Transfection of Antisense Human Transforming Growth Factor-α (TGF-α) Complementary DNA Reduces Growth Rate of A Human Non-Small Cell Lung Cancer Cell Line

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ABSTRACT

The proliferation of human non-small cell lung cancer (NSCLC) cells was demonstrated of being regulated by TGF-α-stimulated autocrine loop. The present study was designed by transfecting NSCLC cell line, NCI-H460, with construct of human coding fragment TGF-α cDNA in the antisense orientation that is the transcriptionally controlled by a strong eukaryotic promoter. The expressed antisense TGF-α transcript is to neutralize endogenous TGF-α expression. The growth rate of the cloned cells H460 with antisense construct selected for G418 resistance were shown reduced compared with those that were transfected with vector alone and the parental cells. In addition, TGF-α transcript level was observed reduced in the cells with antisense TGF-α construct. The result of this work provides a good model for studying the effects of reduction in endogenous TGF-α expression by transfecting antisense construct on anchorage-dependent cell growth *in vitro* and on tumorigenesis *in vivo*.

Key words: Transforming growth factor-α, Antisense cDNA, Non-small cell lung cancer cells

Introduction

Transforming growth factor-α (TGF-α) is a 50-amino acid polypeptide that belongs to epidermal growth factor (EGF) family (Massaugue and Pandiella, 1993) and binds to the EGF receptor (EGFR) with high affinity that activates cell growth by tyrosine phosphorylation of the receptor. The increased EGFR activity in tumorigenesis is attributed to autocrine stimulation by TGF-α, which is produced by a variety of retrovirus, chemical and oncogene-transformed human and rodent cell lines (Coffey *et al.*, 1992; Aaronson, 1993). TGF-α competes with EGF for binding to EGFR due to their structural similarity. In the autocrine hypothesis, TGF-α produced by transformed cells acts on cell surface EGFR to promote unstrained cell proliferation (Salomon *et al.*, 1990; Sporn and Roberts, 1985). Increases in EGFR levels have also been caused by enhanced transcription that acts as an autocrine growth factor receptor for transformed cells. Expression of EGFR and TGF-α in human non-small cell lung cancer (NSCLC) has been reported (Putnam *et al.*, 1992; Rabiasz *et al.*, 1992; Rusch *et al.*, 1993). Previously, we have reported the acquisition of a sensitive autocrine stimulation by TGF-α in the brain metastatic variant of human NSCLC cells by TGF-α antibody neutralization (Fang, 1996).

To further characterize the significance of TGF-α in modulating the growth of human NSCLC cells, a construct with TGF-α in 3' to 5' antisense

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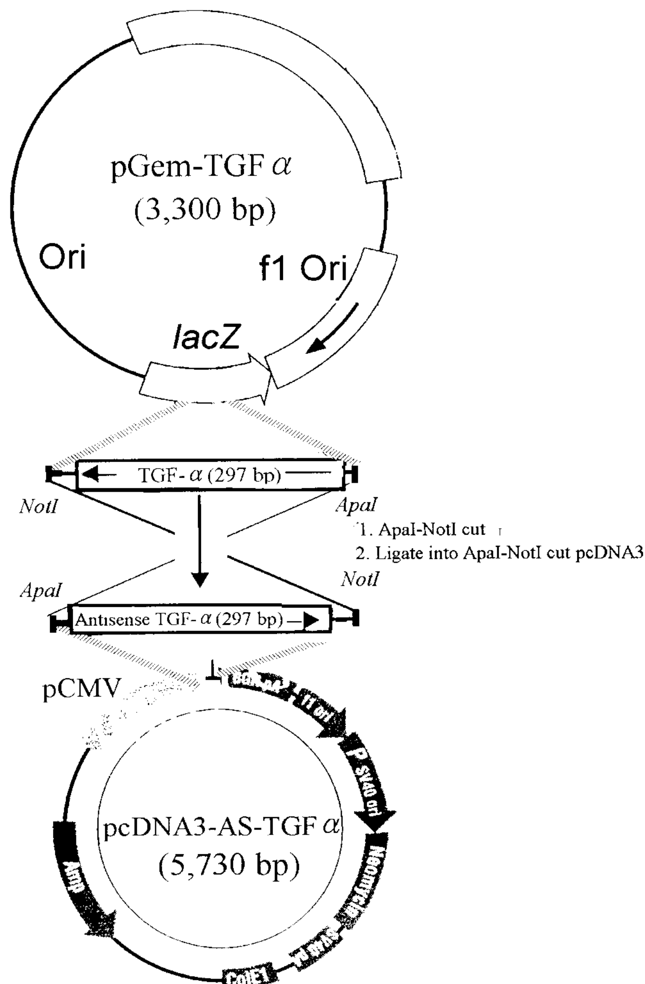


Figure 1. Generation of antisense pcDNA-AS-TGFα construct. Clones from reverse transcriptase polymerase chain reaction-amplified TGF-α cDNA in pGem-T construct was selected, cut by *Not*I-*Apa*I and the fragment ligated into *Apa*I-*Not*I-linearized pcDNA3 vector with TGF-α cDNA in 3'-5' orientation and subcloned.

direction that can be expressed in eukaryotic cells under the cytomegalovirus promoter was produced and transfected into human a NSCLC NCI-H460 cell line.The selected clone with G418 resistance were shown with down-regulated TGF-α transcript and the cell growth rate suppressed. Therefore, the stable cell line with down-regulated expression due to the antisense contruct ought to provide an important model to better understand the interrelationship of other associated oncogenes affected by TGF-α interruption.

Materials and methods

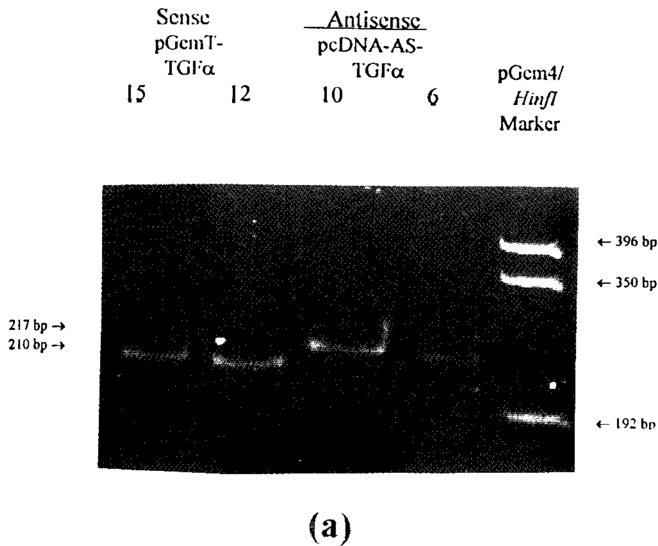
Cell lines

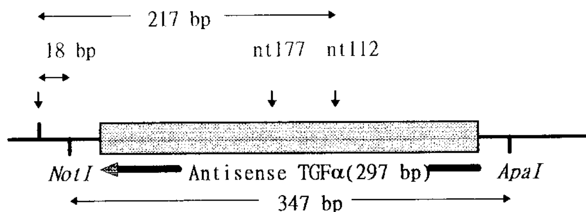
Human lung squamous cell carcinoma cell line NCI-H226, NCI-H322 and large cell carcinoma cell line NCI-H460 (H460) were obtained from Dr. Adi Gazdar (Southwestern Medical Center, Dallas, TX). The cells were grown in RPMI-1640 (Gibco-BRL, Grands Islands, NY) medium supplemented with L-glutamine, sodium pyruvate and 5% heat-activated fetal calf serum (Gibco-BRL, Grands Islands, NY) in a humidified atmosphere of 5% CO_2. The cells were examined free of mycoplasma contamination.

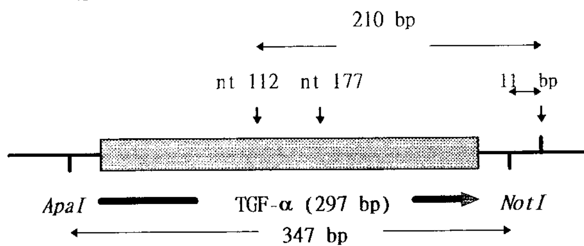
The building of antisense TGF-α cDNA construct.

Total RNA from cell line NCI-H226 was reverse-transcribed with M-MLV reverse-transcriptase (Promega, Madison, WI) in the presence of 30 U RNase inhibitor, 10 μg/ml of random primer (Promega, Madison, WI) and 1 mM dNTP mixture. First strand cDNA was amplified with 0.4 μM of TGF-α primers encompassing nt 35-331 of TGFα cDNA and 0.5 U *Taq* polymerase (Gibco-BRL, Gaithersburg, MD) using an automatic thermal cycler. A 35-cycle polymerase chain reaction that included 95℃ denaturation for 1 min, 45℃ annealing for 1 min and 72℃ extension for 2 min was performed. A 297 bp DNA fragment covering TGF-α cDNA nt 35-331 in exons 1, 2, 3 and 4 (sense primer: 5'-ATGGTCCCCGGCT-GGACA-3' and antisense primer 5'-GGCCTG-CTTCTTCTTCTGGCTGGC-3') (Fang., 1996) were separated in ethidium bromide-stained 0.8% agarose gel. The amplified cDNA fragment was electrophoresis-separated, eluted and cloned into pGem-T vector (Promega, Madison, WI). The cloned TGFα cDNA fragment was confirmed by sequencing and restriction digestion.

The *Not*I -*Apa*I fragment from the pGem-T with TGFα cDNA construct was ligated into the *Apa*I-*Not*I-digested pcDNA3 vector (Invitrogen, San Diego, CA) by T4 DNA ligase (Promega, Madison, WI) and selected after transformation in TOP competent cells. The selected clones were confirmed in 3' to 5' antisense direction of mature TGF-α coding sequence by restriction enzyme digestion.







(b)

Figure 2. Characterization of pGem-TGFα (sense) and pcDNA-AS-TGFα (antisense) constructs. Clones #12, #15 of pGem-TGFα and clones #10, #6 pcDNA-AS-TGFα were digested with *Pst*I restriction enzyme. The *Pst*I-digested fragment for pcDNA-AS-TGFα is longer by 7 bp than that of pGem-TGFα (a) due to an extra 7 bp in distance of pcDNA *Pst*I-*Not*I compared with that of pGemT (b).

Transfection of pcDNA-AS-TGFα into H460 cells

Two micrograms of an antisense construct, pcDNA-AS-TGFα#10, was transfected into 5×10^5 of H460 cells by Lipofectamine method (Gibco-BRL, Gaithersburg, MD) following the protocols provided by the manufacturer and the cells transfected were selected by 400 μg /ml of G418 (Clontech, Palo Alto, CA).

Proliferation assay of pcDNA-AS-TGFα-transfected H460 cells

The selected cultured cells with 70-80% confluency were trypsinized by 0.05% trypsin-0.02% EDTA mixture and 1×10^4 cells were cultured in 6-well plates. The cells were trypsinized at each time point and counted by trypan blue exclusion method.

Results

The generation and analysis of pGem-TGF-α and pcDNA-AS-TGFα constructs

The mature full-length TGF-α cDNA of 297 bp that was amplified from RT-PCR of RNA from NCI-H226 cells was cloned into pGem-T vector and two selected clones, pGem-TGF-α #12 and #15, were confirmed of identical sequence with the published report(Jakowlew *et al*., 1988). The sense TGF-α construct was then digested, eluted and ligated into *Apa*I-*Not*I-linearized pcDNA3 in reverse orientation (Fig 1). Two selected antisense clones, pcDNA-AS-TGFα #10 and pcDNA-AS-TGFα #6, were confirmed of mature TGFα coding sequence in 3' to 5' antisense direction by *Pst*I restriction enzyme digestion. The additional 7 bp obtained from *Pst*I-digested pcDNA-AS-TGFα clones (Fig 2a) as compared with pGem-TGF-α is due to the 18 bp distance from *Pst*I-*Not*I within pcDNA3 as compared with that of 11 bp in pGem-T vector (Fig 2b).

Characterization of the cell line selected from a clone after pcDNA-AS-TGFα transfection

Human NSCLC cell line H460 transfected with antisense TGF-α cDNA was continuously selected in G418 (400 μg/ml) for six weeks. The expanded cells

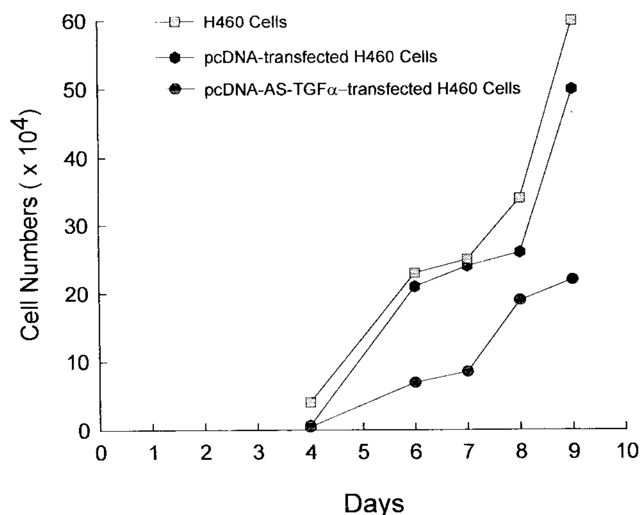


Figure 3. Growth Curves of H460, pcDNA3- and pcDNA-AS-TGFα- transfected H460 cells. The selected clones of 1×10^4 cells of pcDNA3-and pcDNA-AS-TGFα-transfected as well as untransfected H460 cells were cultured in 6-well plates and the cells trypsinized at each time point for counting for nine days. The experiments were repeated twice with the average cells numbers.

from a selected single clone were found to have a longer saturation time (36 h for antisense construct vs. 26h for control with vector only). The cells with pcDNA-AS-TGFα-transfected construct were shown to have a similar but increasing in size compared with control cells. In addition, the growth rate of $1\text{x}10^4$ cells of antisense clone were shown decreased in culture compared with those that were transfected with pcDNA3 vector alone (Fig 3).

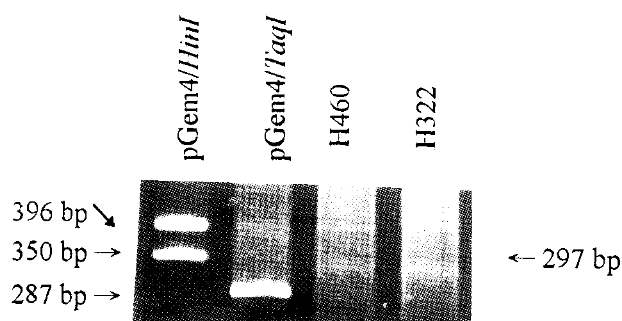
Ten micrograms of total RNA was studied for TGF-α level by reverse transcriptase-mediated polymerase chain reaction in antisense construct-transfected cells and those with vector alone. The expression of TGF-α was shown suppressed in pcDNA-AS-TGFα-transfected H460 cells (Fig 4a) as compared to that of pcDNA3-transfected cells, which displayed detectable level of TGF-α transcript (Fig 4a).

Discussion

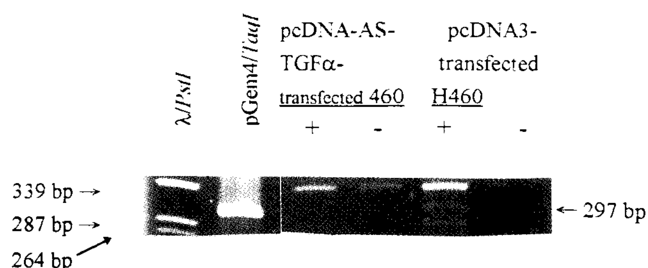
The growth of NSCLC cells were known modulated by TGF-α-mediated autocrine pathway (Putnam *et al.*, 1992). We have demonstrated previously that the growth of NSCLC cells is inhibited as cell-secreted TGF-α is disrupted by antibody neutralization (Fang, 1996). In order to characterize other factors associated with TGF-α disruption, antisense RNA interference on expressed RNA appears to be a rational design. Several methods have been developed to modulate gene expression and regulation of protooncogenes with this objective. Among them, antisense oligonucleotides (Mercola, 1995), antisense RNAs (Eguchi, 1991) and ribozymes (von Ahsen, 1993) have been well-characterized and are currently clinically-trialed as gene therapy protocols. Antisense RNA in their DNA templates can be constructed in expression vectors, carried by plasmid, and produced constitutively in target cells (Prochownik and Erickson, 1992; Zhang, 1995). In this respect, antisense RNAs have an obvious advantage over antisense oligonucleotides, since their templates can be efficiently delivered into target cells, and the active forms can be produced within cells by an effective promoter. Suppression of transcription or translation of antisense RNA protooncogenes can be contributed to: (1) blockage of gene expression; (2) interference of RNA splicing; (3) inhibition of translation; and (4) induction of RNAse III to cleave double-stranded RNA after binding to target RNA (Weintraub, 1990).

In this work, the transfected TGF-α construct was built in antisense orientation that can be driven by CMV promoter in eukaryotic cells. The fully mature TGF-α encoded fragment was cut from the pGemT construct and ligated in 3' to 5' antisene direction into pcDNA3 vector. The selected clones can be characterized by the extra 7 bp due to the additional distance in *Pst*I-*Not*I fragment that appeared pcDNA3 as compared with that of the pGem-T construct.

Human NSCLC cells transfected pcDNA- AS-TGFα were shown to have a suppressed level of TGFα and reduced growth rate in culture compared with cells that were transfected with pcDNA3 vector alone. It is likely that the blockade of TGFα



(a)



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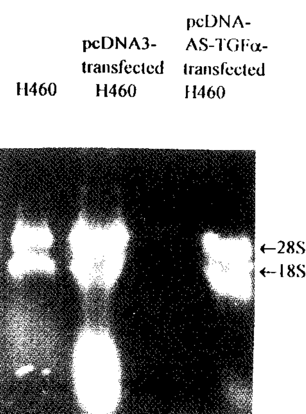

(C)

Figure. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR)-amplified TGF-α for pcDNA3- and pcDNA-AS-TGFα-transfected H460 cells. The RNA for selected cultured cells with 70-80% confluency were extracted and 10 μg RNA amplified by RT-PCR for their TGF-α transcript level. The amplified DNA fragments were resolved by 0.8% agarose gel and stained with ethidium bromide. In (a), H460 and NCI-H322 cells were shown with amplified 297 bp TGF-α cDNA fragment. In (b), the results of RT-PCR of TGF-α cDNA for pcDNA3- and pcDNA-AS-TGFα-transfected H460 cells were shown. In (c), 10 μg of total RNA for H460, pcDNA3- and pcDNA-AS-TGFα-transfected H460 cells were resolved on 0.8% agarose gel to indicate equal RNA used.

expression may indeed decrease the growth of H460 cells. The growth of pcDNA-AS-TGFα-transfected cells began to level off after eight days in culture. The result further demonstrated that TGFα-stimulated autocrine regulation is a contributing factor that helps immortalizing in H460 cells (Roth, 1992). Further aspects due to TGFα disruption are under study.

Antisense oncogene therapies of cancer have been demonstrated to be potentially effective as alternatives to conventional therapy of cancer. The viable cell line established in this work provides a good model for characterizing other growth regulators and phenotypes associated with suppressed endogenous oncogene expression. The instability and low efficiency of cellular uptake in targeting cells as manifested by antisense oligonucleotides can be overcome with antisense construct transfection. In this preliminary work, we have shown the efficacy in reducing proliferation by transfecting human NSCLC cells with antisense TGFα construct. Further characterization as to practical uses of this methodology with better delivery system worthes further attention.

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感染反意第一型轉形生長因子(TGF-α)基因的人類肺癌細胞株降低生長速率

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摘要

本文以人類肺癌鱗細胞株爲材料，萃取RNA後，進行反轉錄聚合酵素連鎖反應所得完整TGF-α cDNA，經純化後，再載入質體，經篩選純系複製後，將TGF-α 基因從質體中切出，構築成反意(anti-sense)TGF-α質體，再感染(transfect)至人類非小細胞肺癌腺細胞株H460，利用此質體得所含強烈啓動子(promoter)表現反意TGF-α基因致使正常(wild-type)的TGF-α RNA無以表現。實驗結果顯示，經反意基因質體感染後細胞株較僅有載體感染後細胞株之生長速率降低，而由反轉錄聚合酵素連鎖反應檢測顯示反意基因感染後細胞TGF-α RNA的表現會降低。此一結果對研究生長因子重要性是一個很有用的模式，也間接提供基因治療研究另一個可能研究方向。

關鍵詞：第一型轉形生長因子、反意基因、人類非小細胞肺癌細胞