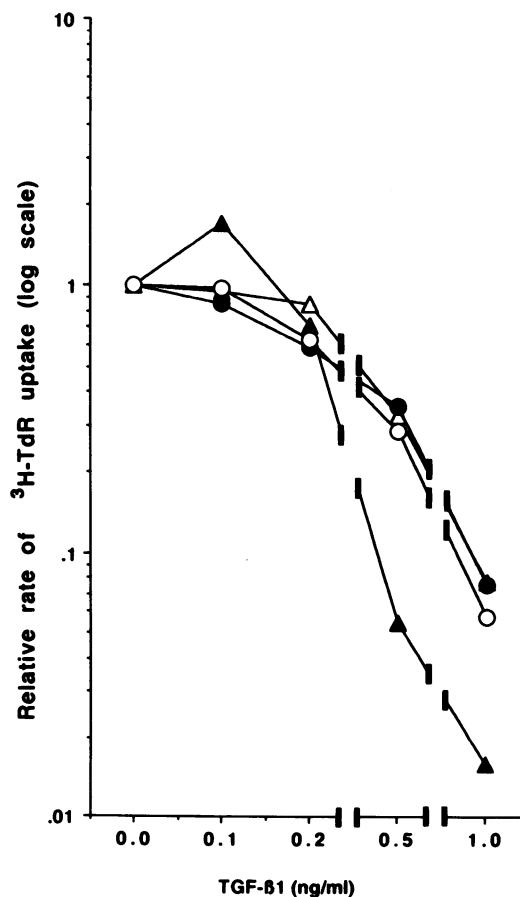
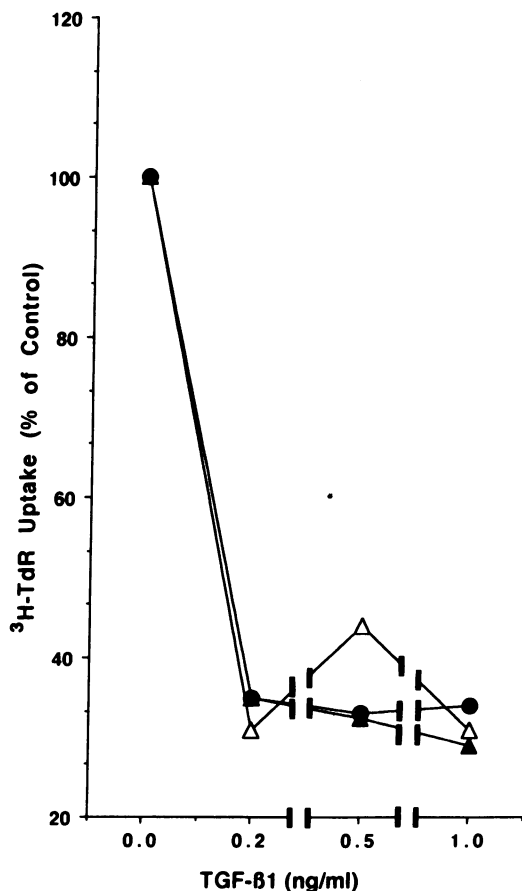


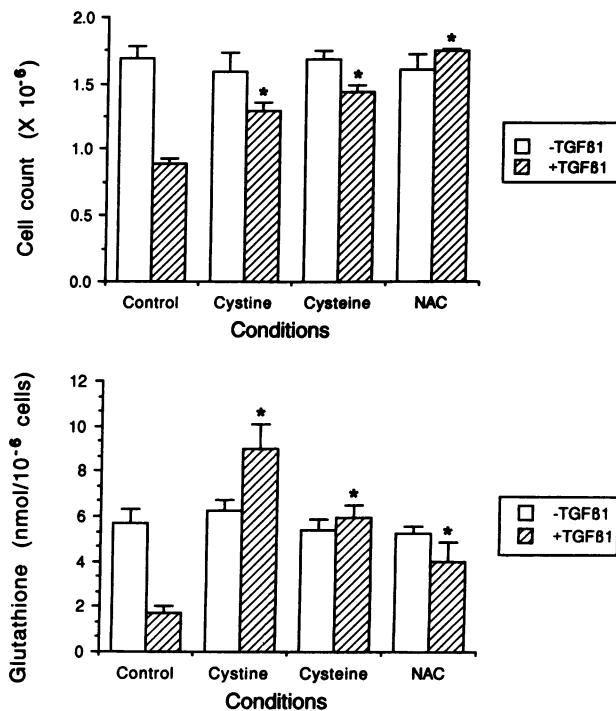
Figure 6. Attenuation of TGF- $\beta$ 1 effect on endothelial cell [ $^3$ H]TdR uptake by cysteine. Cystine and TGF- $\beta$ 1 were added at  $t = 0$  h and [ $^3$ H]TdR uptake was measured at 120–121 h. Cystine: (▲) none; (Δ) 0.2 mM; (●) 0.5 mM; and (○) 1.0 mM. Cultures done as noted in Methods.



**Figure 7.** Assessment of direct interaction of TGF- $\beta$ 1 with cysteine. TGF- $\beta$ 1 at a concentration of 10 ng/ml was preincubated with 0–2 mM cysteine for 2 h. After this preincubation, TGF- $\beta$ 1 was applied to the endothelial cells to give a TGF- $\beta$ 1 concentration of 1 ng/ml. The rate of [ $^3$ H]TdR uptake was measured for 1 h during a 48–49-h interval of incubation of cells with TGF- $\beta$ 1. [ $^3$ H]TdR uptake in control cultures was  $9,800 \pm 250$  cpm per 35-mm dish. Cysteine concentration: (▲) none; (Δ) 1 mM; (●) 2 mM.

amino acid, cystine, had a similar effect as cysteine and *N*-acetylcysteine, (b) preexposure of TGF- $\beta$ 1 to cysteine for up to 48 h failed to influence the subsequent growth inhibitory effect of TGF- $\beta$ 1 as measured by incorporation of [ $^3$ H]TdR by endothelial cells, and (c) cysteine added to cells even 24 h after TGF- $\beta$ 1 partially reversed its effect.

A possible explanation for our observations is that TGF- $\beta$ 1 produces both an early and a delayed effect on endothelial cells. The early effect develops within 24–48 h and is not attenuated by cysteine, *N*-acetylcysteine, or cystine. The delayed effect occurs after 72–96 h, is associated with marked depression of cellular proliferation, and is reversed by early addition of thiol amino acid. Furthermore, as previously pointed out only the delayed effect is associated with desquamation of the cells in the presence of added iron (14). While the early effect is obviously due to the exogenously supplied TGF- $\beta$ 1, the delayed effect appears to be due to autocrine production of TGF- $\beta$ 1 by the cells. A possibly limited influence of the thiol-amino acids on only the autocrine accumulation of TGF- $\beta$ 1 (a relatively late event) may account for the partial, as opposed to total,



**Figure 8.** Effect of cysteine, *N*-acetylcysteine, and cystine on TGF- $\beta$ 1-induced depletion of cellular glutathione and inhibition of proliferation of endothelial cells. Cells were seeded as described in Methods and exposed to TGF- $\beta$ 1 (1 ng/ml in the presence of 1 mM cystine, 1 mM cysteine, and 2 mM *N*-acetylcysteine in 4 ml of medium). After 96 h of incubation, cells were harvested and counted (top) and glutathione levels were measured (bottom). Values are means  $\pm$  SD. \* $P$  < 0.05, compared with control without thiol-amino acid.

reversal of the TGF- $\beta$ 1 effect observed in these experiments. In summary, our results are the first to demonstrate that thiol-amino acid availability and production by mammalian cells may significantly alter the cellular response to the cytokine TGF- $\beta$ 1. The thiol amino acids may thus modulate the effect of TGF- $\beta$ 1 on certain cell types by down-regulating autocrine production of TGF- $\beta$ 1.

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