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Downmodulation of TGF-α Protein Expression With Antisense Oligonucleotides Inhibits Proliferation of Head and Neck Squamous Carcinoma But Not Normal Mucosal Epithelial Cells

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Abstract Interruption of an autocrine growth pathway involving TGF-α and EGFR may inhibit tumor growth and improve survival in head and neck cancer patients. We previously demonstrated that biopsy specimens and established cell lines from patients with squamous cell carcinoma of the head and neck (SCCHN) overexpress TGF-α and its receptor, epidermal growth factor receptor (EGFR) at both the mRNA and protein levels. Protein localization studies showed that TGF-α and EGFR are produced by the same epithelial cells in tissues from head and neck cancer patients further supporting a role for this ligand-receptor pair in an autocrine growth pathway. To confirm that TGF-α contributes to autocrine growth, we examined the effect of down regulation of TGF-α protein on SCCHN cell proliferation. Treatment of 6 SCCHN cell lines with antisense oligodeoxynucleotides targeting the translation start site of human TGF-α mRNA decreased TGF-α protein production by up to 93% and reduced cell proliferation by a mean of 76.2% compared to a 9.7% reduction with sense oligonucleotide (range $P = 0.036$–0.0001). TGF-α antisense oligonucleotide exposure also decreased TGF-α protein levels in normal oropharyngeal mucosal epithelial cells, however their growth rate was not affected. These findings indicate that TGF-α is participating in an autocrine signaling pathway in transformed, but not in normal mucosal epithelial cells, that promotes proliferation. J. Cell. Biochem. 69:55–62, 1998.

Key words: TGF-α; antisense oligonucleotides; head and neck cancer

The prevalence of SCCHN is increasing worldwide [Boyle et al., 1993; Mashberg et al., 1993]. While tobacco and alcohol use are recognized as the most important risk factors for head and neck cancer, the majority of individuals who smoke and drink do not develop SCCHN. The tendency of the upper aerodigestive tract epithelium to undergo transformation in certain patients when exposed to these agents probably reflects genetic alterations in the regional mucosa. This possibility is reinforced by the proclivity of these patients to develop multiple primary tumors sequentially during their lifetime [DeVries and Snow, 1988; Jones et al., 1995]. The "field cancerization" effect of carcinogens such as tobacco and alcohol on the entire squamous epithelial surface is generally invoked to explain this susceptibility to numerous malignancies [Slaughter et al., 1953]. The ability to modulate such genetic changes in the mucosa of the upperaerodigestive tract would provide an

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opportunity to develop novel approaches to preventing and treating these tumors.

Genetic alterations reported in SCCHN tumors and cell lines include amplification of oncogenes such as myc, ras, int-2, and bcl-1 [Yokota et al., 1986; Saranath et al., 1989; Berenson et al., 1989; Somers et al., 1990], mutation of p53 [Boyle et al., 1993], cytogenetic alterations (e.g., involving 3p, 11q, 15p, 17p) [Williams et al., 1993; Maestro et al., 1993; Nawroz et al., 1994], modification of cell cycle proteins (e.g., PRAD1/Cyclin D1) [Shuuring et al., 1992; Callender et al., 1994], as well as up-regulation of growth factors and their receptors [Todd et al., 1989; Rubin Grandis and Tweardy, 1993]. The expression of a number of growth factors has been detected in SCCHN including IGF-II [Thomas et al., 1993], PDGF-A [Thomas et al., 1993], bFGF [Thomas et al., 1993; Schultz-Hector and Haghayegh, 1993], and TGF-α [Todd et al., 1989, 1991; Rubin Grandis and Tweardy, 1993]. However, TGF-α and its receptor, EGFR are the only ligand and receptor pair found to be overexpressed in biopsy specimens and established cell lines from SCCHN patients compared with control normal mucosa from noncancer patients supporting the action of an autocrine regulatory pathway [Rubin Grandis and Tweardy, 1993]. Moreover, the increase in TGF-α and EGFR mRNA appears to be primarily a result of activated gene transcription [Rubin Grandis et al., 1996a]. Further studies in our laboratory show increased TGF-α and EGFR protein expression by these cells and demonstrated that both ligand and receptor are produced by the same epithelial cell populations in biopsy tissues from patients with SCCHN i.e., in both tumor cells and adjacent histologically normal mucosa [Rubin Grandis et al., 1996b]. The present investigation was designed to test the hypothesis that downmodulation of TGF-α would interrupt the autocrine pathway thereby decreasing the proliferation of SCCHN cells. We found that SCCHN cells treated with antisense oligonucleotides targeting TGF-α produced less TGF-α protein, and were growth-inhibited. The same TGF-α antisense oligonucleotide reduced TGF-α protein expression but had no effect on the proliferation of normal mucosal epithelial cells harvested from a series of control patients without cancer, suggesting that the growth-inhibitory effect seen in SCCHN cells is specific for transformed cells.

MATERIALS AND METHODS

Cells

Ten cell lines derived from patients with SCCHN were grown in Dulbecco's Modification of Eagle's Medium (DMEM; Cellgro, Washington, DC) supplemented with 15% fetal bovine serum (GIBCO Laboratories, Grand Island, NY), 100 units/ml of penicillin and 100 units/ml of streptomycin (GIBCO) [Sacchi et al., 1990]. The SCCHN cell lines are part of a large collection established in the Department of Otolaryngology at the University of Pittsburgh [Heo et al., 1989]. Two human leukemic cell lines (HL-60 and NB-4) were maintained in RPMI media (Cellgro) with 10% fetal bovine serum as described [Tkatch et al., 1995] and used as negative controls for the protein assays. Primary cultures were established from oropharyngeal mucosal cells harvested from a series of noncancer patients as described previously [Rubin Grandis et al., 1996b]. Primary cultures from approximately six control individuals were pooled for the oligonucleotide studies.

TGF-α Protein Detection

Cell line supernatants were analyzed with a TGF-α ELISA assay as described (Oncogene Science/Calbiochem). Cell lysates from SCCHN cell lines negative control cell lines (HL-60, NB-4) and primary cultures of normal mucosal epithelial cells were prepared according to the manufacturer's instructions using a hypotonic lysis buffer (100 mM Tris, 1.5 mM EDTA, 100 uM PMSF, 1 ug/ml aprotonin). Total protein concentration was determined as described by the manufacturer (Bio-Rad Protein Assay). Fifty ug of protein per sample was used in the ELISA assay. To determine if treatment of TGF-α-overproducing SCCHN cells with a well-characterized monoclonal antibody directed against EGFR (MAb 425) [Murthy et al., 1990] could stimulate the release of TGF-α into cell line supernatants, five SCCHN cell lines were plated in 24-well polystyrene plates (Falcon) at a density of 3×10^4 cells/well. After 24 h, 200 ul of media containing MAb 425 (10 nM) was added and cells were incubated for an additional 72 h. TGF-α protein in the supernatants of treated and untreated SCCHN cell lines was then measured using the TGF-α ELISA according to the manufacturer's instructions.

Antisense Oligodeoxynucleotide Studies

Phosphorothioated 19-mer oligodeoxynucleotides were synthesized on an Applied Biosystem 394 DNA synthesizer by means of β-cyanothylphosphoramidite chemistry to minimize the degradation of oligonucleotides by endogenous nucleases. The antisense oligonucleotide (5'-CGAGGGGACCATTTTACGG3') was directed against the translation start site (AUG codon) and surrounding nucleotides of the human TGF-α cDNA (nucleotide -7 to +12) [Jacobovits et al., 1988]. The sense oligonucleotide was 5'-CCGTAAAATGGTCCCCTCG-3'.

To determine optimum conditions for oligonucleotide uptake, oligonucleotides were end-labeled with T4 polynucleotide kinase and purified on a NAP-10 column (Pharmacia). Labeled oligonucleotide (1.2×10^6 c.p.m.) was added to several SCCHN cell lines which had been plated at a density of 10^6 cells/60 mm plate in medium containing 5% serum (heat inactivated at 68°C for 1 h) 24 h prior to addition of the oligonucleotide. Following addition of the oligonucleotides, samples were incubated for 1, 24, 48, and 72 h. Supernatant media (1 ml/plate) were removed at each time point and centrifuged to remove cellular debris [Wickstrom et al., 1988]. The cell pellets were rinsed twice with phosphate-buffered saline (PBS) and lysed in 1 ml of Tris-buffered saline containing 1% SDS. The cytoplasmic and nuclear fractions of the cell pellets were analyzed separately. Cells were washed twice in cold 1 X PBS after centrifugation for 1 minute at 4500 rpm and then suspended in buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM DTT). Cells were then centrifuged at 4,500 rpm for 5 min and resuspended in buffer plus Triton X-100 and incubated on ice for 10 min. Cells were then centrifuged at 7,000 rpm for 5 min and the supernatant was stored as cytosol. The pellet was resuspended in buffer (20 mM Hepes pH 7.9, 15 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.1% Triton X-100) and incubated on ice for 30 min with occasional vortexing. The suspension was then centrifuged at 14,000 rpm for 20 min and the supernatant collected as nuclear extract. Uptake of antisense oligonucleotide was determined by liquid scintillation counting of aliquots of the supernatant media, the PBS washes and the aqueous phases of the cell pellets. Percent uptake of oligonucleotide by the cells was calculated as counts in cell pellet divided by total counts added or detected in media + washes + pellets. Percent uptake of oligonucleotide in the nucleus of the cell pellet was calculated by measuring the counts in the nuclear fraction and dividing by total cell counts.

To determine the effect of oligonucleotides on cell growth rates, a series of SCCHN cell lines were plated in medium containing 5% fetal bovine serum at a density of 10^4 cells/microtiter well in 24-well polystyrene plates (Falcon). Normal mucosal epithelial cells were plated in keratinocyte basal medium (KBM) at a density of 10^5 cells/well in six-well polystyrene plates (Corning). After 24 h when the cells had reached 30–40% confluency, the cells were rinsed twice with medium and fresh medium containing increasing concentrations of sense or antisense oligonucleotides was added (3–25 uM in 0.25 ml medium/microtiter well). To determine if exogenous TGF-α could abrogate the effects of the antisense oligonucleotides on SCCHN cell growth rates, human recombinant TGF-α (30 ng/ml) was added along with the oligonucleotides. Cell counts were determined using a hemocytometer in duplicate samples at each time point in each of the experiments using SCCHN cells. Normal mucosal epithelial cell counts were determined in several experiments yielding similar results. Viability of the cells was analyzed by erythrosin B dye exclusion.

To determine the effect of olignucleotides on TGF-α protein expression, SCCHN and normal mucosal epithelial cells were plated as above for growth rate determination. Four days after the addition of oligonucleotides the cells were lysed and total protein was measured as described above. TGF-α protein levels in the cell lysates were determined by the ELISA assay as described above using 50 ug total protein per sample.

RESULTS

TGF-α protein is produced by SCCHN cells, rapidly binds to EGF receptors and can be detected in the supernatant following treatment with antibody to EGFR: To determine if TGF-α protein is produced by SCCHN cell lines, supernatants from 10 SCCHN cell lines known to over-produce TGF-α mRNA [Rubin and Tweardy, 1993] were assayed for TGF-α protein using a commercially available ELISA. There was no detectable TGF-α in any of the cell line supernatants tested (assay detection limit = 10 pg/ml) suggesting that the TGF-α protein may

be, in part, cell-associated. Earlier reports of TGF-α overexpression demonstrated increased membrane-associated TGF-α [Brachman et al., 1989]. To establish that TGF-α was produced in SCCHN cells but remained cell-associated, cell lysates were prepared from each of the 10 cell lines, normal mucosal epithelial cells, as well as two negative control cell lines and the ELISA was repeated. TGF-α protein was detected in all SCCHN and normal cell lysates but not in the negative control cell lysates (range 0.05–4.0 pg/ug total protein; Fig. 1). To determine if blockade of the EGFR could enhance our ability to detect TGF-α in the supernatant, five TGF-α-overproducing SCCHN cell lines were incubated with monoclonal antibody to EGFR, MAb 425 (Fig. 2). TGF-α protein was readily detected in supernatants of all of the SCCHN cell lines examined after treatment with EGFR antibody. These results sugggest that in the absence of blocking antibody, TGF-α is released by cells into the supernatant but is rapidly removed by binding to EGF receptors and internalization.

TGF-α antisense oligonucleotide treatment of SCCHN and normal squamous epithelial control cells decreased TGF-α protein in both but only reduced proliferation of cancer cells. Membrane-associated TGF-α may be stimulating proliferation of adjacent cells such that down regulation of TGF-α would be expected to decrease cell proliferation. A reliable means of targeting TGF-α is by delivery of antisense oligonucleotides. To determine whether oligonucleotides complementary to TGF-α mRNA were efficiently taken up by SCCHN cells in vitro, end-labeled antisense oligonucleotide directed against the translation start site of TGF-α was added to several SCCHN cell lines. Thirty-seven percent of antisense oligonucleotide was taken up by several SCCHN cell lines at 24 h (Fig. 3a and data not shown). Seventy percent of the oligonucleotide in the cell pellet was found in the nuclear fraction demonstrating functional uptake of oligonucleotide by the SCCHN cells (data not shown).

To assess the effects of oligonucleotides on proliferation, six SCCHN cell lines and primary cultures established from normal mucosal epithelial cells harvested from a series of patients without cancer were incubated with oligonucleotides. On days 2, 4, and 6 following the addition of TGF-α-specific sense or antisense oligonucleotides, the cells were visually inspected for percent confluency, assayed for viability by erythrosin B dye exclusion, and counted. Dose-response curves (3–25 uM) were generated in all six SCCHN cell lines (Fig. 3b and data not shown) which revealed that maximum inhibition of growth was achieved in SCCHN cells with 12.5 uM of antisense oligonucleotide. Proliferation of the six SCCHN cell lines was reduced by a mean of 76.2% when treated with antisense

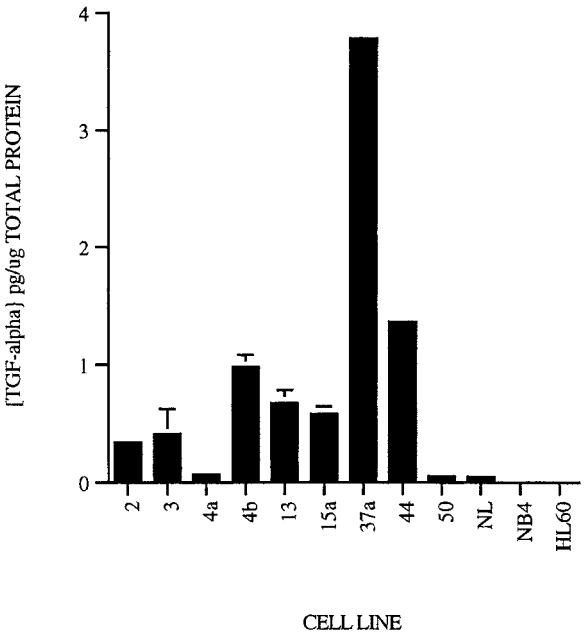

Fig. 1. TGF-α ELISA assay SCCHN cell lysates. Hypotonic cell lysates from 10 SCCHN cell lines and primary mucosal epithelial cells were analyzed to determine the amount of protein per ug of total cell protein. Two hematopoietic cell lines not known to produce TGF-α (HL60 and NB4) were examined as negative controls. The results shown are the mean and standard deviation of three determinations.

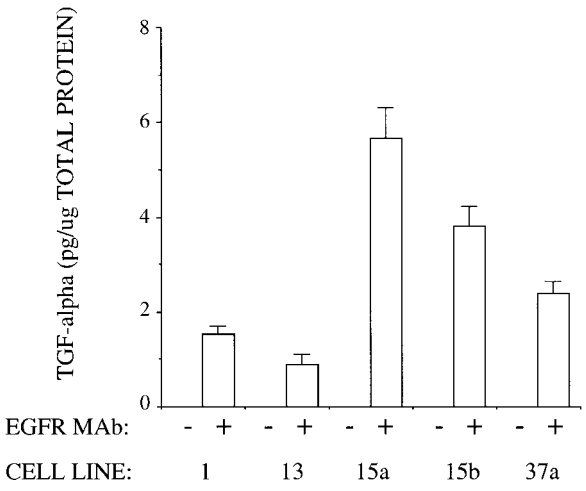

Fig. 2. TGF-α ELISA assay of SCCHN cell line supernatants with and without 3 days of treatment with EGFR Ab (MAb 425, 10nM). The results are the mean and standard deviation of duplicate experiments.

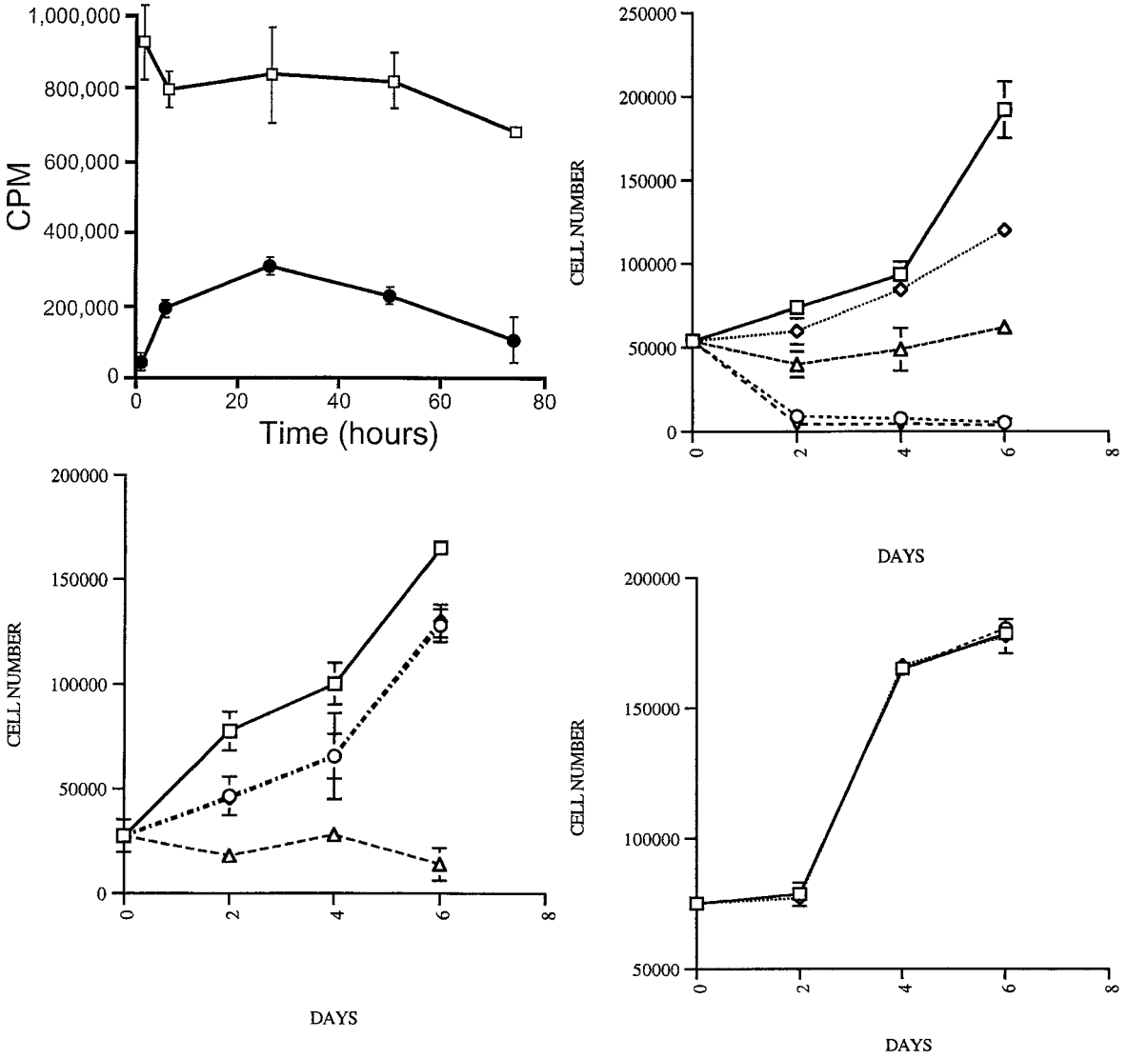

Fig. 3. TGF-α oligonucleotide uptake and effect on SCCHN and normal epithelial cell growth. **A**: A representative SCCHN cell line (PCI 15a) was incubated with antisense oligonucleotide and uptake determined. Total radiolabeled oligonucleotide (cpm) in the supernatant and washes (□) and total radiolabeled oligonucleotide contained within the SCCHN cells (●) are shown and represent the mean and standard deviation and of three determinations. **B**: Demonstrates a dose-response curve of a representative SCCHN cell line (PCI 37a). Cells were exposed to 3 (◇), 6 (△), 12.5 (○), or 25 (∇) uM of antisense oligonucleotides Control cultures (□) did not receive oligonucleotides. Cells were harvested and counted at the times indicated. **C**: A representative SCCHN cell line (15b) was grown without oligonucleotides (□) or in the presence of 12.5 uM sense (○), antisense (△), or antisense oligonucleotides plus recombinant TGF-α 300 ng (ul) (◇). **D**: The growth rate of normal mucosal epithelial cells treated with 12.5 uM TGF-α antisense oligonucleotide (△) is similar to sense-treated (□) and control (●) cells.

oligonucleotides (12.5 uM) compared with 9.7% reduction with sense oligonucleotides (range $P = 0.036–0.001$; Table I). To determine if exogenous TGF-α could "rescue" the SCCHN cells from the growth inhibiting effects of the antisense oligonucleotides, human recombinant TGF-α (30 ng/ml) was added to SCCHN cell cultures along with the oligonucleotides. All six cell lines grown in the presence of exogenous TGF-α demonstrated growth rates comparable to those seen in cells which received sense oligonucleotides and increased proliferation compared with antisense-treated cells (Fig. 3c and data not shown).

To determine if the effects of TGF-α antisense oligonucleotides on cell proliferation were specific for carcinoma cells, the TGF-α antisense oligonucleotide studies were repeated using pri-

mary cell cultures established from normal mucosa harvested from a series of non-cancer patients. In contrast to SCCHN cells, the proliferation of normal mucosal epithelial cells was unaffected by TGF-α antisense oligonucleotide treatment which suggests that the growth inhibitory effect was specific for carcinoma cells (Fig. 3d). Exogenous TGF-α (30 ng/ml) had a small (Ć30%) mitogenic effect at 2 and 4 days on the growth rate of normal mucosal epithelial cells.

To establish that the inhibition of SCCHN cell proliferation by antisense oligonucleotide resulted from a decrease in TGF-α production, we measured TGF-α protein in SCCHN cell lysates following exposure to oligonucleotides using the TGF-α ELISA assay. These studies demonstrated a 93% reduction of TGF-α protein in the cell lysates following incubation of SCCHN cells with TGF-α-specific antisense oligonucleotides but not with sense oligonucleotides. Although the growth rate of normal mucosal cells was unaffected by TGF-α antisense oligonucleotide treatment, TGF-α protein expression was also downmodulated in these cells treated with TGF-α antisense oligonucleotides by 89.3% (Fig. 4 and data not shown). These results indicate that although TGF-α protein is produced by normal mucosal epithelial cells, it is not critical for their proliferation.

TABLE I. Effect of TGF-α Antisense Oligonucleotides on SCCHN Proliferation*

Cell line	Mean % growth inhbition with TGF-α antisense oligonucleotide [12.5 uM]	Mean % growth inhibition with TGF-α sense oligonucleotide [12.5 uM]	*P* Value
15a	70%	11%	0.012
50	61%	13%	0.036
15b	83%	1%	0.000
1483	73%	12%	0.0001
37a	94%	8%	0.0001
13	76%	13%	0.0001
Control mucosal cells	<5%	<5%	n.s.

*SCCHN cell lines and control normal mucosa cells were incubated with TGF-α antisense or sense oligonucleotides (12.5 uM0 for 6 days. Cell counts were performed on day six by erythrosin B dye exclusion and the mean percent growth inhibition was calculated. Statistical analysis was performed using the Student's *t-test*.

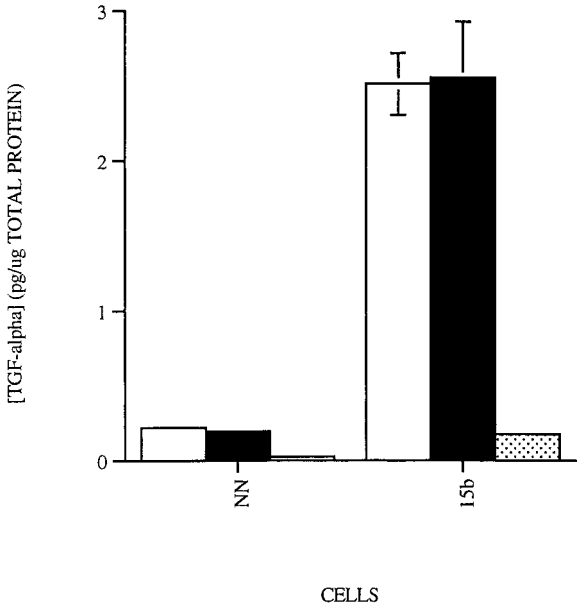

Fig. 4. TGF-α ELISA assay of SCCHN cell lysates (PCI 37a) and normal squamous epithelial cells. Cells were harvested and lysed following growth in control medium (□) or in medium containing TGF-α sense oligonucleotide (■) or in medium containing antisense oligonucleotide (▨) for 4 days.

DISCUSSION

These studies demonstrated that TGF-α is participating in an autocrine growth pathway in SCCHN involving proliferation. ELISA assays showed that the increased TGF-α protein produced by head and neck cancer cells binds rapidly to EGF receptors but can be detected in the supernatants following treatment with antibody against EGF receptor. Treatment of a series of head and neck cancer cell lines with antisense oligonucleotides targeting the translation start site of TGF-α mRNA resulted in decreased TGF-α protein production and inhibition of proliferation. In contrast, the growth rate of normal mucosal epithelial cells harvested from a series of non-cancer patients was unchanged when treated with the TGF-α antisense oligonucleotides despite downmodulation of TGF-α protein suggesting that the effects of TGF-α on cell growth are specific for transformed cells.

When a growth factor and its receptor are produced by the same cells, they may participate in an autocrine growth pathway. The acquisition in vitro of such an autocrine growth mechanism has enabled cells of several lineages to be transformed from the normal to malignant phenotype [Sporn and Roberts, 1985]. A variety of growth factors and their receptors have been postulated to contribute to malignant transformation including bombesin and its receptor in small cell carcinoma of the lung

[Minna, 1989], IGF-1 and its receptor in glioblastoma [Trojan et al., 1992, 1993], IL-6/IL-6R in certain myeloma cell lines [Schwab et al., 1991] and IL-2/IL-2R in T-cell leukemia [Waldmann et al., 1993]. TGF-α has been reported as an autocrine growth factor for several human colon carcinoma cell lines [Sizeland and Burgess, 1992; Karnes et al., 1992; Ziober et al., 1993]. Studies using TGF-α antisense oligonucleotides or expression vectors have reported growth inhibition in transformed epithelial cells from prostate, breast, liver, and colon [Duddy et al., 1995; Howell et al., 1995; Rubenstein et al., 1996; Cammilleri et al., 1996]. We have previously shown that TGF-α and EGFR protein are produced by both basal and suprabasal cells in the histologically normal mucosal epithelium from patients with SCCHN known to be at high risk for subsequent tumor formation and at the advancing margin of SCCHN tumors, a region of increased proliferation [Rubin Grandis et al., 1996a]. In SCCHN cells, TGF-α protein is both cell-associated and released. It has been well described that many TGF-α-synthesizing cells exhibit transmembrane TGF-α precursors at their cell surface [Derynck, 1992]. Moreover, it is estimated that the presence of transmembrane TGF-α at the cell surface is actually more common than the release of soluble TGF-α into the medium [Derynck, 1992]. The transmembrane isoforms of TGF-α appear to be capable of interacting with EGF receptors on neighboring cells without the actual release of ligand into the supernatant [Brachman et al, 1989; Wong et al., 1989]. In SCCHN cells this transmembrane growth factor may be stimulating the growth of adjacent cells by binding to their over-expressed EGF receptors. Treatment of SCCHN cells with an antibody which blocked ligand binding to EGFR, decreased the available receptors for endogenous ligand, thereby enabling the detection of TGF-α released from SCCHN cell lines into the supernatant. In the absence of antibody, release of TGF-α and its subsequent binding to receptors on the same or adjacent cell and internalization appears to be too rapid and irreversible preventing us from detecting TGF-α in the culture medium. TGF-α antisense oligonucleotide exposure did not affect normal epithelial cell growth despite being able to decrease TGF-α protein expression in these cells. These findings raise the possibility that TGF-α may play a different role in normal versus transformed epithelium, i.e., rather than stimulating proliferation, TGF-α in the normal cell may be important for epithelial cell differentiation. This hypothesis is supported by TGF-α localization studies which demonstrated TGF-α protein in the suprabasal layers of the epithelium, and not in the basal layer of normal mucosa from control patients without cancer [Rubin et al., 1996b]. Alternatively, EGFR, not TGF-α, protein levels may be the limiting factor driving normal epithelial proliferation in this ligand-receptor system. We have previously reported the detection of only minimal amounts of EGF receptors in normal squamous mucosa from control patients in the suprabasal without cancer [Rubin et al., 1993; Rubin Grandis et al., 1996b]. Although the precise role of TGF-α in the physiology of normal mucosal epithelial cells has yet to be determined, our findings suggest that the proliferation of SCCHN cells is much more sensitive to down regulation of TGF-α than the normal mucosal epithelial cell. Therefore, inhibition of TGF-α synthesis and/or activity may represent a tumor-specific target for the development of SCCHN chemopreventive strategies.

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