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Modulation of Pro-Epidermal Growth Factor, Pro-Transforming Growth Factor α and Epidermal Growth Factor Receptor Gene Expression in Human Renal Carcinomas[1]

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ABSTRACT

We have analyzed the expression of the genes for the precursors of epidermal growth factor (pro-EGF) and transforming growth factor α (proTGF-α) as well as for the EGF receptor in tissue specimens of a large number of adult patients with renal cell carcinoma. Since normal kidney tissue was available from the same patients we could directly compare the expression of these genes in tumors with that in adjacent normal renal tissue. Our experiments reveal underexpression of the proEGF gene in all tumors analyzed (21 of 21) and overexpression of the genes for proTGF-α (33 of 33 analyzed) and EGF receptor (22 of 23 analyzed) in tumor samples, when compared with normal kidney tissue. The expression of the proTGF-α gene appeared to depend on grade and differentiation of the tumor, since well differentiated tumors (grade 1) expressed more proTGF-α mRNA than the adjacent normal tissue but significantly less than poorly differentiated tumors (grade 2 or 3), which are the most aggressive ones. In none of these tissue specimens did we find, by Southern analysis, amplification of the proTGF-α or EGF receptor gene. Therefore, overexpression of these genes must be due to another effect, perhaps an alteration of their mRNA turnover. Although the EGF receptor gene (c-*erb*B1) is overexpressed in nearly all carcinomas analyzed, there was no linear coexpression with the proTGF-α gene. In contrast, transcription of the proEGF gene was completely turned off in tumor tissue. Although we have found by restriction fragment length polymorphism analysis, in one of three tumor samples, evidence for a somatic mutation within the proEGF gene, we do not know yet, due to the limited number of Southern analyses, whether this somatic mutation is causally involved in the decrease of proEGF mRNA expression and, hence, is representative of renal cell carcinoma. To our knowledge, this is the first observation on primary tumor tissue in humans that upon malignant transformation the gene for a polypeptide growth factor gene is underexpressed.

INTRODUCTION

The uncontrolled growth of tumor cells may be due in part to the ability of transformed cells to produce a variety of different polypeptide growth factors which are involved in the autostimulation of growth (autocrine stimulation hypothesis) of these cells (1). Alternatively, altered susceptibility to physiological growth-regulating polypeptides (altered signal transduction hypothesis) could account for the malignant phenotype. Thus, for example, a structurally modified growth factor receptor has been described in human leukemic cells, which could be crucial for leukemogenesis.[5]

EGF[6] is a 53-amino acid single-chain polypeptide with a molecular weight of 6045, which acts through a specific cell surface receptor (c-*erb*B1). Receptor activation leads to a variety of biochemical events which cause stimulation of DNA replication, cell division, or differentiation of normal mesenchymal and epithelial (2) as well as malignant cells (3). Although EGF is detectable in many adult mammalian tissues such as pancreas, gut, bone marrow, and mammary and salivary glands (4, 5), one major site of production is the kidney (6, 7). EGF is produced as a large precursor protein (proEGF), which can exist as a biologically active transmembrane protein (8, 9). Because of this localization and its similarity to the LDL receptor, a dual function as a precursor for EGF and a membrane receptor for a hitherto unknown ligand has been postulated (10). The cDNA for human proEGF has been cloned (8) and the gene was mapped to chromosome 4q25–27 (11).

The EGF receptor is a glycoprotein with kinase activity, which catalyzes autophosphorylation at tyrosine residues (12). The gene for this protein is localized on chromosome 7 p12–13 (13).

Another ligand for the EGF receptor is TGF-α (14). This structurally related polypeptide was originally identified by its ability to induce reversibly the transformed phenotype of kidney fibroblasts. TGF-α shares about 35% homology with EGF, with conservation of all six cysteine residues. Cloning of the TGF-α cDNA has revealed that this polypeptide is also synthesized as a larger precursor molecule (proTGF-α), an integral membrane glycoprotein of 160 amino acid residues. The corresponding gene has been mapped to chromosome 2 p13 (15). The 50-amino acid TGF-α derives from the extracellular part of the precursor by proteolytic cleavage. Proteolytic processing, however, is not mandatory for biological activity, since the precursor protein itself can elicit a biological response in the target cell (16, 17). The amino acid sequences of TGF-α of rodents and humans are highly conserved throughout evolution, with about 90% identity, hinting at an important biological function. However, little is known about the physiological function of TGF-α. Fetal tissues and transformed cells (18) have been identified as major sources of TGF-α; since fetal tissues do not express EGF (19) but, nevertheless, express EGF receptor (20), it has been suggested that TGF-α is the embryonic form of EGF that is ectopically expressed in transformed tissues (21). Although TGF-α expression has also been shown in some normal adult tissues such as human skin epidermis (22) and activated macrophages (23), in certain experimental systems aberrant expression of TGF-α can lead to transformation (24, 25).

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[6] The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor α; RFLP, restriction fragment length polymorphism; cDNA, complementary DNA.

Renal adenocarcinoma in humans is an aggressive malignancy which arises from the renal tubular epithelial cell (26). Nephrectomy is the treatment of choice upon diagnosis; however, at that time most tumors have already micrometastasized and no effective chemotherapy or hormonal therapy exists for this tumor (27). In order to gain insight into the molecular mechanisms which underly this malignancy, we have analyzed renal tissue samples, from adult patients who underwent radical nephrectomy, for the expression and structure of the genes for the precursors of EGF and TGF-α and their common membrane receptor.

MATERIALS AND METHODS

Tissue Samples. Human renal cell carcinoma and adjacent normal kidney tissues from untreated patients were obtained at the time of surgical resection, immediately frozen in liquid nitrogen, and stored at −80°C until RNA and DNA extraction. Histological diagnosis and nuclear grading was provided by the Department of Pathology, Technical University Munich.

cDNA Probes. The following probes were used: a 4.8-kilobase cDNA of proEGF (phEGF116), kindly provided by Graeme Bell, University of Chicago; a 1.0-kilobase cDNA of proTGF-α (pSP65-C17N5), kindly provided by Rik Derynck, Genentech Inc. (South San Francisco, CA); and a 1.8-kilobase cDNA of the EGF receptor (c-*erb*B1) (pHER-A64-1), commercially available from the American Type Culture Collection, (Richmond, VA).

Isolation of RNA and Northern Blot Analysis. Total cellular RNA was extracted from 1 g of frozen tissue using the guanidine isothiocyanate/cesium chloride method (28). Polyadenylated RNA was purified by oligothymidylate chromatography (29). The integrity of each sample was verified by electrophoresis on agarose gels. The concentration of RNA was determined spectrophotometrically at 260 nm. RNA samples were ethanol precipitated and stored at −20°C. Polyadenylated RNA was glyoxylated at 50°C for 60 min, electrophoresed (4–5 μg) on 1% agarose gels (29), and transferred to nylon membranes (Hybond N; Amersham) according to the instructions of the manufacturer. The membranes were exposed to UV light for 5 min and deglyoxylated at 80°C for 2 h. Prior to hybridization, the integrity of the RNA was confirmed by staining the nylon membranes with methylene blue. Membranes were prehybridized for 90 min and then hybridized with nick-translated ^{32}P-labeled cDNAs (specific activities, 0.8–1.0 × 10^8 cpm/μg) in sealed plastic bags for 36 h, either at 56°C in the presence of formamide (29) or at 65°C in the absence of formamide (30). Membranes were then washed under high stringency conditions and exposed to Kodak XAR-5 film at −80°C for 3–8 days, using two intensifying screens.

Isolation of DNA and Southern Blot Analysis. High molecular weight genomic DNA was isolated from 1 g of frozen tissue by standard methodology (29). Integrity of the DNA was evaluated by electrophoresis on a 0.8% agarose gel, by comparison with uncut λ DNA. Ten μg of genomic DNA were digested with restriction endonucleases (*Eco*RI or *Taq*I, purchased from GIBCO or BRL), electrophoresed on 1% agarose gels, and transferred to nylon membranes (Amersham). Hybridization and autoradiography were carried out as described (29).

RESULTS

Primary Tumor Samples. RNA and/or DNA samples were available from 33 randomly selected patients undergoing radical nephrectomy for renal cell cancer. Twenty-one normal tissue/tumor sample pairs were analyzed for proEGF expression, 23 for EGF receptor expression, and 33 for proTGF-α expression. The age of the patients ranged from 41 to 79 years; 8 were women and 25 were men. Twenty-five of the tumors were grade 1 and 8 were grades 2 and 3.

Expression and Structure of the ProEGF Gene. Four representative examples of Northern blot analysis of proEGF mRNA expression are shown in Fig. 1. A specific 5.0-kilobase transcript was identified in all normal kidney samples analyzed (21 of 21) but in none of the carcinomas (0 of 21). Digestion of cellular genomic DNA with *Eco*RI and subsequent hybridization with the proEGF probe showed that the restriction fragment length pattern was identical in two specimens when tumor and normal tissue were compared (Fig. 2). In a third sample, however, a RFLP was observed, indicating a somatic mutation in the proEGF gene (Fig. 2).

Expression and Structure of the proTGF-α Gene. Northern blot analysis using proTGF-α cDNA revealed that a specific 4.8-kilobase proTGF-α mRNA could be detected in carcinoma tissue of all patients investigated (33 of 33) but not in the normal parts of the same kidney. The level of TGF-α mRNA expression appeared to depend on the histological grade and differentiation status, since less well differentiated tumors produced more proTGF-α mRNA (Fig. 3). Because overexpression of the proTGF-α gene could be due to gene amplification of rearrangements, we prepared genomic DNA from 16 samples of renal cell carcinoma and normal kidney tissue for comparative Southern Blot analysis. Digestion of cellular DNA with *Taq*I and subsequent hybridization did not reveal evidence for amplification of the gene or any abnormal digestion patterns indicative of rearrangements (Fig. 4).

Expression and Structure of the EGF Receptor (c-*erb*B1) Gene. Fig. 5 shows representative Northern blot analyses of the EGF receptor mRNA in five paired tissue samples. Typically, a major 10-kilobase transcript was identified; in some of the samples there were additional 4.6- and 3.3-kilobase transcripts present. There was a wide range of variation in the level of expression of this gene among the normal kidney tissues, but it was consistently elevated in almost every tumor sample, as compared to its normal counterpart (22 of 23). In a few of the carcinoma samples with strongly elevated EGF receptor mRNA levels, there was also enhanced proTGF-α expression (data not

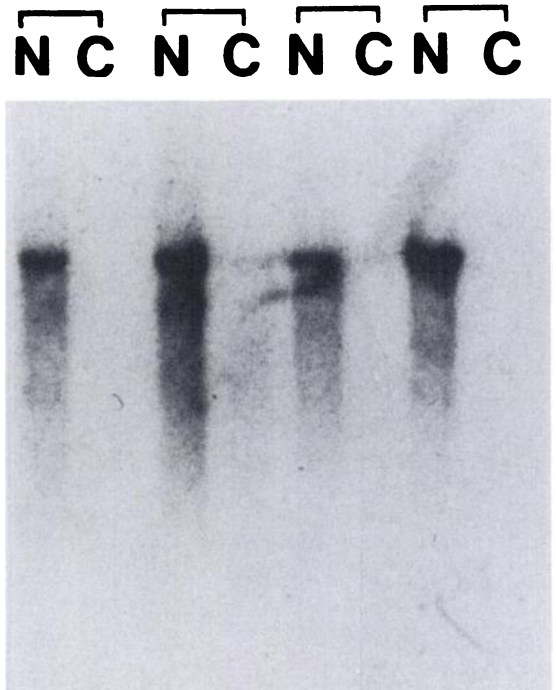

Fig. 1. Northern blot analysis of mRNA derived from renal cell carcinoma (*C*) and adjacent normal tissue (*N*) from four patients, using the proEGF cDNA. Each lane contains 5 μg polyadenylated RNA. Transcript size was 5.0 kilobases, as determined by using 28S and 18S rRNA as molecular weight markers.

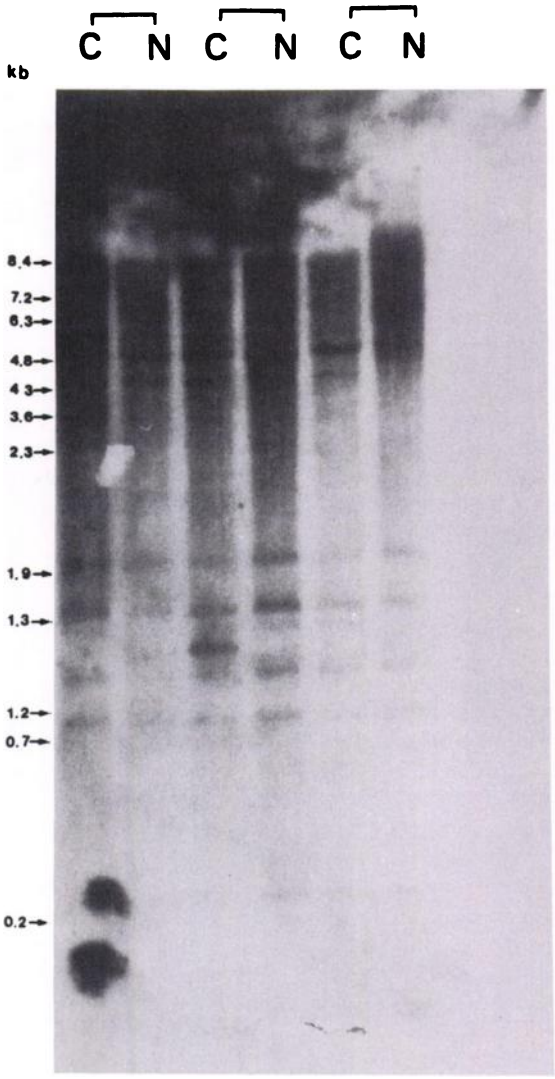

Fig. 2. Southern blot analysis of genomic DNA derived from renal cell carcinoma (*C*) and adjacent normal tissue (*N*) from three patients using the proEGF cDNA. Ten μg *Eco*RI-digested DNA were loaded in each lane.

shown). Southern analysis of the EGF receptor gene by digestion with *Eco*RI and subsequent hybridization did not reveal evidence for major rearrangements or RFLPs (Fig. 6) in three paired samples.

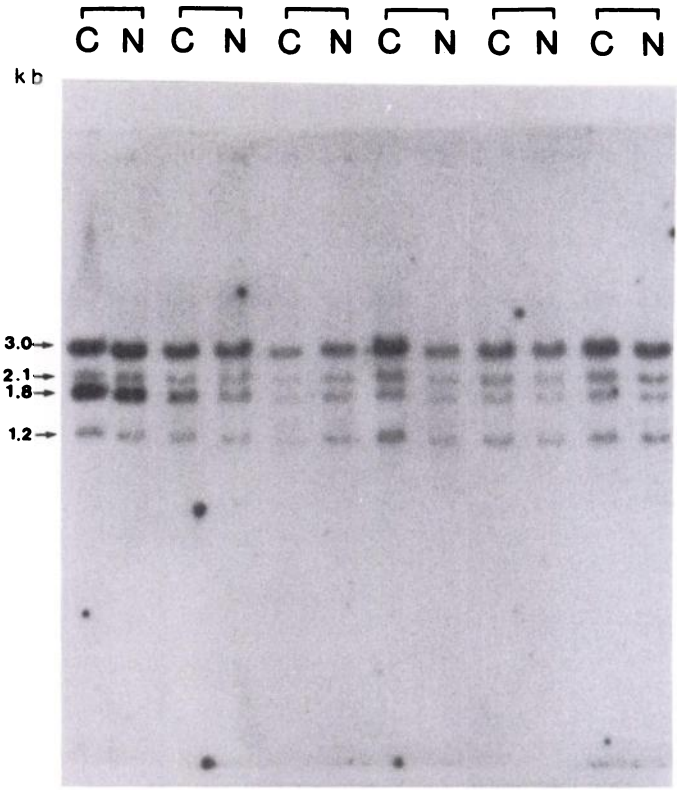

Fig. 4. Southern blot analysis of genomic DNA derived from renal cell carcinoma (*C*) and corresponding normal tissue (*N*) from six patients, using the proTGF-α cDNA. Fourteen μg *Taq*I-digested DNA were loaded in each lane.

DISCUSSION

Several polypeptide growth factors, including EGF and insulin-like growth factor I, have been implicated in the regulated renal growth following tubular necrosis (31). Very little, however, is known about the precise role of each individual factor in the regulatory network which determines the pattern of normal growth (32). Renal carcinoma arises from the abnormal proliferation and differentiation of epithelial cells; polypeptide growth factors such as interleukin 6 (33) and transforming growth factor β (34) have been implicated as growth-stimulatory or -inhibitory molecules for renal carcinoma cells under *in vitro* conditions. Kidney tissue as well as urine from patients with renal cell cancer contain increased amounts of basic fibroblast

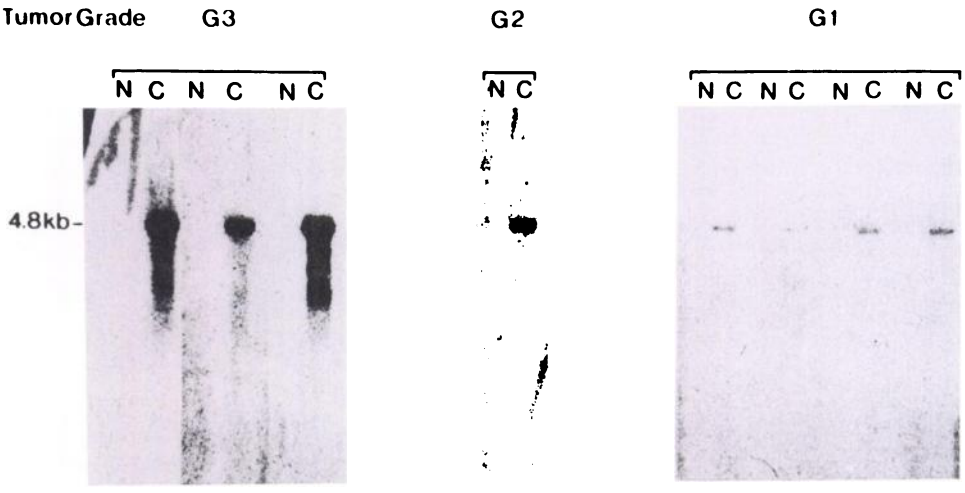

Fig. 3. Northern blot analysis using the cDNA for proTGF-α. Five μg mRNA from tumor tissue (*C*) and corresponding normal kidney tissue (*N*) were loaded in each lane. This figure shows representative examples of tumors of three different histological grades and differentiation status.

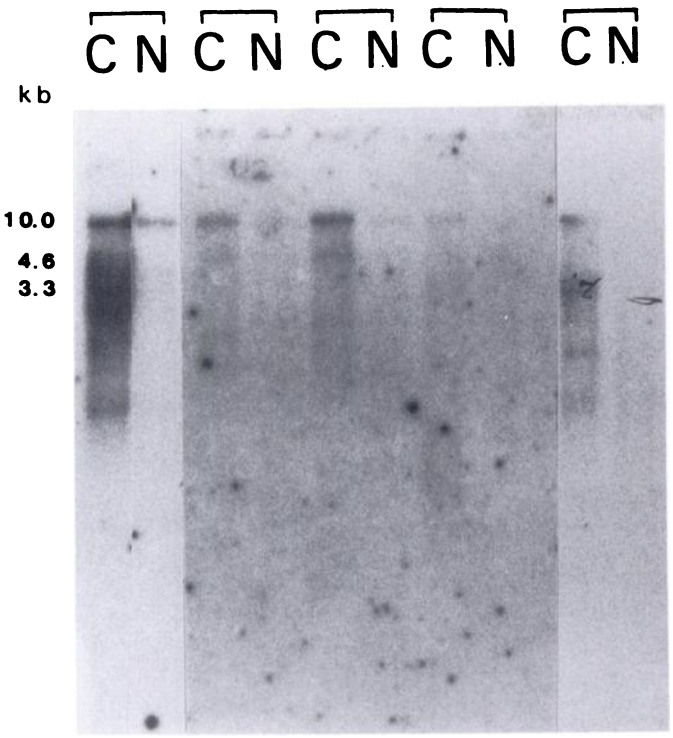

Fig. 5. Northern blot analysis of five representative pairs of renal cell carcinomas (*C*) and normal tissue (*N*), using the EGF receptor cDNA.

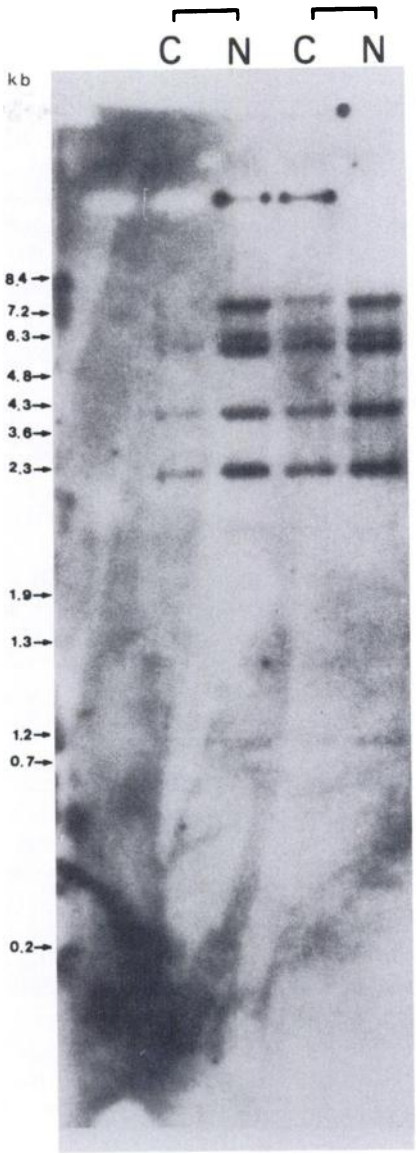

Fig. 6. Southern blot analysis of genomic DNA derived from renal cell carcinoma (*C*) and adjacent normal tissue (*N*) from two patients, using the EGF receptor cDNA. Ten μg *Eco*RI-digested DNA were loaded on each lane.

growth factor activity (35). Again, very little is known about the potential *in vivo* role of these and other polypeptide growth factors. Moreover, no systematic *ex vivo* study on a larger series of human tumor samples is available, which is a prerequisite to establish a participation and to pinpoint the function of each individual regulatory polypeptide factor.

In this report we have shown that expression of the proEGF gene was abolished in all of the carcinomas studied (21 of 21). Since an additional function for proEGF as a receptor for a hitherto unknown ligand has been postulated (10), it could well be that down-regulation of proEGF gene expression renders the kidney cell unresponsive to this unknown ligand. Recently, it has been reported that in an animal model proEGF mRNA expression is down-regulated in renal cell injury caused by *cis*-platinum infusion (36). At the same time secretion of EGF into urine is reduced. Since there are many similarities between response to cell injury and tumor formation (37, 38), the dramatic decrease of proEGF mRNA levels in renal cell cancer as well as renal injury can point to mechanisms common to both situations. Using Southern analysis in three tumor samples, we identified a RFLP for *Eco*RI in one patient; normally the proEGF gene is not polymorphic for *Eco*RI (39). Whether this somatic mutation is representative of renal cell carcinomas is uncertain at the moment. Again, the fact that proEGF mRNA levels were decreased in all tumor samples studied (21 of 21) strongly indicates that this gene or its product may be involved in the pathobiochemistry of renal cell cancer. Our results are in contrast to immunohistochemical studies (40), which have reported positive staining for EGF of the epithelium in normal renal parenchyma but in addition positive cytosolic staining in human renal carcinomas. This discrepancy may be explained by the utilization of a polyclonal antibody against murine EGF.

We demonstrate enhanced expression of the proTGF-α mRNA in human primary renal cell carcinomas of all 33 adult patients investigated, when compared with normal tissue of the same kidney. These data confirm the original observation made by Derynck *et al.* (41), extend our earlier results (42), and are in agreement with recent observations (43) that describe proTGF-α mRNA elevation in 5 of 5 renal carcinomas.

Our data on a larger series of primary renal tumors strongly support the concept that TGF-α, although found in some normal cell types, is predominantly expressed in transformed cells. This idea is further corroborated by our additional observation that the level of proTGF-α mRNA expression inversely correlates with the differentiation status of the tumor. Thus, well differentiated carcinomas express much less proTGF-α than undifferentiated tumors. However, since the total number of grade 2 and grade 3 tumors (8 tumors) is low compared to the grade 1 tumors in our series (25 tumors), more samples have to be analyzed before a general conclusion can be drawn. Overexpression of the proTGF-α gene may play a crucial role in promoting or maintaining uncontrolled growth of these carcinomas. TGF-α could stimulate renal growth either directly by auto- or paracrine action upon epithelial cells or indirectly by stimulation of endothelial cells; angiogenesis is an important step in tumor progression (44). TGF-α induces angiogenesis *in vivo*, being a more potent angiogenic stimulus than EGF (45).

Correlation of hypervascularity and increasing dedifferentiation has been shown for renal carcinomas (46).

EGF does not seem to be part of an autocrine cycle in normal renal epithelial cells, since it is localized in the apical portions (47) whereas the EGF receptor is found in the basolateral area of these cells. Because EGF is unidirectionally transported from the basolateral to the apical area of these cells (48), it seems more likely that EGF-like domains in extracellular matrix proteins such as laminin (49) serve as physiological stimulators of the EGF receptor. Whether the role of overexpressed TGF-α in renal carcinomas is to substitute for such a matrix protein stimulator is unclear.

Since the proTGF-α gene was overexpressed in all 33 tumor specimens, we did restriction enzyme analysis in 16 paired normal/tumor samples but could not find any evidence for amplification or rearrangement of this gene. Hence, another effect such as a change in gene regulation leading to enhanced transcription or an increased stability (half-life) of the proTGF-α mRNA could be responsible.

Because proTGF-α can exhibit biological activity upon adjacent cells without being proteolytically processed to low molecular weight TGF-α (16, 17), further discrimination by Western blotting between large molecular weight pro-TGF-α, which is restricted in its action to the adjacent cell, and low molecular TGF-α, which may diffuse through the pericellular space to more distant cells, will be necessary.

Analysis of EGF receptor gene expression in 24 of the 33 tumor samples available revealed enhanced levels of mRNA in all but one of the tumor specimens, when compared to the corresponding normal part of the kidney. This is a much higher percentage than the 48% observed in another report (50) but about the same in a more recent study (51). That this overexpression of the EGF receptor mRNA leads to enhanced translation into protein is supported by saturation analysis experiments using radioiodinated EGF (52). In a few of the tumor samples with elevated EGF receptor mRNA levels, there was increased coexpression of proTGF-α. Again, this implies a key role of this gene in renal cell tumorigenesis. *In situ* hybridization studies are required to analyze whether the same or different cell types are responsible for the increased expression of these two genes.

The genetic alterations observed were consistent in practically all renal carcinomas analyzed. This suggests that underexpression of proEGF and overexpression of TGF-α and the TGF-α/EGF receptor may play a critical role in the development of all types of renal cell carcinomas. The fact, however, that this malignancy displays different cell types (46) implies that alterations of additional genes must be present to confer the specific phenotype to each individual subtype of renal cancer.

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