Expression of Transforming Growth Factor β Ligand and Receptor Messenger RNAs in Lung Cancer Cell Lines

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Abstract
Specific cDNA probes for transforming growth factor βs (TGF-βs) 1, 2, and 3 and TGF-β types I, II, and III receptors were used to study expression of the mRNAs of the different TGF-β ligand and TGF-β receptor isoforms in cultured non-small cell lung cancer (NSCLC) cells and small cell lung cancer (SCLC) cells. Expression of TGF-β1 mRNA was detected in both cell types using Northern blot hybridization, with the level of expression of this mRNA being higher in several NSCLC cell lines. In addition, expression of TGF-β2 and TGF-β3 mRNAs was also detected in NSCLC and SCLC cells but at levels that were lower than that of TGF-β1 mRNA. Besides expression of a 3.4-kilobase (kb) TGF-β3 transcript, a smaller 2.8-kb TGF-β3 transcript was detected in some NSCLC and SCLC cells. TGF-β1 and TGF-β2 proteins were detected in the conditioned media of NSCLC and SCLC cells, with the levels being higher in several NSCLC cells than in SCLC cells. Expression of TGF-β types I and II receptor mRNAs was also detected in most NSCLC and SCLC cells, with expression of a 5.5-kb type I receptor mRNA being higher than that of a 5.5-kb type II receptor mRNA in both cell types. In contrast, a 6-kb TGF-β type III receptor mRNA was detected in only some NSCLC cells and could not be detected in the SCLC cells examined. Also, there was an inverse relationship between the level of expression of the 5.5-kb TGF-β type I receptor mRNA and that of the 6-kb TGF-β type III receptor mRNA. Addition of TGF-β1 and TGF-β2 proteins resulted in an increase in the mRNAs for TGF-βs 1 and 2 and an increase in the amount of TGF-β1 protein in some NSCLC cells, indicating that these cells are responsive to TGF-β and its effects. At the same time, a differential change in expression of the 2.8- and 3.4-kb TGF-β3 transcripts was detected in some lung cancer cells following the addition of TGF-β1 and TGF-β2. Also, addition of TGF-β1 to NSCLC cells inhibited colony formation of some of these cells in soft agarose in a dose-dependent manner. These results show that some lung cancer cells are responsive to the effects of TGF-β and suggest that TGF-β may have a role in controlling the proliferation of these cells.

Introduction
Several different growth factors have been identified in the lung including TGF-β. The TGF-βs are a group of structurally related polypeptides that exert multiple effects in various cell types (1–3). The TGF-βs, acting as multifunctional signaling molecules, are elements of intricate biological pathways that, in conjunction with other growth factor molecules, provide the basis for intercellular and autacellular communication in higher animals. The TGF-βs are synthesized as latent precursors that undergo proteolytic cleavage to produce biologically active dimeric polypeptides of M, 25,000 that can bind TGF-β receptors and initiate TGF-β-related functions. The cloning of human TGF-β1 has led to the identification of four other forms of TGF-β (TGF-βs 2, 3, 4, and 5) and the definition of a larger gene family comprising several other structurally related, but functionally distinct, proteins. Thus far, three species of TGF-β have been isolated in mammalian cells, TGF-βs 1, 2, and 3, which have similar binding characteristics and biological activities (4, 5). The original narrow definition of TGF-β, in terms of induction of a transformed phenotype in mesenchymal cells (6, 7), has now been extended by the knowledge that TGF-β affects many different types of cells. TGF-β has been found to exhibit both stimulatory and inhibitory effects on growth and development. The nature of the action of TGF-β on a particular target cell is dependent on several parameters, including the cell type, its state of differentiation, the growth conditions, and on the presence or absence of other polypeptide growth factors. For example: (a) TGF-β1 stimulates the proliferation of normal rat kidney fibroblasts in the presence of EGF in soft agarose but inhibits the proliferation and antagonizes the mitogenic action of EGF on these cells in monolayer culture; (b) TGF-β1 stimulates the proliferation of fibroblasts from very early human fetuses but inhibits the proliferation of fibroblasts derived from older fetuses; (c) TGF-β1 stimulates differentiation of some cells, including bronchial epithelial cells and prechondrocytes, while it inhibits differentiation of other cells including adipocytes and myoblasts; and (d) TGF-β1 can either stimulate or inhibit the proliferation of Fischer rat 3T3 cells transfected with a myc gene in soft agarose, depending on whether platelet-derived growth factor or EGF are also present (reviewed in Refs. 1 and 2).

Receptors for the TGF-βs have been shown in virtually all normal epithelial and mesenchymal cells examined, as well as in several malignant cells (4, 8, 9). At least nine different proteins which can bind TGF-β have been identified (10, 11). High affinity binding has been observed with three molecules, termed TGF-β types I, II, and III receptors, all of which have been cloned (12–15). The TGF-β types I and II

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3 The abbreviations used are: TGF-β, transforming growth factor β; EGF, epidermal growth factor; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; kb, kilobase(s); SELISA, sandwich enzyme-linked immunosorbent assays.
receptors (M₁, 60,000–70,000 and 85,000–110,000, respectively) have binding constants of 5–50 pm (16, 17), while that of the type III receptor (M₂, 200,000–400,000) is lower (30–300 pm) (18). TGF-β1 and TGF-β3 bind with slightly higher affinity than TGF-β2 does, but in general, all three receptors exhibit comparable activities using several bioassays (19).

Although TGF-β has been detected in several cancer cell types, few studies have been conducted in lung cancer including NSCLC and SCLC. Those that have been conducted have focused mainly on TGF-β1 in either NSCLC cells (20–22) or SCLC cells (23, 24). None of these studies has examined and compared the different TGF-β isoforms in NSCLC and SCLC cells. In addition, little is known about the TGF-β receptors in lung cancer cells.

In the present study, we have examined the expression of the mRNAs for three TGF-β ligands (TGF-β1, 2, and 3) and three TGF-β receptors (types I, II, and III) in NSCLC and SCLC cells. We demonstrate coexpression of the mRNAs for the TGF-β ligands and TGF-β receptors at different levels in these cells. We identify a 2.8-kb TGF-β3 transcript in some lung cancer cells that seems to be regulated differently from the 3.4-kb TGF-β3 transcript that is expressed in many types of cells. We also identify mRNAs for TGF-β types I, II, and III receptors in NSCLC and SCLC cells and show an inverse relationship between the levels of expression of a TGF-β type I receptor mRNA and a TGF-β type III receptor mRNA in NSCLC cells. In addition, we show inhibition of some NSCLC cell proliferation by TGF-β1. Our results suggest that some lung cancer cells are responsive to the effects of TGF-β and that TGF-β may be able to play a role in controlling the proliferation of these lung cancer cells.

Results
Expression of TGF-β mRNAs and Proteins in Lung Cancer Cell Lines. The expression of the mRNAs for TGF-β1, 2, and 3 in lung cancer was investigated using NSCLC cells and SCLC cells. To examine expression of the TGF-β mRNAs in NSCLC cells, 3 identical Northern blots of total RNA from 10 NSCLC cell lines were prepared (Fig. 1). Hybridization of one blot with a radiolabeled cDNA probe for TGF-β1 showed a single transcript of 2.5 kb in 5 of the 10 NSCLC cell lines (Fig. 1A), which is the expected size for a human TGF-β1 transcript (20). These include NCI-H157, a squamous cell carcinoma; NCI-H727, a carcinoma; NCI-H838 and NCI-H1264, two adenocarcinomas; and NCI-H1299, a large cell carcinoma. The highest levels of expression of TGF-β1 mRNA were detected in NCI-H727, NCI-H838, NCI-H1264, and NCI-H1299. Exposure of this blot for a longer time also showed a transcript of 2.5 kb in the five other NSCLC cell lines including NCI-H23 and NCI-H322, two adenocarcinomas; NCI-H226, a squamous cell carcinoma; NCI-H460, a large cell carcinoma; and NCI-H720, a carcinoma (data not shown). Hybridization of an identical blot with a radiolabeled cDNA probe for TGF-β2 showed four transcripts of 3.7, 4.0, 5.1, and 5.8 kb in 3 of the 10 NSCLC cell lines (Fig. 1B), which are the expected sizes for human TGF-β2 transcripts (25). These include NCI-H727, NCI-H838, and NCI-H1264, which are the cell lines that also showed high levels of expression of TGF-β1 mRNA. Only two transcripts for TGF-β2 mRNA of 5.1 and 5.8 kb were detected in NCI-H720. No hybridization was detected to TGF-β2 mRNA in the six other NSCLC cell lines, even after prolonged exposure. Hybridization of a third identical blot with a radiolabeled cDNA probe for TGF-β3 showed a transcript of 3.4 kb in three of the NSCLC cell lines (Fig. 1C), which is the expected size for a human TGF-β3 transcript (26). These include NCI-H727, NCI-H838, and NCI-H1264. In addition to the 3.4-kb TGF-β3 transcript, a smaller transcript of 2.8 kb for TGF-β3 was also detected in NCI-H727. No hybridization was detected to TGF-β3 mRNA in the six other NSCLC cell lines, even after prolonged exposure. As a control, the gels were stained with ethidium bromide and photographed to ensure that equal amounts of RNA had been applied to the gels (Fig. 1D).

Expression of the TGF-β mRNAs was also examined in SCLC cell lines including four classic SCLC cell types that exhibit histological features typical of SCLC cells such as small size, nuclear molding, necrotic areas, crush artifact, and deposition of basophilic granular DNA on elastic fibrils and one variant cell type displaying mixed small cell-large cell characteristics such as large size, large nuclei, prominent nucleoli, and distinct cytoplasmic borders (27). Hybridization of one blot with a radiolabeled cDNA probe for TGF-β1 showed expression of TGF-β1 mRNA in the classic SCLC cell lines NCI-H209, NCI-H510, and NCI-N592, with expression of TGF-β1 mRNA being about 8-fold higher in NCI-H510 than in the other two SCLC cell lines where expression was detected (Fig. 2A). No expression of TGF-β1 mRNA was detected in the classic SCLC cell line NCI-H345 or in the variant SCLC cell line NCI-N417 even after prolonged exposure (Fig. 2A). Hybridization of identical blots with radiolabeled cDNA probes for TGF-β2 and TGF-β3 showed expression of TGF-β2 mRNA in NCI-H345 and NCI-N592, with the 5.1-kb TGF-β2 mRNA in NCI-H592 being about 10-fold higher than that in NCI-H345, and of TGF-β3 mRNA in NCI-N417 and NCI-N592 (Fig. 2, B and C). In addition to the 3.4-kb TGF-β3 transcript, a second 2.8-kb TGF-β3 transcript was detected in NCI-N417 (Fig. 2C). No hybridization to TGF-β3 mRNA was detected in the other SCLC cell lines, even after prolonged exposure. As before, the gels were stained with ethidium bromide and photographed to ensure that equal amounts of RNA had been applied to the gels (Fig. 2D).

Table 1 shows a comparison of the relative steady-state levels of expression of TGF-β1, TGF-β2, and TGF-β3 mRNAs in NSCLC and SCLC cell lines obtained using densitometry of the autoradiographs in Figs. 1 and 2. In general, the level of expression of the mRNAs for TGF-β1, 2, and 3 is higher in several of the NSCLC cell lines than in the SCLC cell lines. In the NSCLC cell lines, TGF-β1 mRNA predominates in abundance over TGF-β2 mRNA and TGF-β3 mRNA. In contrast, there is no predominant TGF-β mRNA species in the SCLC cell lines.

Using SELISA specific for TGF-β1 and TGF-β2, the amounts of TGF-β1 and TGF-β2 proteins in the conditioned medium of several of the NSCLC and SCLC cell lines were determined (28). The amounts of TGF-β1 and TGF-β2 proteins were found to be higher in the NSCLC cells including NCI-H157, NCI-H727, NCI-H838, and NCI-H1299 compared to the SCLC cells including NCI-H209, NCI-H345, NCI-N417, NCI-H510, and NCI-N592 (Table 2). The amounts of TGF-β1 and TGF-β2 ranged between 20–480 pm for TGF-β1 and 12–40 pm for TGF-β2 in these NSCLC cells. The amounts of TGF-β1 protein in the NSCLC cells NCI-H23, NCI-H460, and NCI-H720 were lower than in the other NSCLC cells, reflecting the lower level of expression of TGF-β1 mRNA in these NSCLC cells (Fig. 1).
Expression of TGF-β mRNAs in NSCLC cell lines. Total RNA (10 μg) isolated from exponentially growing subconfluent NSCLC cell lines [Lane 1, NCI-H23; Lane 2, NCI-H157; Lane 3, NCI-H226; Lane 4, NCI-H322; Lane 5, NCI-H460; Lane 6, NCI-H720; Lane 7, NCI-H727; Lane 8, NCI-H818; Lane 9, NCI-H1264; and Lane 10, NCI-H1299] was electrophoresed on a 1% agarose-formaldehyde gel and transferred to a Nytran filter as described in “Materials and Methods.” Hybridization was performed with 32P-labeled random-primed cDNA probes (A) TGF-β1, (B) TGF-β2, and (C) TGF-β3 as described in “Materials and Methods.” The blots were exposed for 3 days. D, the ethidium bromide staining pattern of one of the gels showing 18S and 28S rRNA.

Expression of TGF-β Receptor mRNAs in Lung Cancer Cell Lines. In addition to investigating the expression of the TGF-β isoforms in NSCLC and SCLC cells, we also examined the expression of TGF-β types I, II, and III receptors in these cells. To examine expression of the TGF-β types I, II, and III receptor mRNAs, the blots that were used to investigate expression of the TGF-β1, 2, and 3 mRNAs were dehybridized, exposed to film to ensure complete dehybridization, and then hybridized with cDNA probes for each of the TGF-β receptors. Hybridization of one blot containing RNA from NSCLC cells with a radiolabeled cDNA probe for a TGF-β type I receptor (plasmid ALK-5) showed a major transcript of 5.5 kb in 8 of the 10 cell lines (Fig. 3A), which...
is the expected size for this human TGF-β type I receptor mRNA (14). These include NCI-H23, NCI-H157, NCI-H226, NCI-H322, NCI-H460, NCI-H720, NCI-H1264, and NCI-H1299. The highest levels of expression of this TGF-β type I receptor mRNA were in NCI-H157, NCI-H226, NCI-H322, NCI-H460, and NCI-H720. In addition, minor transcripts of 2.8 and 4.2 kb were also detected in NCI-H226, NCI-H322, NCI-H460, and NCI-H1299. A low level of expression of this TGF-β type I receptor mRNA was also detected in NCI-H727 and NCI-H838 after prolonged exposure (data not shown). Hybridization of an identical blot with a radiolabeled cDNA probe for a second TGF-β type I receptor (plasmid tsk 7L) showed a single transcript of 3.5 kb in all of the NSCLC cells examined (Fig. 3B), which is the expected size for this TGF-β type I receptor transcript (14, 15). The highest levels of expression of this TGF-β type I receptor mRNA were in NCI-H23, NCI-H460, NCI-H1264, and NCI-H1299. Hybridization of an identical blot with a radiolabeled cDNA probe for TGF-β type II receptor showed a single transcript of 5.5 kb in all of the NSCLC cell lines examined (Fig. 3C), which is the expected size for a human TGF-β type II receptor transcript (13). The level of expression of the TGF-β type II receptor mRNA was higher in NCI-H157, NCI-H460, NCI-H727, NCI-H838, and

**Table 1** Summary of the relative expression of TGF-β mRNAs in lung cancer cell lines

<table>
<thead>
<tr>
<th>NCI cell line</th>
<th>mRNAs (Relative densitometric values)</th>
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<tbody>
<tr>
<td></td>
<td>TGF-β1</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
</tr>
<tr>
<td>H23</td>
<td>1+</td>
</tr>
<tr>
<td>H157</td>
<td>5+</td>
</tr>
<tr>
<td>H226</td>
<td>1+</td>
</tr>
<tr>
<td>H322</td>
<td>1+</td>
</tr>
<tr>
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<td>1+</td>
</tr>
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<td>1+</td>
</tr>
<tr>
<td>H727</td>
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</tr>
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<td>25+</td>
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<td>30+</td>
</tr>
<tr>
<td>H1299</td>
<td>12+</td>
</tr>
<tr>
<td>SCLC</td>
<td></td>
</tr>
<tr>
<td>H209</td>
<td>1+</td>
</tr>
<tr>
<td>H345</td>
<td>ND</td>
</tr>
<tr>
<td>N417</td>
<td>ND</td>
</tr>
<tr>
<td>H510</td>
<td>8+</td>
</tr>
<tr>
<td>H592</td>
<td>1+</td>
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**Table 2** Expression of TGF-β1 and TGF-β2 proteins in NSCLC and SCLC cell lines

<table>
<thead>
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<th>NCI cell line</th>
<th>TGF-β1 (pm)</th>
<th>TGF-β2 (pm)</th>
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<tbody>
<tr>
<td>H23</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>H157</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>H460</td>
<td>20</td>
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</tr>
<tr>
<td>H720</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>H727</td>
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<td>16</td>
</tr>
<tr>
<td>H838</td>
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<td>12</td>
</tr>
<tr>
<td>H1299</td>
<td>480</td>
<td>ND</td>
</tr>
<tr>
<td>H209</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>H345</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>N417</td>
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<td>ND</td>
</tr>
<tr>
<td>H510</td>
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<td>ND</td>
</tr>
<tr>
<td>H592</td>
<td>50</td>
<td>ND</td>
</tr>
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in NCI-H27. No hybridization with TGF-β type III receptor mRNA was detected in the other NSCLC cell lines, even after prolonged exposure.

Expression of the TGF-β receptor mRNAs was also examined in SCLC cell lines. Hybridization of one blot containing RNA from SCLC cell lines with a radiolabeled cDNA probe for a TGF-β type I receptor (plasmid ALK-5) showed a transcript of 5.5 kb in all of the SCLC cell lines examined including NCI-H209, NCI-H345, NCI-N417, NCI-H510, and NCI-N592 (Fig. 4A). The level of this transcript was similar in each of the SCLC cell lines. Hybridization of an identical blot with a radiolabeled cDNA probe for a second TGF-β type I receptor (plasmid tsk 7L) showed a transcript of 3.5 kb in four of the five SCLC cells examined including NCI-H209, NCI-H345, NCI-N417, and NCI-H510 (Fig. 4B). The level of this transcript was highest in NCI-H345. No expression of this TGF-β type I receptor mRNA was detected in NCI-N592, even after prolonged exposure. Hybridization of an identical blot with a radiolabeled cDNA probe for TGF-β type II receptor showed hybridization to a transcript of 5.5 kb in all of the SCLC cell lines examined including NCI-H209, NCI-H345, NCI-N417, NCI-H510, and NCI-N592 (Fig. 4C). The level of expression of TGF-β type II receptor mRNA was highest in NCI-H345 and NCI-N592. Hybridization to a fourth identical blot with a radiolabeled cDNA probe for TGF-β type III receptor showed no hybridization to any of the SCLC cell lines, even after prolonged exposure (Fig. 4D).

**Regulation of TGF-β mRNAs by TGF-β1 in Lung Cancer Cells.** To determine whether lung cancer cells can be regulated by TGF-β, we have also extended our studies to examine the levels of steady-state expression of the different TGF-β mRNAs in NSCLC and SCLC cells treated with TGF-β1. Exponentially growing cultures of NSCLC and SCLC cells were cultured with TGF-β1 for 24 h in Si medium containing 0.1% BSA. The amount of TGF-β added was based on previous studies (29, 30). The treatment time was based on preliminary experiments which showed similar changes between 16 and 48 h of treatment with these growth factors (data not shown). The NCLC cell lines NCI-H157, NCI-H327, NCI-H345, and NCI-H510 and the SCLC cell lines NCI-H209, NCI-H345, and NCI-H510 were selected to be initially treated with TGF-β1 because of the detectable basal levels of expression of TGF-β1 in these cells (Figs. 1A and 2A). Use of NCI-H23, NCI-H226, NCI-H322, NCI-H460, NCI-H720, NCI-H345, and NCI-N417 would require the purification of poly(A)+ RNAs from these cells in order to detect TGF-β1 mRNA in a timely manner. The four NSCLC cell lines selected are also examples of different kinds of NSCLC, with NCI-H157 being a squamous cell carcinoma, NCI-H327 being a carcinoid, NCI-H345 being an adenocarcinoma, and NCI-H510 being a large cell carcinoma. Total RNA was extracted from these cells, and Northern blot analysis was performed with radiolabeled random primed TGF-β ligand and TGF-β receptor cDNA probes as before. Within 24 h of the addition of TGF-β1, there were 3- to 5-fold increases in the level of expression of TGF-β1 mRNA in NCI-H157, NCI-H327, and NCI-H1299 (Fig. 5A); no change in expression of TGF-β1 mRNA was detected in NCI-H838 following the addition of TGF-β1. Expression of TGF-β2 mRNA increased in NCI-H327 following the addition of 10 ng/ml TGF-β1 to these cells (Fig. 5B), while no change in expression of this mRNA was detected in NCI-H838 or NCI-H1299. Expression of TGF-β2 mRNA was below the level of detection in NCI-
H157 before and after the addition of TGF-β1. In contrast to the increases that were detected in TGF-β1 mRNA in the NSCLC cells following addition of TGF-β1, there was an 8-fold decrease in expression of the 3.4-kb TGF-β3 mRNA in NCI-H727 (Fig. 5C); no change was detected in the level of the 3.4-kb TGF-β3 mRNA in NCI-H838 or NCI-1299. Expression of the 3.4-kb TGF-β3 mRNA was below the level of detection in NCI-H157. In contrast, expression of the 2.8-kb TGF-β3 mRNA increased approximately 5-fold in NCI-H157 and 8-fold in NCI-H727 and NCI-H1299 following the addition of TGF-β1. No expression of the 2.8-kb TGF-β3 mRNA was detected in NCI-H838 before or after addition of TGF-β1 to these cells. The cell lines NCI-H23, NCI-H226, NCI-H322, NCI-H460, and NCI-H720 were also treated with TGF-β1 and examined for expression of TGF-β1, 2, and 3 mRNAs; expression of the three TGF-β mRNAs was below the level of detection both before and after treatment with TGF-β1 in these cell lines (data not shown). Treatment of SCLC cell lines including NCI-H209, NCI-H510, and NCI-N592 with TGF-β1 resulted in a 1.5- to 3-fold decrease in the expression of the TGF-β3 mRNA only in NCI-H510 (Fig. 5G), while no change was detected in the mRNAs for TGF-β1 and TGF-β2 in any of these cells (Fig. 5, E and F). Other SCLC cell lines including NCI-H345 and NCI-N417 were also treated with TGF-β1; none of these additional SCLC cell lines showed any change in the levels of expression of TGF-β1, 2, or 3 mRNAs (data not shown). When these blots were probed with TGF-β types I, II, and III receptor cDNA probes, no change in expression of the corresponding TGF-β receptor mRNAs in the NSCLC and SCLC cells was seen after treatment with exogenous TGF-β1 (data not shown).

**Regulation of TGF-β mRNAs by TGF-β2 in Lung Cancer Cells.** In addition to examining the levels of expression of the different TGF-β mRNAs in NSCLC cells with TGF-β1, we also examined expression of the TGF-β mRNAs in NSCLC cells treated with TGF-β2. As with TGF-β1, exponentially growing cultures of NSCLC cells were cultured with TGF-β2 for 24 h in S1T medium containing 0.1% BSA. Total RNA was extracted from NCI-H157, NCI-H727, NCI-H838, and NCI-H1299 cells, and Northern blot analysis was performed with radiolabeled random-primed TGF-β ligand and TGF-β receptor cDNA probes as before. Within 24 h of the addition of TGF-β2, there was an increase in expression of TGF-β1 mRNA in NCI-H157, NCI-H838, and NCI-H1299 (Fig. 6A), with the increases in TGF-β1 mRNA being approximately 5-fold and 3-fold in NCI-H157 and NCI-H1299, respectively, and approximately 2.5-fold in NCI-H838. No change in expression of TGF-β1 mRNA was detected in NCI-H727 following the addition of TGF-β2. Following the addition of TGF-β2, expression of TGF-β2 mRNA increased approximately 3- and 2-fold in NCI-H727 and NCI-H838, respectively (Fig. 6B). Expression of TGF-β2 mRNA could not be detected in NCI-H157 and NCI-H1299. In contrast to the increase in expression of the mRNAs for TGF-β1 and TGF-β2, following the addition of TGF-β2, expression of TGF-β3 mRNA decreased approximately 2-fold in NCI-H727 (Fig. 6C); no change was detected in TGF-β3 mRNA in NCI-H838 and NCI-1299. TGF-β3 mRNA could not be detected in NCI-H157. Expression of TGF-β3 mRNA was also examined in the SCLC cell lines NCI-H209, NCI-H510, and NCI-N592 following the addition of TGF-β2. No change was detected in the mRNAs for TGF-β1, 2, and 3 in these cells (data not shown). When these blots were probed with TGF-β types I, II, and III

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**Fig. 4.** Expression of TGF-β receptor mRNAs in SCLC cell lines. Total RNA (10 μg) isolated from SCLC cell lines (Lane 1, NCI-H209; Lane 2, NCI-H345; Lane 3, NCI-N417; Lane 4, NCI-H510; Lane 5, NCI-N592) was analyzed as before with (A) TGF-β type I receptor (plasmid ALK-5), (B) TGF-β type I receptor (plasmid tsk 7L), (C) TGF-β type II receptor, and (D) TGF-β type III receptor cDNA probes. The blots were exposed for (A) 2 days, (B) 10 days, (C) 10 days, and (D) 10 days. Ethidium bromide staining pattern of one of the gels showing 18S and 28S rRNA.
receptor cDNA probes, no change in expression of the corresponding TGF-β receptor mRNAs was seen after treatment with exogenous TGF-β2 (data not shown).

**Regulation of TGF-β1 Protein by TGF-β1 and TGF-β2 in NSCLC Cells.** Expression of TGF-β1 protein was also examined in NSCLC cell lines following treatment with TGF-β1 and TGF-β2 using a SELISA specific for TGF-β1 (28). After treatment with TGF-β1, an increase in the level of TGF-β1 protein was observed in the conditioned medium of NCI-H157, NCI-H727, and NCI-H1299, while no change was detected in the level of this protein in NCI-H838 (Fig. 7A). A SELISA assay specific for TGF-β2 was also performed on the conditioned medium following the addition of TGF-β1 to these cells. No change was detected in the amount of TGF-β2 protein secreted into the medium of these NSCLC cells (data not shown). After treatment of these NSCLC cells with TGF-β2, an increase in the level of TGF-β1 protein was observed in the conditioned medium of NCI-H157, NCI-H838, and NCI-H1299, while no change was detected in the level of TGF-β1 protein in NCI-H727 (Fig. 7B). No change was detected in the amount of TGF-β2 protein secreted into the conditioned medium of these NSCLC cells following the addition of TGF-β2 (data not shown). SCLC cell lines were not examined because the levels of TGF-β1 and TGF-β2 proteins were very low in untreated cells (Table 2), and an examination of expression of their TGF-β1 and TGF-β2 mRNAs following treatment with TGF-β1 did not show an increase in these mRNAs (Fig. 5, E and F).

**Effect of TGF-β1 on Proliferation of NSCLC Cells in Soft Agarose.** The effect of TGF-β1 on NSCLC cell proliferation in soft agarose was determined. Table 3 shows that when colonies >120 μm in diameter were counted, colony number was reduced by the addition of 0.1, 1, and 10 ng/ml TGF-β1 to NCI-H157, NCI-H727, and NCI-H1299 cells in a dose-dependent manner. Colony formation was not affected by 1 or 10 pg/ml TGF-β1 in these cell lines. Colony number was also reduced in NCI-H345 but only after the addition of 10 ng/ml TGF-β1. No change in colony number was detected in NCI-H838 after the addition of TGF-β1 at the concentrations tested.

**Discussion**

Previous studies have used immunohistochemistry and in situ hybridization to localize and demonstrate the temporal
Fig. 6. Effect of addition of TGF-β2 on TGF-β mRNA expression in NSCLC cell lines. Total RNA (10 μg) isolated as before from NSCLC cell lines NCI-H157 (Lanes 1–3), NCI-H727 (Lanes 4–6), NCI-H838 (Lanes 7–9), and NCI-H1299 (Lanes 10–12) was cultured for 24 h with: no addition (Lanes 1, 4, 7, and 10); 1 ng/ml TGF-β2 (Lanes 2, 5, 8, and 11); or 10 ng/ml TGF-β2 (Lanes 3, 6, 9, and 12). Total RNA was then analyzed as before with TGF-β1 (A), TGF-β2 (B), and TGF-β3 (C) cDNA probes. The blots were exposed for 1 day (A), 5 days (B), or 8 days (C). D, ethidium bromide staining pattern showing 18S and 28S rRNA.

and spatial distribution of the TGF-βs in embryonic and adult lungs (31–34). The demonstration of TGF-β mRNAs and proteins in the lung lends strong support to the hypothesis that the TGF-βs are able to regulate important processes relevant to normal lung physiology. However, little is known about the TGF-β proteins and their corresponding mRNAs in lung cancer. In this study, we show expression of the mRNAs for TGF-βs 1, 2, and 3 and TGF-β types I, II, and III receptors in cultured human lung cancer cell lines. While the number of cell lines that were examined in our study is relatively small, those cell lines that were selected for study are representative of different types of NSCLC and SCLC and serve as subjects for an initial investigation. We show that the levels of expression of the mRNAs for TGF-β1, 2, and 3 and TGF-β1 and TGF-β2 proteins are higher in many of the NSCLC cells than in the SCLC cells that were examined. As in several previously examined normal and transformed mammalian cells, the mRNAs for TGF-β1 and TGF-β2 showed the expected sizes in NSCLC and SCLC cells. However, in addition to the 3.4-kb TGF-β3 mRNA (26), a second TGF-β3 transcript of 2.8 kb was detected in some NSCLC and SCLC cells. This 2.8-kb TGF-β3 mRNA transcript has also been recently reported in two other SCLC cell lines (23). To our knowledge, this 2.8-kb TGF-β3 mRNA has not been reported in any other cell lines. We have shown that the 2.8-kb TGF-β3 mRNA is affected by treatment with TGF-β1, and changes in expression of this transcript occur either in parallel to or distinct from those that occur with the
specific to lung cancer cell types. Normal lung and tumor lung biopsy specimens will have to be analyzed for expression of the 2.8-kb TGF-β3 mRNA to determine its extent and localization in tissue samples. Further studies will be needed to learn more about the mechanism of regulation of this mRNA in lung cancer.

In this study, we also show expression of the mRNAs for TGF-β types I, II, and III receptors in both NSCLC and SCLC cells. While there seems to be no correlation between the level of expression of the mRNAs for the TGF-β ligands and the TGF-β receptors in either the NSCLC or SCLC cells, specific TGF-β receptor mRNAs predominate in these lung cancer cell types. For example, the level of TGF-β types I and II receptor mRNAs predominates over that of TGF-β type III receptor mRNA in most NSCLC cells examined, and the level of TGF-β type I receptor mRNA predominates over that of TGF-β types II and III receptor mRNAs in most SCLC cells examined. There appears to be an inverse relationship between the level of expression of the 5.5-kb TGF-β type I receptor mRNA and that of the 6-kb TGF-β type III receptor mRNA in the NSCLC cells examined. While the function of the TGF-β type III receptor is not completely understood, it has been suggested that the TGF-β type III receptor, rather than participating directly in signal transduction, might act to control the access of TGF-β to the TGF-β types I and II signaling receptors (11, 13, 35, 36). Also, while TGF-β type III receptor has been detected in mesenchymal, epithelial, neuronal, and other cell types (11), it has been shown that this receptor is not expressed in certain types of myoblasts, endothelial, epithelial, and hematopoietic cells that nevertheless respond to TGF-β (18, 37, 38). Preceding studies performed using mink lung epithelial cells have implicated the TGF-β types I and II receptors as being directly involved in signal transduction and to act as mediators of responsiveness to TGF-β (11, 13, 14, 39, 40). Several studies have also shown that the loss of expression of TGF-β types I or type II receptors corresponds to loss of responsiveness to TGF-β (17, 41, 42). Our study has shown that while some NSCLC cells, like NCI-H727 and NCI-H838 (that do not show expression of the 5.5-kb TGF-β type I receptor mRNA), are still able to respond to TGF-β; in this case, expression of the mRNAs for TGF-β types I, II, and III is affected by treatment of NCI-H727 and NCI-H838 with exogenous TGF-β. In these two cell lines, while the 5.5-kb TGF-β type I receptor mRNA that was readily detectable, in several of the other NSCLC cell lines, it could not be detected until after prolonged exposure; another TGF-β type I receptor mRNA (3.5 kb) could be detected in these two cell lines using another TGF-β type I receptor cDNA probe. In addition, this other TGF-β type I receptor cDNA probe was also able to detect a 3.5-kb TGF-β type I receptor mRNA in several other NSCLC and SCLC cells as well. Thus, all of the lung cancer cell lines examined showed expression of TGF-β types I and II receptor mRNAs. It may be that the TGF-β types I and II receptors in some lung cancer cells are able to bind and respond to TGF-β because of the relatively high level of expression of the TGF-β type III receptor mRNA. It has been shown that the ability of the TGF-β type II receptor in L6 myoblasts to bind TGF-β can be increased by forcing expression of the otherwise missing TGF-β type III receptor (12). Whereas NCI-H838 also shows expression of the TGF-β type III receptor mRNA, its level of expression is 12-fold lower than that of NCI-H727, and this lower level of expression may not allow NCI-H838 to respond to TGF-β in the same manner as NCI-H727. Also, expression of a

**Fig. 7.** Production of TGF-β1 protein upon treatment with (A) TGF-β1 and (B) TGF-β2 in lung cancer cell lines. NSCLC and SCLC cell lines were cultured for 24 h with 1 ng/ml TGF-β1 (□) or with 10 ng/ml TGF-β1 (■) or without TGF-β1 (△) as described in “Materials and Methods.” Acid-activated conditioned media were assayed in specific sandwich ELISA assays using neutralizing antibodies against TGF-β1 and TGF-β2. Mean (bars, SE) of four analyses is indicated.
3.5-kb TGF-β type III receptor transcript could be detected in NCI-H727 but not in NCI-H838. It may be that this transcript may encode an additional TGF-β receptor protein that may participate in the signal transduction mechanism. Further studies are needed to examine the TGF-β receptor proteins and determine how they are modulated in these lung cancer cell lines.

In addition to examining expression of the mRNAs for the TGF-β ligands and receptors in lung cancer cells, we have also examined the ability of TGF-β1 to inhibit the proliferation of these cells. We have shown that TGF-β1 inhibits the growth of some NSCLC cells in soft agarose. TGF-β1 has been shown to be one of the most potent polypeptide growth inhibitors known for a wide variety of cell types including selected cell types of mesenchymal and myeloid origin, as well as nearly all epithelial, lymphoid, and endothelial cells (43–45). TGF-β1, TGF-β2, and TGF-β3 are equipotent in the degree to which they inhibit cell proliferation (19). Inasmuch as several of the NSCLC cell lines have specific mRNAs for the TGF-β receptors and synthesize and secrete TGF-β into the cell culture medium, all of the required elements exist for an inhibitory autocrine pathway of growth control in these lung cancer cells. Blocking antibody studies using specific antibodies to TGF-β1, TGF-β2, and TGF-β3 could help to establish the importance of TGF-β in a potential inhibitory autocrine pathway in these cells. Future studies will have to be performed to examine whether the TGF-β proteins that are secreted into the conditioned medium of the NSCLC and SCLC cells are latent or active. If the secreted TGF-β proves to be latent, as preliminary data suggest, these tumor cells may be unable to activate the latent TGF-β and thus may be refractory to the growth inhibitory effects of endogenous TGF-β. This may be related to their aberrant malignant growth behavior. The amount of exogenous TGF-β1 that was effective in inhibiting colony proliferation in soft agarose was higher than the amount of TGF-β1 measured in the conditioned media. Future studies will have to be performed using specific TGF-β antibodies to determine whether the endogenous TGF-β in lung tumor specimens may be higher in some localized cells so that a potential autocrine pathway could function in specific cells. It also remains to be determined in further studies whether expression of one or more of the TGF-β isoforms can be increased in the amount and/or activity by chemical and biological agents in these lung cancer cells so that proliferation of these malignant cells can be controlled.

In addition to its role as an inhibitor of the proliferation of epithelial cells, TGF-β, being a multifunctional growth factor, can participate in other activities that are important to the study of lung cancer. For example, TGF-β serves to increase the accumulation of extracellular matrix by activating gene transcription to increase synthesis and secretion of matrix proteins and their receptors and decrease synthesis of proteolytic enzymes that degrade matrix proteins and increase synthesis of protease inhibitors that block the activity of these enzymes. TGF-β has also been shown to mediate cell invasiveness, immune cell function vascularization, and angiogenesis.

All of these processes are important in tumor formation. Thus, in addition to understanding the role of TGF-β in controlling the proliferation of lung cancer cells, there are potential therapeutic reasons for understanding expression of the TGF-βs and their receptors in lung cancer. It is possible that TGF-β might be able to affect tumor formation by acting on surrounding stromal elements, recruiting inflammatory cells, and participating in neovascularization and elaboration of connective tissue. Further studies will be needed to examine the role of TGF-β in the proliferation of lung cancer.

### Materials and Methods

#### Cell Culture.
Human lung cancer cell lines (NCI-H157, -H209, -H226, -H322, -H345, -N417, -H460, -H510, -N592, -H720, -H727, -H838, -H1264, and -H1299) were cultured in serum-supplemented medium at 37°C (RPMI 1640; Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Gibco; Refs. 46 and 47). The NSCLC cell lines were adherent and were split weekly 1:20 by using trypsin/EDTA (Gibco). The SCLC cells were cultured as floating aggregates and were split weekly by dilution 1:1 in fresh medium. Routinely, the cells showed greater than 90% viability, were Mycoplasma free, and were used when they were in exponential growth phase.

For treatment of cells with TGF-β1, cells were washed with PBS and incubated with SIT medium (RPMI 1640 containing 5 μg/ml bovine insulin, 10 μg/ml human transferrin, and 3 × 10⁻⁶ M sodium selenite) and 0.1% BSA. Conditioned medium was collected, and cells were harvested for RNA extraction. Cell debris was removed by centrifugation, protease inhibitors were added, and the medium was stored at −70°C until assayed.

Human TGF-β1 and porcine TGF-β2 were obtained from R & D Systems (Minneapolis, MN). Bovine insulin, human transferrin, and sodium selenite were purchased from Sigma Chemical Co. (St. Louis, MO).

#### RNA Extraction and Northern Blot Analysis.
Total RNA was extracted from lung cancer cell lines by the method of Chirgwin et al. (48). For Northern blot analysis, equal amounts of RNA (10 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde, transferred to "Nytran" filters (Sleicher and Schuell, Keene, NH), UV cross-linked, and baked for 3 h. Ethidium bromide (33
μg/ml was included in both the gels and running buffers to visualize the positions of rRNAs by UV illumination after electrophoresis. Blots were hybridized with 32P-labeled (3000 Ci/mmol; Amersham, Boston, MA) random-primed probes at 65°C according to Church and Gilbert (49), and then exposed for various times at −70°C using an intensifying screen. Densitometry of autoradiograms was performed using an LKB Ultrascan laser densitometer.

**cDNA Probes.** Hybridization was performed using the following cDNA probes: 0.9-kb Xba-HindIII fragment of rat TGF-β1, plasmid pRTGFβ1 (50); 1.0-kb Xba-HindIII fragment of mouse TGF-β2, plasmid pMTGFβ2 (51); 0.75-kb Xba-HindIII fragment of mouse TGF-β3, plasmid pMTGFβ3 (52); 1.0- plus 1.2-kb EcoRI fragments of human TGF-β type I receptor, plasmid ALK-5 (14); 1.7-kb EcoRI fragment of mouse TGF-β type I receptor, plasmid tsk 7L (15); 1.7-kb HindIII-BamHI fragment of human TGF-β type II receptor, plasmid H2-3FF (13); and a 5-kb HindIII fragment of rat TGF-β type III receptor, plasmid R3-OFF (12).

**Quatiation of TGF-β 1 and 2.** TGF-βs 1 and 2 in conditioned medium were assayed by SELISAs using both chicken and rabbit neutralizing polyclonal antibodies against native TGF-βs (R & D Systems) and mouse monoclonal antibodies (Genzyme, Cambridge, MA) as described by Danelipour et al. (28). These antibodies have been shown to react strongly with their native TGF-β antigen and only weakly with the other TGF-β isoforms by the manufacturer. Briefly, conditioned medium used in the SELISAs was precipitated with trichloracetic acid, centrifuged, and resuspended in cold ethanol (1:1, v/v). Following centrifugation, the pellet was lyophilized to dryness and dissolved in buffer (4 mM HCl, 150 mM sodium chloride, and 0.5 mg/ml BSA) overnight at 4°C. Following centrifugation, the supernatants were added to the SELISAs at eight 2-fold dilutions and quantitated based on a standard curve using human TGF-β1 and porcine TGF-β2 (R & D Systems).

**Proliferation in Soft Agarose.** Proliferation studies were performed using the agarose cloning system described by Mahmoud et al. (53). Briefly, the base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in 6-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, growth factor, and 2 x 10^5 single viable cells. For each cell line and growth factor concentration, triplicate wells were plated. After 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet was added, and after 16 h at 37°C, the plates were screened for colony formation. The number of colonies larger than 120 μm in diameter was counted using an Omnicon image analysis system.

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**References**


