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Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines

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Abstract

Enhanced expression of the epidermal growth factor receptor (EGFR) or its ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) can increase signalling via receptor-mediated pathways which may lead to excessive proliferation and cellular transformation. Such autocrine regulation of growth has been demonstrated for prostate cancer cell lines in culture but its role in prostate cancer *in vivo* has not been established. To assess the potential of such a mechanism, we have examined the pathway components in prostate carcinomas (CaP) in comparison with non-malignant benign prostatic hyperplasias (BPH). In the present study, we investigate the dosage, structure and expression of EGF, TGF-α and EGFR genes in a series of 34 human prostate samples and 3 prostate cancer cell lines. All of the samples contained transcripts from each of the genes. The expression of pre-pro-TGF-α mRNA and pre-pro-EGF mRNA were significantly higher in CaP ($n = 13$) than BPH ($n = 21$) specimens ($p < 0.05$). The androgen-responsive prostatic carcinoma cell line, LNCaP, expressed high levels of EGF mRNA while the androgen-independent DU145 and PC-3 cell lines expressed high levels of TGF-α mRNA and EGFR mRNA. In general, overexpression of these mRNAs was not associated with amplification or detectable gene rearrangment; only DU145 cells demonstrated any alteration in these genes, with apparent amplification of the TGF-α gene. Relative to BPH, all prostate carcinomas and cell lines studied had elevated levels of mRNA for one or both mRNA coding for the ligands for EGFR. Thus enhanced expression of the ligands and co-expression of the EGF receptor are frequent events in human prostate tumors, consistent with the cell culture data supporting autocrine growth regulation via EGFR-mediated pathways. (Mol Cell Biochem **126:** 151–158, 1993)

Key words: prostate carcinomas, benign prostatic hyperplasia, epidermal growth factor receptor, epidermal growth factor, transforming growth factor alpha

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Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein consisting of an extracellular binding domain and an intracellular domain that exhibits tyrosine kinase activity upon stimulation by epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α) [1–3]. Binding of EGF or TGF-α to EGFR initiates a signal transduction process that results in the stimulation of cellular proliferation [4, 5]. There is evidence that the EGF receptor system is involved in oncogenic transformation of cells. Overexpression of EGFR can transform cells in a ligand-dependent manner. The v-erb B oncogene codes for a product homologous to the cytoplasmic domain of the EGFR [6]. TGF-α is produced by many transformed cells and has been identified in several tumor cell lines [7–9]. The amplification of EGFR gene has been documented in some tumors, such as squamous cell carcinomas, malignant gliomas, breast, gastric and renal carcinomas [7–12]. These investigations implicate the EGFR and its ligands, EGF and TGF-α, in regulating the growth of many malignant cell types [13–15].

The response of human prostate cancer cells to growth factors is poorly understood. Although it is clear that androgens play a crucial role in supporting prostate tumor growth [16–19], the limited success of androgen deprivation therapies in controlling prostate cancer progression suggests a role for other growth control pathways [20]. Human EGF has been found in prostatic fluid [21] and in extracts of prostate tissue, including benign prostatic hyperplasias (BPH) and prostate carcinomas (CaP) [22, 23]. Both EGF and TGF-α have been detected in the human prostatic cell lines (DU145, PC-3 and LNCaP) [18, 24–26]. EGFR has been demonstrated biochemically and immunocytochemically in human BPH and CaP tissue [27, 28]. Elevated EGFR mRNA levels have been detected by using RNase protection assay in prostatic carcinoma tissue and cell lines (DU145 and PC-3) [30]. Many investigators have proposed that some transformed cells are able to overcome normal growth restraints by producing growth factors which act through autocrine mechanisms [13, 14]. For prostate cancer cell lines, autonomous growth involving TGF-α and the EGFR pathway has been demonstrated [29, 37, 38]. The findings indicate that enhanced expression of the ligands in the presence of the EGFR contribute to growth of prostate cancer cells in culture. Were this mechanism also operating in human prostate tumors *in vivo*, then overexpression of the corresponding genes should be evident in patient samples.

In order to address this question, we examined the structure, dosage and expression of the genes for EGF, TGF-α and EGFR in a series of 34 surgical samples of prostate tissue and three human prostatic carcinoma cell lines.

Materials and methods

Cell lines

The human prostatic cell lines DU145, PC-3, and LNCaP [31–33] were grown as monolayer cultures in essential media supplemented with 10% heat-inactivated bovine serum and 1% antibiotic-antimycotic solution (Gibco Laboratories, Grand Island, New York). Cells were harvested at early confluence.

Prostate tissue

Benign prostatic hyperplasia tissue and carcinomatous prostate tissue was obtained at the time of surgery, and graded prior to freezing at – 70° C. The cancer samples were categorized according to their state of glandular differentiation using the Gleason system [34].

RNA preparation

Total RNA was extracted from human prostate tissues and cell lines by a guanidinium isothiocyanate procedure and centrifugation through a cesium chloride gradient [35]. Poly A^+ RNA was selected using oligo dT cellulose chromatography [36].

Genomic DNA preparation

Genomic DNA from cell lines was isolated by proteinase K digestion followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation [35].

RNA analysis

For dot blot analysis, total RNA was bound to nitrocellulose in aliquots containing 10 μg, 5 μg, 2 μg, 1 μg RNA. Each sample was represented on duplicate dots.

For Northern analysis, 15 μg of poly A^+ selected RNA was separated by electrophoresis on 1% agarose/formaldehyde gels and blotted onto nitrocellulose membrane [35].

DNA analysis

For genomic Southern blots, 10 μg of genomic DNA was digested with either PvuII or PstI, separated by electrophoresis on a 1% agarose gel and transferred to nitrocellulose membrane.

Hybridization

RNA dot blots, Northern blots and genomic Southern blots were sequentially incubated with cDNA probes for EGFR (# 57346, ATCC, Rockville, MD), TGF-α (# 59950, ATCC, Rockville, MD) and EGF (received from Dr. G.I. Bell) [39] labeled by nick translation with α-^{32}P-dCTP (specific activity of 1×10^8 dpm/μg). RNA dot blots also were incubated with 28S ribosomal RNA probe; Northern blots were hybridized also with a probe for β-actin (both probes were gifts from Dr. R. Matusik). Southern blots were incubated with prolactin (# 31721, ATCC. Rockville, MD), growth hormone (# 31389, ATCC, Rockville, MD), pip (received from Dr. R.P.C. Shiu) [40] and D2S5 (# 57170, ATCC, Rockville, MD) cDNA probes.

Hybridizations were performed at 42° C overnight, in 50% deionized formamide, 5 × Denhardt's solution, 0.1% SDS, 5 × standard saline phosphate-EDTA, and 200 μg/μl of denatured salmon sperm DNA. Blots were washed twice for 15 min at room temperature in 2 × standard saline citrate, 0.1% SDS and twice for 15 min at 65° C in 0.1 × standard saline citrate, 0.1% SDS [35]. Blots were exposed to X-OMAT film at – 70° C with intensifying screens. Autoradiographs were quantitated by scanning densitometry.

Results

In order to assess the potential for autocrine regulation of growth via EGFR-mediated pathways in human prostate tumors, we examined the expression of mRNAs encoding EGF, TGF-α and EGFR. Total RNA was isolated from samples of prostate carcinoma (n = 13), BPH (n = 21) and three of prostatic carcinoma cell lines. RNA from the cell lines was used in both Northern analysis and RNA dot blots to verify the specificity of the DNA probes in assessing the relative expression of each tran-

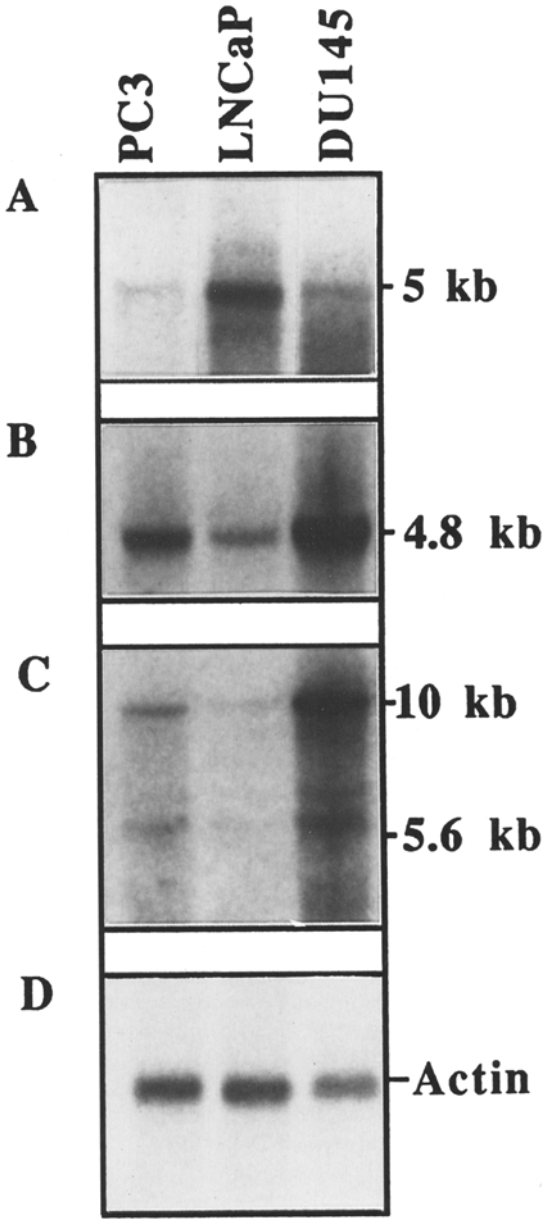

Fig. 1. Northern blot analysis of mRNA (15 μg/lane) from the prostatic carcinoma cell lines. α-^{32}P-labeled cDNA probes for: A, EGF; B, TGF-α; C, EGFR and D, actin were hybridized sequentially to the blot. A, EGF; B, TGF-α; C, EGFR and C, β-actin. Molecular weights of the transcripts are indicated at right in kilobases.

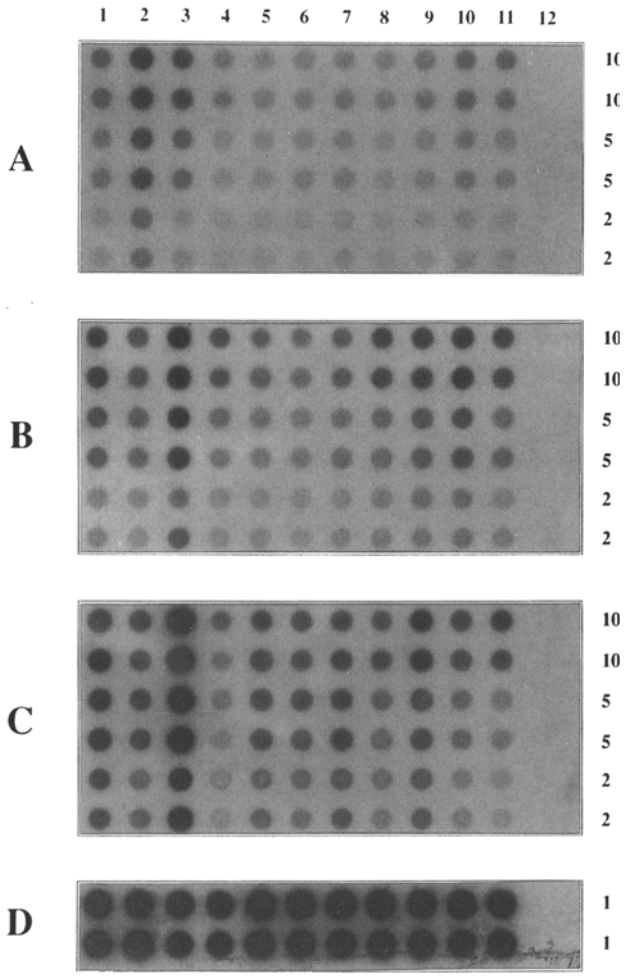

Fig. 2. Representative dot blot analysis of RNA isolated from human prostate tissue and prostatic cell lines. Hybridization of α-^{32}P-labeled cDNA probes (A, EGF; B, TGF-α; C, EGFR; and D, 28s rRNA) to samples of total RNA ranging from 10 µg to 2 µg (1 µg in D for the rRNA probe). Lanes contain RNA from: 1, PC-3; 2, LNCaP; 3, DU145; 4–7, BPH tissue; 8–11, CaP tissue, 12, yeast tRNA.

script. RNA from the patient samples was only sufficient for RNA dot blots.

The presence of authentic transcripts of each gene was confirmed by Northern analysis of poly A$^+$ RNA from the three cell lines. As shown in Fig. 1 both the 10.8 Kb and 5.6 Kb transcripts of EGFR mRNA were clearly detected in all three cell lines. No aberrant transcripts of any of the genes were found. The LNCaP cell line contained the most mRNA for EGF, while mRNAs for TGF-α and EGFR were higher in DU145 and PC-3 cell lines.

The Northern analysis of poly A$^+$RNA was in agreement with the RNA dot blot of total RNA from the cell lines (Fig. 2, lanes 1–3). Relative expression was determined by densitometric scanning and regression analysis of the dilution series on the dot blot. Among the cell lines, the steady state level of EGF mRNA was highest in LNCaP cells, TGF-α and EGFR mRNAs were higher in DU145 and PC-3 cells (Fig. 3).

Having confirmed that RNA dot blots can be used to assess the relative expression of the EGF, TGF-α and EGFR transcripts, we next sought to evaluate their levels in patient samples. We examined 13 prostate carcinomas with Gleason scores ranging from 4 to 9 (higher

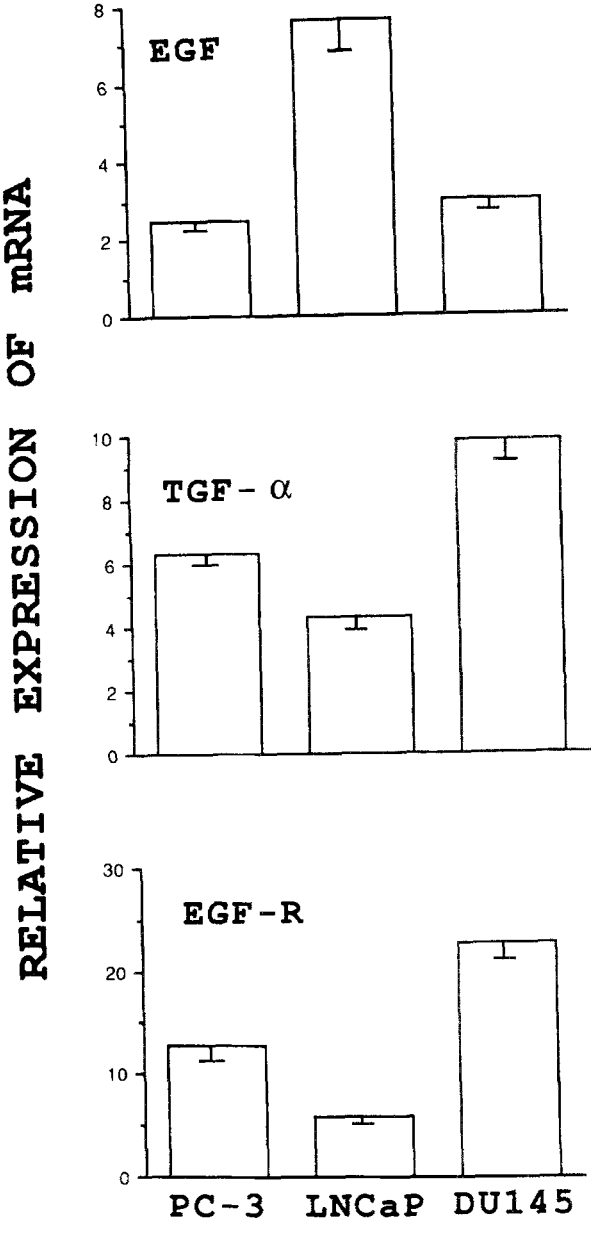

Fig. 3. Relative expression of EGF, TGF-α and EGFR mRNA in total RNA from prostatic carcinoma cell lines. The relative abundance of each mRNA was determined by densitometric scanning of autoradiographs of RNA dot blot. Mean ± S.E. for five aliquots of each cell line are represented by the bars.

numbers indicating greater de-differentiation). The tissue examined were 70% or more carcinoma. Only pathology assessments were available on this series of samples; clinical staging was not available. For comparison, non-malignant BPH samples from 21 patients were examined. Densitometric analysis of the RNA dot blots indicate that, relative to BPH samples, carcinoma samples contain significantly higher levels of EGF mRNA and TGF-α mRNA ($P < 0.05$) (Fig. 4).

In a previous study using RNase protection, we demonstrated that mRNA for EGF receptor is higher in CaP than BPH tissues [30]. A similar trend was observed in the present study, although significance was not reached with the smaller sample size. While the carcinoma samples demonstrated enhanced expression of the ligand transcripts and, in some cases, the receptor mRNA, the highest levels of these mRNAs were found in the cell lines.

To ascertain whether this overexpression is associated with amplification or rearrangement of TGF-α, EGF and EGFR genes in the prostate carcinoma cell lines, genomic Southern blots were performed. In addition to hybridization with TGF-α, EGF and EGFR cDNAs, blots were hybridized also with prolactin cDNA probe, to quantitate loading of DNA (data not shown). To control for possible aneuploidy involving any of chromosomes 2, 4 and 7, the Southern blots were also hybridized with other unique probes for each of these chromosomes which carry the EGF, TGF-α and EGFR genes, respectively. The Southern blots indicate a normal diploid complement for each of chromosomes 2, 4 and 7 in all cell lines (data not shown).

No gross rearrangement of the EGF, TGF-α or EGFR genes was observed (Fig. 5). The two high molecular weight fragments of the EGFR gene which appear in the PC-3 lane represent polymorphic alleles rather than gene rearrangement, since the pattern is seen only in Pst I-restricted DNA. With other restriction enzymes (EcoR I, Hind III and Pvu II), all three cell lines show identical Southern blot bands for the EGFR gene (data not shown). In DU145 cells, there appears to be low level amplification of the TGF-α gene estimated to be < 5-fold (Fig. 5). No other gene amplification was evident.

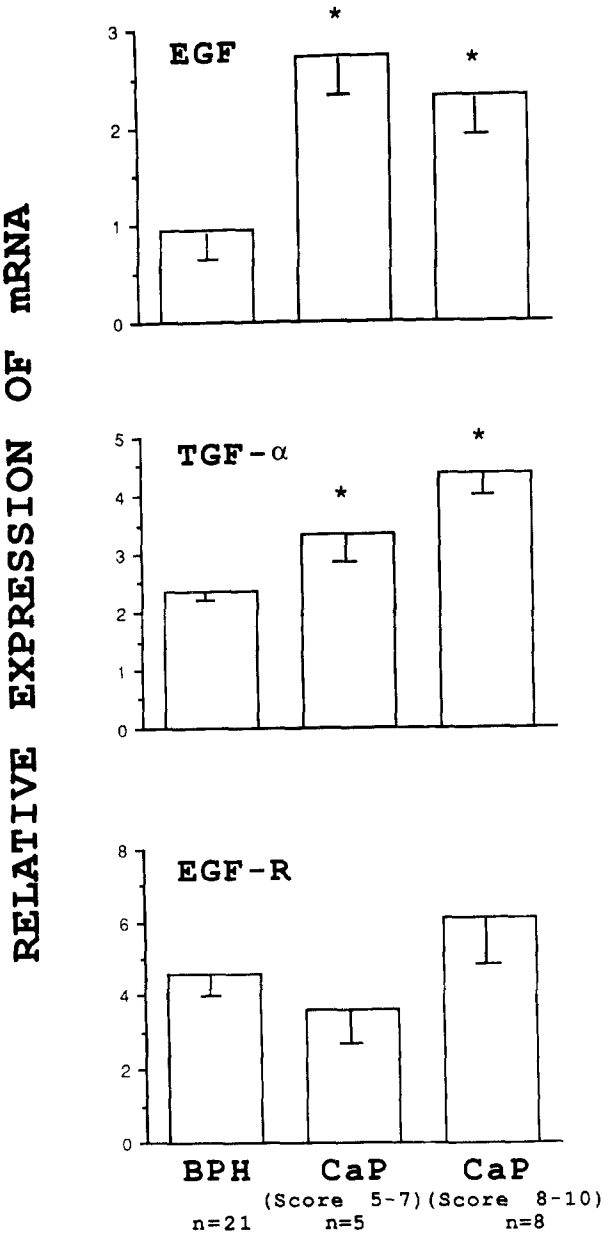

Fig. 4. Relative expression of EGF, TGF-α and EGFR mRNA in total RNA from prostate tissue. The relative abundance of each mRNA was determined by densitometric scanning of autoradiographs of RNA dot blots. BPH, benign prostatic hyperplasia. CaP (score 5–7), carcinoma with a combined Gleason score between 5 and 7 inclusive. CaP (score 8–10), carcinoma with a combined Gleason score of 8 or greater (poorly differentiated). Means ± S.E. are represented by the bars. Significant differences ($p < 0.05$) from levels in BPH are indicated by *.

Discussion

Relapse following androgen-ablation therapy in men with metastatic prostate cancer is a serious clinical problem. The mechanisms by which prostate cancer cells grow in response to non-androgenic signals is only beginning to be defined. Several groups have investigated the possible role of autocrine growth regulation involving EGFR-mediated signal transduction. TGF-α has been shown to be mitogenic in DU145 and LNCaP cells [25, 26, 37]. EGFR expression has also been demonstrat-

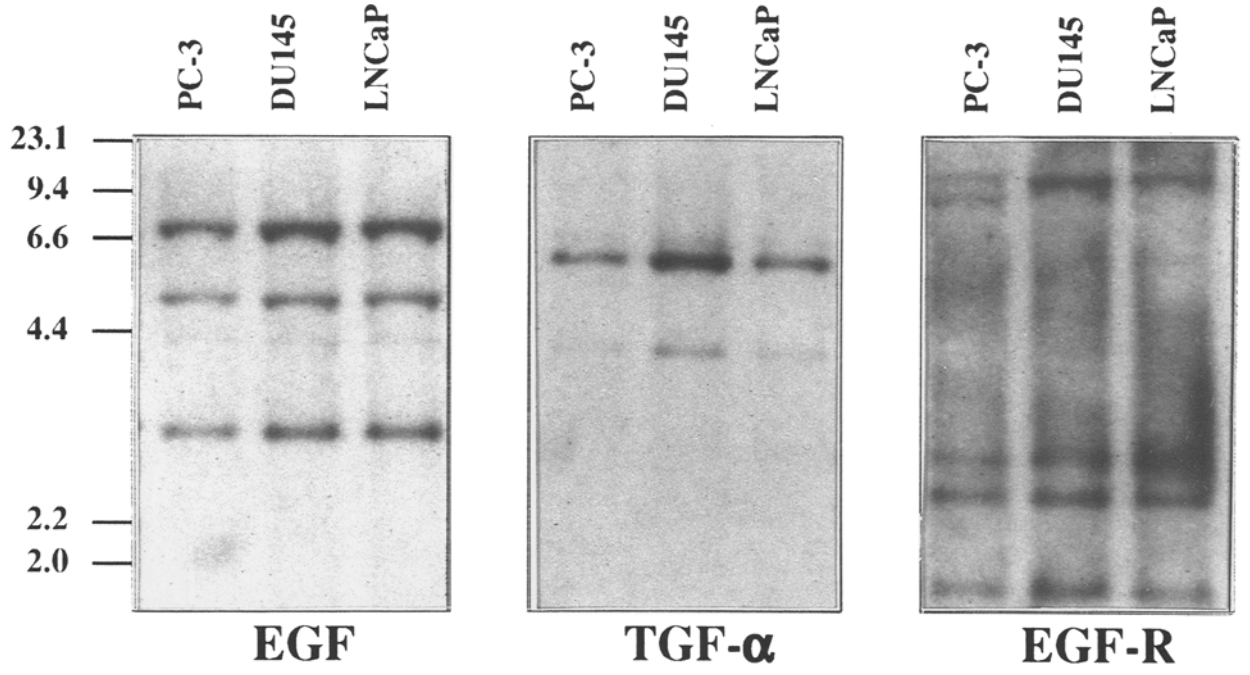

Fig. 5. Genomic Southern analysis of DNA from the prostatic carcinoma cell lines. Genomic DNAs (10 μg/lane) were digested with Pst I, fractionated by gel electrophoresis and transfered to nitrocellulose membrane. α-^{32}P-labeled cDNA probes for EGF, TGF-α and EGFR were hybridized sequentially to the blot. Molecular weight markers are indicated at left in kilobase pairs.

ed in prostatic cancer cell lines [24–26, 30]. More recently, blockade of EGFR-mediated signal transduction has been shown to reduce growth of androgen-independent PC-3 cells [29] and DU145 cells [38]. Although autocrine growth regulation via this pathway appears to explain the androgen-independence of these cell culture model systems, it is not yet clear how important such regulation is for prostate cancer *in vivo*.

To address this question, we evaluated the expression of EGF, TGF-α and EGFR genes in a series of prostate tissues and in three prostatic carcinoma cell lines. Both pre-pro TGF-α and pre-pro EGF mRNAs are significantly elevated in prostate carcinomas compared to benign hyperplasias. Among the cell lines it is interesting to note that the highest levels of TGF-α mRNA are seen in the two androgen-independent cell lines, DU145 and PC-3. The androgen-responsive LNCaP cell line expresses low levels of TGF-α but high levels of EGF mRNA. Part of the progression to hormone independence may involve a switch in the predominant ligand from EGF to TGF-α. Among the cell lines, neither aberrant mRNAs nor gene rearrangements were evident. Only in DU145 cells was a modest level of gene amplification seen for the TGF-α gene (< 5-fold). These data, therefore, suggest that overexpression of EGF and TGF-α is a frequent alteration in prostate tumor cells but likely does not relate to gene amplification. The overexpression may be due to altered transcriptional or post-transcriptional control of the mRNA levels. If the levels of mRNAs observed reflect the levels of processed ligands, then tumor growth stimulation by autocrine mechanisms like those seen in cell cultures are highly possible *in vivo* as well.

In relation to the EGF receptor, all prostate samples expressed transcripts. The elevated EGFR mRNA levels seen in the cell lines were not associated with either gene amplification or rearrangement. This is in contrast to other human cancers; amplification of EGFR DNA has been associated with breast, squamous cell carcinomas, gastric and renal carcinomas [7–12]. In our study, all samples demonstrated co-expression of the EGFR mRNA and the ligand mRNAs. It is unclear whether overexpression of the ligands and the receptor is necessary for autocrine growth *in vivo* or whether enhanced levels of either are sufficient. In transfection experiments, enhanced expression of either EGFR [41] or TGF-α or EGF [42, 43] was sufficient to induce transformation. In our study, it is clear that EGF or TGF-α expression appear enhanced even when the expression of the receptor mRNA is modest.

Our results extend the observations of others regarding autocrine regulation of androgen-independent pros-

tate cancer cells in culture to an examination of prostate tumor tissues *in vivo*. The data support the involvement of EGFR-mediated growth in the phenotype of prostate carcinoma primarily through the quantitative modification of ligand and receptor expression.

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