Mar.Üniv.Ecz.Der.,
6 (2), 99-115 (1990)

J.Pharm.Univ.Mar.,
6 (2), 99-115 (1990)

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AUTOCRINE EFFECT OF TRANSFORMING GROWTH FACTOR - ALPHA IN HUMAN PROSTATE CANCER CELLS

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SUMMARY

We have investigated transforming growth factor - alpha(TGF- α) and its receptor, epidermal growth factor receptor (EGF- R), expression in androgen - dependent human prostatic cancer cell line (LNCaP) transfected withTGF-α cDNA expression vector. Three transfected clones were tested in serum free media whether TGF-α has the transforming capacity to induce androgen-independent phenotype by creating an autocrine growth loop.

Transfectants were shown to be inducible with 10^{-7} M Cd^{++} without toxic effect. In the presence of inducer, all clones not only express high levels of TGF-α mRNA but also produce and secrete high levels of (8-10 fold) biologically active TGF-α which can successfully compete with EGF for binding to EGF-R. After induction only one clone (LH1) had increased cloning in soft agar two fold above that in non-induced controls. High levels of TGF-α produced by the clones had little or no impact on EGF-R mRNA transcription. LNCaP had low levels ($3\text{-}4x10^4$) of EGF-R binding sites/cell. Transfectants showed less/no binding sites prior to suramin (1mg/ml) stripping of receptors from the high levels of TGF-α produced by them. But, after the suramin treatment low levels ($2\text{-}3x10^4$) of EGF-R sites/cell can be recovered.

Thus, TGF-α produced by the clones down regulates the EGF-R in an autocrine fashion and promotes a differential growth response tightly coupled to the level of occupancy of EGR-R in serum free media.

ÖZET

TGF-α ve EGF reseptörünün androjen hormonuna bağımlı insan prostat kanseri (LNCaP) hücrelerindeki etkisi incelendi. Transfeksiyon yolu ile LNCaP hücrelerine TGF-α cDNA vektörü aktarıldı ve 3 klon izole edildi. Klonlardaki TGF-α 'nın hücrelerin fenotipini değiştirme ve hormona bağımlılıktan kurtarabilme kapasitesi araştırıldı. Klonlarda sentezlenen TGF-α nın EGF reseptörünü baskıladığı ve dolayısı ile hormonsuz ortamda hücrelerin büyümesini TGF-α ortamdaki miktarına ve EGF-R'ne bağlanabilme kapasitesine bağımlı olarak otokrin biçimde kontrol ettiği görüldü.

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INTRODUCTION

Transforming growth factor alpha(TGF-α) is a potent mitogen that is secreted as a mature 50 amino acid polypeptide (1). TGF-α structurally and functionally resembles epidermal growth factor (EGF) and induces a mitogenic response by binding to and activating the tyrosine kinase activity of the 170 kd cell surface EGF receptor (2-4).

TGF-α activity was first described in the media of retrovirally transformed cells (5,6) and has since been implicated in neoplastic transformation. In addition, TGF-α plays a significant role in the normal growth and development such as liver (7) and mammary gland (8,9). Enhanced production of TGF-α and EGF-R is frequently found in human cancer and malignantly transformed cultured cells (10), including those of the liver (11,12), mammary epithelium (13) and pancreas (14,15). Augmented TGF-α levels have also been detected in the urine of cancer patients (16). Significantly, cultured cells transfected or infected with highly active TGF-α expression vectors were shown to become transformed (17-19). These findigns support the autocrine hypothesis whereby a transformed cell can both secrete and bind a specific growth factor, thereby becoming growth factor independent (20).

In general, hormone dependent cancers such as breast, endometrial, prostate, lymphoma and leukemia are regulated by host-derived streoid hormones. In case of breast cancer, estrogen induces growth stimulator, TGF-α and IGF- I like activity, but inhibits secretion of growth inhibitory TGF- ß thus, regulates the cancer cells in an autocrine fashion *in vitro* (21). Recent studies on the androgen dependent LNCaP prostate cancer cells showed that they have an autocrine growth loop, involving TGF-α and its receptor (22).

We were interested in the transforming capacity of TGF-α to induce phnotypic changes. This paper shows that elevated levels of TGF-α produced by LNCaP cells transfected with TGF-α cDNA vector down regulates its receptor in an autocrine fashion in serum free media. However, TGF-α alone was not sufficient to transform cells from androgen-dependent to -independent stage.

MATERIALS AND METHODS

Cell Lines

LNCaP cells were obtained originally according to J.Horoszewicz (23) and were confirmed to be LNCaP cells cytogenetically as described

by J.Weng-Peng (NCI) LNCaP passages 20-49 were used for the experiments. All cell lines were negative for mycoplasmic contamination, NRK 49F normal rat kidney fibroblast obtained from R.Lupu.

Anchorage-dependet growth assay

Cell lines were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere and cultured in IMEM (Biofluids, Rockville, MD) growth medium containing sodium phenol red 10 mg/L, gentamicin 25 MCG/L, glutamin 2 mg/L and supplemented with 5 % FBS in a Falcon tissue culture flasks (Becton & Dickinson Comp., Oxnard, CA) unless stated otherwise. Upon confluence cells were detached using tyripsin versene (Biofluid, Rockville, MD) passed trough a 20 gouge syringe and passaged at 1:20 dilutions for continued growth. When needed cells were seeded in 12 well Costar plates (Costar Corp.Cambridge, MA). After 48h, incubation media were changed to defined serum free media (SFM) containing 2mg/L human fibronectin (Collaborative Res. Inc. Bedford, MA), 2mg/L human transferrin and trace elements 1x solution (Sigma Chem. Corp.st. Lois, MO), HEPES 20mM (Gibco Lab.Grant Island N.York), Glutamin 2 mM (Biofluids, Rockville, MD) at final concentrations, and cells were incubated further 24h before treated with human recombinant TGF-α 1.8 nM (Bachem Fine Chemicals Inc. Torrence, CA) or Cadmium sulfate 10^{-7} M (Sigma Chem. Corp. St. Louise, MO) always in prewarmed fresh serum freemedia. The cells were harvested by using 1x PBS containing 0.2 g/L EDTA and counted using a coulter counter in isoton III solution (Coulter Elect. Inc. Hialeah, Florida).

Ancorage-independent growth assay

Soft agar cloning assays were carried out as previously described (24) using a 1 mL buttom layer of IMEM without phenol red, containing 0.6 % Noble agar (Difco, Detroit, MI) and 10 % CCS in 35 mm Costar tissue culture dishes. 0.8 mL top layer of IMEM containing test samples, 0.36 % noble agar, 10 % CCS and cells to be tested ($1x10^4$ cells/plate unless otherwise stated) were added after the solidification of bottom layer. Each sample was plated in triplicate. All samples were sterilized by filtration using a 0.22 µm Millex CU milipore (Milipore Corp. Bedford, MA) filter before plating. Plates were incubated in a humidified 5 % CO_2 atmosphere at 37°C and were counted at 18-20 days of incubation with Amicon 3600 image analysis system (Artex Systems Corp. Farmingdale, N.York). Colonies greater than 60 µm in diameter were scored as positive.

Transfection of TGF-α cDNA into LNCaP cells

Plasmids were constructed using standart recombinant DNA techniques by F.Kern. 925 basepair-EcoR1 fragment containing the complete coding region of the TGF-α precursor peptide was inserted into the EcoR1 site of pMT-TGF1 expression vector placing the cDNA under the control of human metallothionein II A promoter. LNCaP cells were either transfected with plasmids containing sequences coding for resistance to G418 (Neomycin) or cotransfected with additional plasmid pMT-TGF1 containing TGF-α cDNA sequences. Cotransfected G418 resistant clones were initially screened for the production of appropiately sized TGF-α mRNA and three clones, designated to be, LH1, LH2 and LH9 used in this study.

Preparation of conditioned media (CM)

Cells were grown to approximately 80 % cofluency in IMEM with 5 % FBS medium, then washed and incubated 18h in defined SFM. Later, changed to prewarmed SFM for further 48h incubation, where indicated Cd^{++} 10^{-7} M added to the media for inducing metallothionine promoter. Finally, medium was collected and centrifuged at 4°C for 10min at 450xg, in the presence of protease inhibitors (phenymethylsulfonylfloride, 1 mM; leupeptin, 0.5 µg/mL; pepstain 1 µg/mL; aprotinin 0.1 % v/v at final concentrations) and filtered through a 0.45 µm Nalgane filter. The medium was concentrated 1/50 volume at 4°C in an Amicon concentrator (Amicon Corp. Danver, MA) using a YM5 membrane filter and dialyzed using Spectra/por Mr 3000 cut off tubing (Spectrum Med. Ind Los ange-les, CA) against 100 volumes of water for 48h at 4°C. Before use in NRK or RIA assays, it was sterilized on Amicon centreprep Mr 3000 centrifugal devices as described in manufacturer's instructions. Concentrated CM were assayed at various dilutions for TGF-α like activity. DNA was quantinated using a fluorescence method (25) from the aliquots of pooled cells harvested from the same flasks which CM had been collected.

Estimation of TGF-α like activity in CM

TGF-α like activity was determined by radioimmunoassay (RIA) in reference to a standard curve generated using human recombinant TGF-α bought from Bachem. The RIA kit obtained from Biotope inc. (Redmond, WA), uses a polyclonal anti-rat TGF-α antiserum which does not cross-react with human EGF. Aliquots of CM were reduced with 40 nM dithiothreitol and denatured by immersion in a boiling water bath for 1 min (26). Assays were done in duplicates according to the manufacturer's protocol.

Estimation of transforming activity

The biological transforming activity in the CM was assayed by its ability to stimulate anchorage-independent growth of NRK 49F cells in soft agar (27). Using 2.5 mL, 5 mL, 10 mL equivalent of concentrated CM, aliquots were plated with $3x10^3$ NRK cells/plate in the presence of 10 % CCS. Standart curves using hrTGF-α were constructed and IMEM, without cells, concentrated under the same conditions were used as control for background growth. Plates were incubated and counted as described in anchorage-independent growth after 14 days of incubation.

Northern blotting and RNase protection analyses

The cells used for RNA extraction were the same cells which CM had been collected. They were harvested by PBS containing 0.2 g/L EDTA, washed with PBS, an aliquout of it kept for DNA assay and total RNA was extracted by direct aplication of 4 M guanidium isothiocyanate (GIT) to the cells. Total cellular RNA from the GIT treated cells were obtained by centrifugation throuh a CsCl cushion as described in Maniatis (28). After centrifugation, RNA was further purified by sodium acetate (0.3 M pH 5.2) precipitation and phenol/chloroform extraction. Purified RNA was quantitated specrophotometrically and qualitated electrophoretically on 1.1 % agarose with 2.2 M formaldehyde gels and stained with EtBr (2 mg/mL). After confirmation of equal loading (30 µg/lane) of RNA samples, electrophoretically fractioned RNA on gel was blotted onto nitrocellulose filters using 20xSSC (1xSSC is 0.15 M sodium citrate) and hybridized to uniformly ^{32}P- labelled riboprobe (Promega Corp. Madison, WI) transcribed with SP6 RNA polymerase from Xba I restricted pGEM4 plasmid containing 925bp EcoRI fragment of TGF-α according to the manufacturer's instructions. Blotted filter was preyhbridized in buffer (50 % formamide; 5xSSC, 5 mM sodium phosphate pH 6.5, 0.1 % SDS; 1 mM EDTA; 0.05 % BSA; 0.05 % Ficoll; 0.05 % PVP and 200 µI denatured salmon sperm DNA pH 6.5) at 58°C for 4h. Afterwards, the filter was incubated in fresh hybridization buffer containing $6x10^6$ dpm riboprobe at 58°C for further 18h. Then the filter was washed with three changes of preheated 0.1xSSC for a total of 1 hour at 65°C. To reduce the nonspecific binding of the probe, the filter was washed three times in 2xSSC and incubated for 15 min at room temperature in 2xSSC containing 1 µg/mL RNase A and finally washed in 0.1xSSC-0.1 % SDS for 30min at 50°C.

Total cellular RNA for RNase protection assay was prepared as above. The EGF-R probe for RNase protection assay was a ^{32}P-labelled 383bp Apal/Clal fragment in pGEM7z(f) linearized with Pvull and transcribed with SP6 RNA polymarase according to promega-riboprobe protocol. Sixty micrograms of total RNA was hybridized with $5x10^4$ cpm ribo-

probe for 16h at 50°C. Samples were then digested with 40 µg/mL RNase A for 30 min at 28°C. The RNase digestion is terminated by the addition of both proteinase K and SDS. After extraction once against phenol-chloroform-isoamyl alcohol (25:24:1), the samples were precipitated with 1 µg RNA in absolute ethenol. Pallets were boiled in loading buffer and electrophoretically fractionated in 6 % polyacrylamide gel containinig 7M urea. A *in vitro* constructed size and loading riboprobe marker designated 36P4 was used as an internal control of the assay. Finally, gels were dried and exposed at -70°C to a Kodak x-omatic AR film (Eastman Kodak, Rochester, NY.) between two chronex quanta III intensifying screens (Dupont, Wilmington, DE.).

EGF receptor binding assay

Whole cell EGF-R analysis was performed basically as described by Krupp (29).Except the prostatic cell lines were grown into subconfluent monolayers in falcon flask in 5 %FBS, IMEM medium then changed to SFM for 18h incubation. Media were changed for the second time, prewarmed fresh SFM with/without cadmium and suramin 1mg/mL either alone or in combination was added for 48h treatment. Afterwards, cells were washed 2 times with ice-cold 1xPBS, harvested with PBS containing 0.2 g/L EDTA and pelleted at 800rpm at 4°C for 5 min, resuspended in binding buffer (IMEM without phenol red, 50 mM HEPES, 0.1 % BSA pH 7.4), counted and distributed into ice-cold assay tubes $1x10^5$ cells/tube in triplicate and incubated 30min in ice. Then the cells were centrifuged, pellets were resuspended in binding buffer containing increasing concentrations of (1-10nM) ^{125}I-mouse EGF were (Spec. act. ^{125}Ci/mmol) in the presence/ absence of a 100 fold excess of unlabeled EGF. Cells with radioactive EGF were incubated at 4°C for 3h with gentle shaking. After the incubation, they were rinsed three times with ice-cold binding buffer and extracted with 0.1 M NaOH with 0.1 % SDS and measured with Packard gamma counter (Packard Instruments, Downers Grove, IIIinois). Data analyzed by direct linear plot (30); necessary preliminary estimates were derived from linear transformation of specific binding data.

Determination of tumorigenessis and hormone dependent growth in vivo

Subconfluent monolayers were incubated further 48h in the presence and absence of Cd^{++} 10^{-7} M and harvested in th presence of PBS with mechanical agitation, centrifuged at 800 rpm for 5 min and washed twice with PBS. Resuspended in media and an aliquot was counted. The volume of the cell suspension was adjusted to yield viable cell count of $5x10^6$ cell/0.5 mL per inoculation site. Cell viability was estimated by the ability to exclude trypan blue. Inoculations were made with and

without the presence of basement membrane (Matrigel) support, kindly provided by E.Tompson. Cells ($5x10^6$) were inoculated subcutaneously into 3 sites on each flank of ovariectomized NCI BALB/c Nu/Nu athymic nude mie with/without hormone supplements. Tumors were measured after 14-20 weeks of inoculation and their pathology was verified microscopically.

RESULTS

Expression of TGF-α mRNA in transfectants

LNCaP tranfectants LH1, LH2, LH9, and LNCaP-Neo were constructed, as described in materials and methods and screened for the production of appropriately sized TGF-α mRNA. They contain the TGF-α under the control of inducible metallothionein promoter and optimal induction was obtained at 10^{-7} M Cd^{++} concentration after 48h treatment in SFM without the toxic effect. The location of the relevant mRNA species on the northern blot analyses corresponded to the predicted TGF-α mRNA sizes of 1.4 kb, just under the 18s RNA (Fig.1). MCF-7 clone H8(31) was used for a positive control of the assay because it constitutively express TGF-α mRNA. The levels of TGF-α mRNA was considerably increased after the treatment in all three clones (Fig1). All lines were equally loaded as estimated from its EtBr stained prehybridization profile and from the outoradiographs of the blot prior to RNase A treatment. Furthermore LNCaP, LNCaP-Neo controls and the other transfected clones did not have high base line levels of specific TGF-α mRNA after the RNase A treatment (Fig.1).

Secretion of TGF-α into CM

Using a specific anti-TGF-α antibody, which does not give a cross reaction with EGF, equivalent amounds of CM were analyzed by RIA to confirm the fact that the secreted material in CM was indeed TGF-α . In ağreement with the northern blot analysis, all LNCaP cell lines produced higher levels of TGF-α immunoreactive material after the inducement (Table 1). The LNCaP induced with 10^{-7} M Cd^{++} had no change in the immunoreactive TGF-α production as expected. The clonal variations of TGF- production was reflected in all assays.

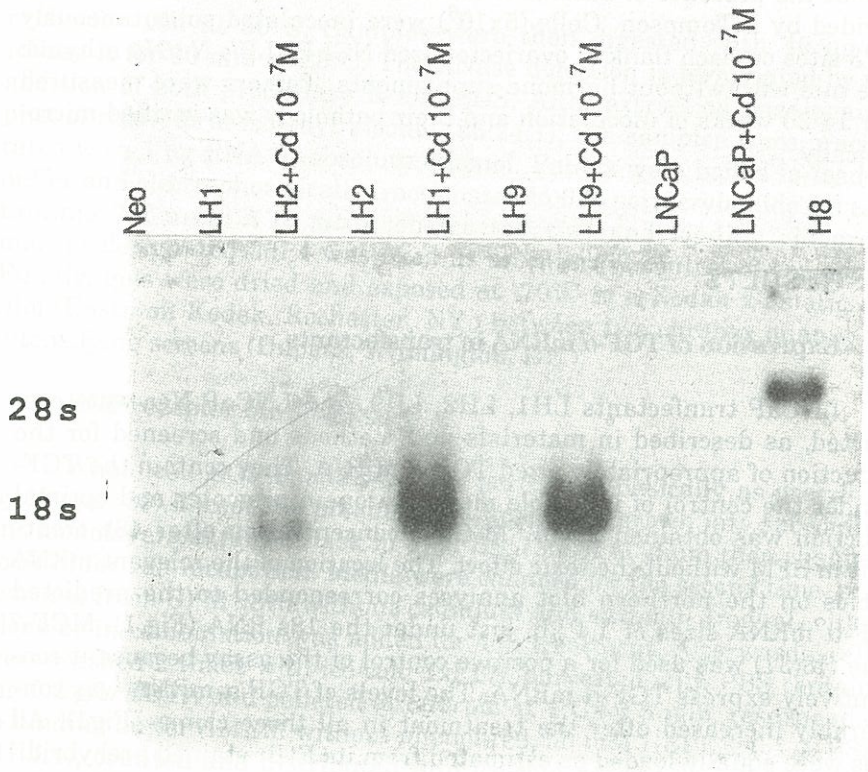

Fig-1: Expression of TGF-α mRNA in LNCAP cell lines. Northern blot analysis of 30μg of total RNA hybridized to TGF-α riboprobe as in materials and methods. Lines represents 1- LNCaP-Neo, 2- LHI, 3- LH2+Cd 10^{-7}M, 4LH2, 5- LH1+Cd 10^{-7}M, 6- LH9, 7- LH9+Cd 10^{-7}M, 8-LNCaP, 9- LNCaP+Cd 10^{-7}M, 10- MCF- 7 H8 total RNA samples. Cells were induced in SFM for 48h prior to RNA extraction. MCF-7 H8 cells were included as positive control and LNCaP and LNCaP-Neo cells were used as negative controls. EtBr staining of the gel indicated that samples were undergrated and comparably loaded.

The biological activity of the material in CM was confirmed by the ability of CM from LNCaP and the cloned cells to induce anchorage-independent growth of NRK 49F cells. When compared to the parent LNCaP, transfected cell lines had 2-3 fold greater baseline colonies (Fig.2). After 10^{-7} M Cd^{++} induction of the clones the number of colonies were increased as proportional to the amount of immunoreactive TGF-α found in CM. In the presence of cadmium, LH1 and LH9 had increased TGF-α production as seen in RIA which is also reflected in 15 and 10 fold increase in the number of NRK colonies formed respectively (Fig.2).

Table-1: Detection of TGF-α like activity in CM by radioimmunoassay. Concentrated media from LNCaP and transfected clones (with/without Cd10 $^{-7}$ M induction for 48h) were assayed for TGF-α like activity using RIA kit.

Cell Lines	RIA TGF- Concentrations
Media control	< 0.01 nM
LNCaP-Neo	0.09 nM
LNCaP	1.6 nM
LNCaP+Cd 10^{-7}M	1.7 nM
LH1	< 0.1 nM
LH1+Cd 10^{-7}M	> 10.0 nM
LH2	0.8 nM
LH2+Cd 10^{-7}M	5.0 nM
LH9	0.65 nM
LH9+Cd 10^{-7}M	> 10 nM

Expression of EGF- R mRNA and EGF-R Binding Characteristics

Since variations in TGF-α production is reflected in m RNA levels, it is possible that variations in the autocrine loop may occur at the level of the receptor for this growth factor. Thus, EGF-R expression was examined in all clones by RNase protection assay (Fig. 3). The higher level of EGF-R mRNA expression after induction is observed in one clone. LH9. The others had little change in their baseline mRNA levels, after the loading corrections calculated using 36P4 probe (Fig. 3). Thus, showing that TGF-α production had little/no impact on EGF-R mRNA transcription.

In several other systems, either TGF-α or oncogene transformation has been shown to cause loss of detectable EGF-R binding probably due to down regulation induced by an increas in TGF-α production (31-34). Therefore, EGF-R binding characteristics were determined for LNCap and its transfectants.

In order to measure only cell surface receptor binding and avoid obscuring receptor internalization and degradation of ^{125}I-EGF receptor complexes, all binding assays were performed at 4°C. LNCap and transfected cell lines treated with/without cadmium and suramin prior to binding assays as described in materials and methods. Suramin is known to inhibit the binding of growth factors (EGF, PDGF, TGF- α and

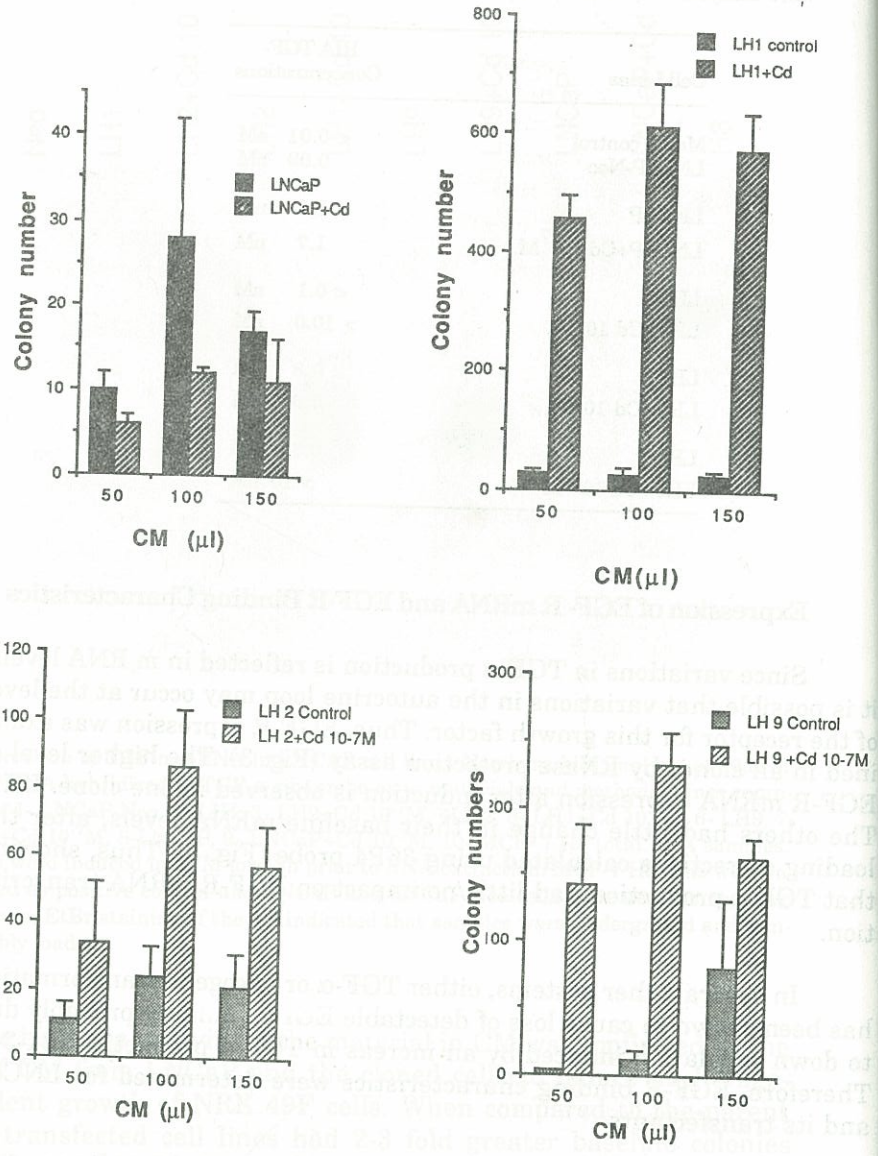

Fig-2 : Biological transforming activity of TGF-α in CM. Assayed by induction of NRK 49F fibroblast cloning in soft agar. Each of the LNCaP cell lines graphs were drawn after the subtraction of background clonies as estimated from The CM blanks.

β) to their receptors. Its binding effect can be reversed in the increased concentrations of the growth factor (35). As shown in Table 2, LNCap cells have approximately 35-40x10^3 total binding sites per cell, treatment with cadmium and suramin alone, or in combination did not effect the numbers. However, transfected clones under the same conditions have variable profile. Such as, the high TGF-α producing clones LH1 and LH9 have less binding sites. The reduction in the receptor number has probably resulted from the change in the recycling/degradation ratio due to higher amount of available ligand. Because the reduction of EGF-R mRNA was not observed in high TGF-α producing clone LH9 in RNase protection assay.

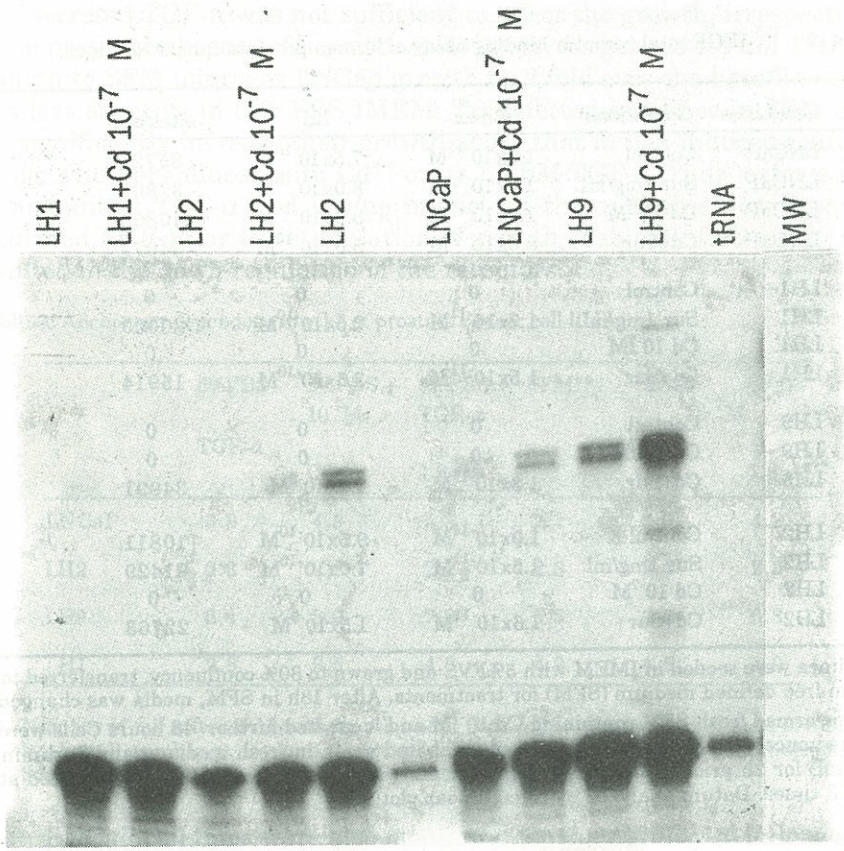

Fig - 3 : RNase protection analysis of EGF receptor mRNA expression by LNCap and transfected cell lines. Total RNA was isolated, hybridized to EGF-R and 36P4 riboprobes, digested and electrophoresed as in materials and methods. The bands at proximately 141bp represent the protected portion of the EGF-R probe. The bands around 100bp represent the 36P4 probe protected controls indicating the amounts of RNA used for all the cell lines. For control of nonspecific hybridization of riboprobe 60 μg tRNA hybridized to both probes and digested with RNase A.

All uninduced cell lines had low levels of EGF recepor mRNA and low levels of EGF-R binding sites/cell. When they were induced, EGF-R mRNA levels showed little change, this was not mirrored in the binding sites. In the presence of inducer, there were no detectable EGF-R binding sites/cell, Suramin 1mg/ml treatment for 2h was sufficient for the recorvery of most EGF receptors of LH1, LH2 and LH9 cell lines, although they had less numbers then parent LNCap (Table 2). Undetectable EGF-R sites in the absence of suramin was due to higher level TGF-α present in clones than parent LNCap. TGF-α at LNCap cell lines appears to have down regulating effect on the EGF receptor via autocrine loop. The absence of detectable levels of EGF-R in the cloned cell lines indicates that the levels of secreted TGF-α could be sufficient to induce some biological effect mediated by this receptor. The next step was, to assess the TGF-α effect on mitogenecity and tumorogenesis.

Table -2 : ^{125}-IEGF total receptor binding assay of human prostate cancer cell lines.

Cell Lines	Conditions*	Bmax	Kd	sites/cell
LNCaP	Control	$1.3x10^{-11}$M	$7.5x10^{-10}$M	35721
LNCaP	Sur 1mg/mL	$1.7x10^{-11}$M	$8.0x10^{-10}$M	37800
LNCaP	Cd 10^{-7}M	$1.3x10^{-11}$M	$8.0x10^{-10}$M	40865
LNCaP	Cd+Sur	$8.0x10^{-12}$M	$5.0x10^{-10}$M	34071
LH1	Control	0	0	0
LH1	Sur 1mg/mL	$1.7x10^{-11}$M	$2.5x10^{-10}$M	20000
LH1	Cd 10^{-7}M	0	0	0
LH1	Cd+Sur	$1.5x10^{-11}$M	$3.5x10^{-10}$M	15914
LH9	Control	0	0	0
LH9	Cd 10^{-7}M	0	0	0
LH9	Cd+Sur	$1.3x10^{-11}$M	$1.8x10^{-9}$M	34091
LH2	Control	$1.0x10^{-11}$M	$9.5x10^{-10}$M	10811
LH2	Sur 1mg/ml	$1.5x10^{-11}$M	$7.0x10^{-10}$M	21429
LH2	Cd 10^{-7}M	0	0	0
LH2	Cd+Sur	$1.6x10^{-11}$M	$1.5x10^{-9}$M	22763

*Cell lines were seeded in IMEM with 5%FVS and grown to 80% confluency, transferred to serum free defined medium (SFM) for treatments. After 18h in SFM, media was changed to prewarmed fresh SFM containing Cd 10^{-7}M and incubated further 48 hours Cells were washed once with prewarmed SFM and incubated again in fresh media or/and Suramin 1mg/mL for 2h prior to the assay. Experiments with LNCaP and LH1 were repeated at least 3 times. Datum analyzed by direct linear plot(30).

Growth response of Prostatic cancer cell lines to TGF-α

The anchorage dependent growth in monolayers in serum free media for LH9 and LH1 cell lines were approximately 5 fold when com-

pared to the LNCaP and LH2 cell lines which had only 2 fold growth at the same conditions (Table 3). LNCaP and transfected cell lines were tested for their response to various concentrations of cadmium (10^{-6} to 10^{-9} M) for the induction of TGF-α mRNA (on Northern blots) and for the growth yield (on monolayers). Addition of 10^{-6} M Cd^{++} to the media for the induction resulted in the highest amount of TGF-α mRNA, however at this conccentration cadmium had a toxic effect on cell monolayer growth. On the other hand 10^{-8} and 10^{-9} M Cd^{++} did not change either cells TGF-α mRNA levels or the growth conditions over the uninduced baseline levels. Therefore, 10^{-7} M Cd^{++} was chosen as the optimal inducing concentration in all experiments (data not shown). Treatment of cells with 10^{-7} M Cd^{++} either in SFM or 5 % FBS IMEM had not significantly change the stimulation of growth in LNCaP and LH2 cells, indicating that secreted TGF-α was not sufficient to effect the growth, irrespective of the presence/absence of hormones. However, exogenous 1.8 nM TGF-α additon to SFM improves LNCap growth by 2 fold over the base line but it is less effective in 5 % FBS IMEM. Transfected cell lines in SFM did not significantly increase their growth above that in non-induced control levels, whether induced with Cd^{++} or by 1.8 nM TGF-α. Thus, extra added amount of TGF-α had low/no impact on the available but already saturated EGF-R for the stimulation of growth (Table-3). Rather, it has resulted in the down regulation of the receptors.

Table-3: Anchorage dependet growth* of prostate cancer cell lines

	5%FBS	5%FBS + 10^{-7}M Cd	5%FBS + TGF-α 1.8nM	SFM**	SFM+ 10^{-7}M Cd	SFM+ 10^{-7}M 1.8nM
	TGF-α					
LNCaP	3.8	4.3	5.2	2.1	2.0	4.2
LH2	2.3	2.3	2.2	2.3	2.4	2.3
LH9	6.4	8.0	7.8	5.3	7.5	6.3
LH1	2.8	3.5	4.8	4.9	4.5	4.0

* Number of cell affter 6 days of growth under the indicated growth conditions divided by the cell number at day zero to arrive fold increase of growth at a given condition.
** Serum Free Media

LNCaP cell lines were also monitored for their ability to induce anchorage-independent growth in soft agar, which is closely related to cells transforming capacity to induce transformed phenotype. The soft agar colony formation assays were conducted under 2 different conditions (Table 4). In all conditions the prostatic cancer cell lines tested, formed baseline colonies of approximately LNCaP (300), LH1 (100), LH9 (100) and LH2 (150) in soft agar plates.

Table-4 : TGF- α stimulated ancorage independent growth* of human prostate concer cell lines

	LNCaP		LH1		LH2		LH9	
	Cd^{++} 10^{-7} M	TGF-α 1.8nM	Cd^{++} 10^{-7} M	TGF-α 1.8nM	Cd^{++} 10^{-7} M	TGF-α 1.8nM	Cd^{++} 10^{-7} M	TGF-α 1.8nM
	**	**	**	**				
Pretreated	0.5± 0.3	1.7± 0.5	2.2± 0.5	1.1± 0.2	0.97	1.03	0.31	1.94
Not-Pretreated	0.50	1.34	0.17	1.26	0.21	1.11	0.69	1.40

* Number of colonies formed after treatments were divided with the untreated contol colony number to arrive the numbers in fold growth.
** Represents ± SD. n=3.

First protocol for cell inducement was under pretreated conditions. Cells were grown in the usual growth conditions (as in materials and methods) and changed to 5 % CCS for 18h, then changed again to fresh medium containing 10^{-7} M Cd^{++} incubated for 48h prior to soft agar plating, only 1.8 nM TGF-α added exogenously to soft agar where indicated. Results showed that all cell lines had no significant colony growth with the exceptions of LNCaP and LH9 which are grown in the presence of exogenously added 1.8 nM TGF-α. Furhermore, when the same cells were induced for TGF-α production with Cd^{++} prior to plating, all cell lines had no significant colony growth with the exception of LH1 clone which had 2 fold increase over the non-induced control (Table 4. top line). Second protocol, considered the fact that continual stimulation of cells with Cd^{++} during the growth of colonies could result in the higher number of colonies. Thus, cells were grown as above, but without Cd^{++} stimulation in 5 % CCS, then Cd^{++} and TGF-α were added to the soft agar plates directly for continual stimulation during the incubation. Results suggested that continued Cd^{++} stimulation of TGF-α in the soft agar did not improve the cells' transforming capacity (Table 4, bottom line), rather it had a toxic effect.

We have also failed to observe either tumor formation or the growth of LNCaP in the absence of hormones both *in vitro* (Table 3) and *in vivo*. Furthermore, induction of transfectants with $Cd^{++}10^{-7}$M for 48h prior to injection was also not sufficient for the tumor formation *in vivo* until LH1 and LNCaP cells were induced and coinjected with basemant membrane (matrigel) support,. then few sizeable tumors were obtained. Currently, tumorogenesis in the presence/absence of hormone with inducers and matrigel is under investigation.

So far, all growth results confirm the EGF-R studies, indicating that over production of TGF-α in prostatic cancer cell lines behave in an autocrine fashion and down regulates its receptor whereby curtails its growth stimulation. This is analogous to the observations published that differential growth response to EGF/TGF is more important then the enhanced production of TGF-α, when determining the transition from normal to malignant human breast epithelium (36).

DISCUSSION

Polypeptides such as growth factors differentiation factors and hormones are crucial components of the regulatory system that coordinates development and transformation of multicellular organisms. Many of these factors mediate their pleiotropic actions by binding to and activating cell surface receptors with an intrinsic protein tyrosine kinase activity or receptor kinases. The biological activity of TGF-α is widely reported to be proportional to its binding to and activating the tyrosine kinase activity of the 170 kd cell surface EGF receptor (2-4). Enhanced production of active TGF-α and EGF receptor is frequenly found in human cancers (10) and cultured cells transfected with highly active TGF-α expression vectors become malignantly transformed (17). It appears that the ligand-induced activation of the kinase domains and its signaling potential are mediated by receptor olgomerization (37) which is an universal phenomenon among growth factor receptors. Oligomerization can be induced by monomeric ligands such as EGF/TGF-α that leads into conformational changes in EGF receptors.

Data peresented on human prostatic cancer cell line LNCaP transfected with active TGF-α expression vector shows that in the presence of inducer, all clones express high level of TGF-α mRNA, also produce and secrete biologically active TGF-α which competes with EGF for binding to EGF-R. However, elevated levels of TGF-α production has little impact on the EGF-R mRNA transcription. Furhermore, there is no increase in the number of receptor sites when TGF-α production was elevated. Although, there is a correlation between the levels of TGF-α produced by cells and the occupancy of EGF-R sites per cell (i.e. When TGF-α levels were elevated, there is no detectable sites). The deprivation of system from its ligand by suramin allows detection of receptors in the cells. Thus, it can be concluded that all transfectants without induction had low levels of EGF-R mRNA and low number of EGF-R sites/cell, when they were induced withCd^{++} EGF-R mRNA showed little change but there were no detectable EGF-R sites/cell. Undetectable EGF-R in the absence of suramin was due to the peresence of high level of TGF-α present. Therefore, overproduction of TGF-α appears to down regulate the prostatic cancer cell lines' EGF receptors. Thus, pointing to an auto-

crine control for the ligand and its receptor in turn, curtailing the growth stimulation effect seen in other malignant cell lines.

In conclusion, one can say that additional studies *in vitro* and *in vivo* are needed on transfected human prostate cancer cells which will take account firstly the androgen effect and secondly, the routing of ligand after binding to the receptor for confirming these findings and clarifying the role of TGF-α autostimulatory loop in this saturated system.

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(Received December 10, 1990)