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LABORATORY INVESTIGATION

Immunochemical study of a transforming growth factor-α-related protein in the chicken kidney

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Immunochemical study of a transforming growth factor-α-related protein in the chicken kidney. A number of polypeptides are involved in renal growth and physiology. Both transforming growth factor-α (TGF-α) protein and mRNA are expressed in kidney cells during embryonic and adult stages, and exert mitogenic activity on kidney cells in culture. We studied the immunolocalization of a TGF-α-related protein at the ultrastructural level and found it in the basolateral membranes of dark cells from distal tubules of the chicken kidney. By Western blotting techniques, we identified a protein complex composed of at least two TGF-α immunoreactive subunits of 40 and 88 kDa, respectively. Both subunits were sensitive to elastase digestion, and released TGF-α immunoreactive products. In addition, TGF-α immunoreaction was found in primary culture of chicken kidney cells. These findings suggest that the TGF-α-related protein complex plays a very specific role in proliferation and/or differentiation of kidney cells.

The kidney is a target organ for a number of polypeptide growth factors. Certain of these peptides interact with sensitive renal cells as classical endocrine hormones. Others are produced within the kidney and exert actions on the cell of origin or on adjacent cells in an autocrine, paracrine or juxtacrine fashion. Some growth factors have been shown to regulate renal metabolic or transport processes *in vitro* and to control glomerular filtration and renal plasma flow when administered to humans or experimental animals [1, 2].

Growth factors are believed to mediate adaptations in renal function that occur in chronic reduction of functional renal mass and to promote regeneration following acute renal injury. Moreover, the growth of the kidney that occurs under several conditions is accompanied by enhanced expression of one or more growth factors within the organ, suggesting a causal relationship between the two events. Thus, expression of EGF, IGF-I and HGF are enhanced in the setting of compensatory renal growth [3, 4].

Furthermore, growth factors regulate renal growth and development during embryogenesis. Heparin-binding FGF-like factors produced by metanephric kidney may play a role in nephron vascularization [5] and the transient presence of NGF-receptors during differentiation of metanephroi in culture suggests a role for NGF in metanephrogenesis [6]. It is known that IGF-I, IGF-II, TGF-α and TGF-β are essential for growth and development of rat metanephric anlage *in vitro* [7–9], activating or inhibiting proliferation or differentiation of cells.

TGF-α mRNA is expressed consistently in human and rat renal cell carcinoma [10, 11]. However, the presence of TGF-α mRNA in normal kidney is controversial. Thus, Lee et al [12] detected mRNA in the normal rat kidney, whereas others failed to find it [13, 14].

TGF-α exerts mitogenic activity in several types of cultured renal epithelial cells. Argilés et al [15] showed stimulatory and inhibitory effects for TGF-α on proliferation of a human renal adenocarcinoma cell line depending on cell status. TGF-α also enhances renal cell differentiation [16].

TGF-α has been shown to inhibit phosphate uptake in OK cells [17] and stimulate transport of bicarbonate and volume in addition to phosphate in proximal convoluted tubules [18].

TGF-α is synthesized as part of a larger transmembrane precursor (pro-TGF-α) from which the mature TGF-α-form is released by the action of a specific elastase-like protease [19, 20]. Both TGF-α secreted to the extracellular medium and membrane anchored pro-TGF-α are biologically active through the binding to EGF-R [21–23].

Our knowledge *in vivo* of the sites of growth factor synthesis and actions in the kidney is rudimentary. The presence of TGF-α immunoreactivity has been reported previously in mesonephric distal tubule cells from day 8 to day 20 of embryonic development (ED) and in metanephric distal tubule cells from day 14 of ED to the adult [24]. Other authors also have reported TGF-α immunoreactivity in adult human and rat kidney [11, 25]. Therefore, the kidney seems to be a good source and model for further investigation and characterization of TGF-α synthesized *in vivo*. The aim of this study was to enhance our knowledge of the cell biology of TGF-α in the chicken kidney. Thus, we determined the ultrastructural localization of TGF-α in nephrons of the chicken kidney. In addition, we characterized a TGF-α-related protein found on kidney blots. Furthermore, we achieved preliminary insight in the study of its physiological role in growth and differentiation in primary and secondary culture of chicken kidney cells.

Methods

Light immunohistochemistry

Kidneys from adult chicks were fixed for 24 hours in Bouin's fluid. The samples were processed routinely for embedding in paraffin and 4 μm-thick serial sections were cut.

After deparaffinization and hydration, some sections were

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treated with 3% hydrogen peroxide, 10% methanol for five minutes, and rinsed in 0.01 M PBS, pH 7.2, for 3 × five minutes. The slides were incubated at room temperature with normal rabbit serum [1:30 diluted in 0.01 M PBS, pH 7.2, 0.1% bovine serum albumin (BSA), 0.01% sodium azide (NaAz)] for 30 minutes to block nonspecific protein binding, and overnight at 4°C with mouse monoclonal anti-TGF-α (0.5 μg/ml in PBS-BSA-NaAz, Oncogene-Science Cat. No. GF10; referred to as mAb), as the first layer. The anti-TGF-α recognizes a human and rat TGF-α epitope (residues 34 to 50 of the mature TGF-α). It does not show cross reactivity with EGF [26]. We also incubated serial sections with a mouse monoclonal anti-calbindin antibody (1:800, Sigma Immunochemicals, ref. C8666). In some cases, sections were incubated with a mixture of antibodies anti-TGF-α and anti-calbindin, and the corresponding serial section with either anti-TGF-α or anti-calbindin. The sections were then rinsed in PBS and incubated for 30 minutes at room temperature with peroxidase-conjugated rabbit anti-mouse IgG; washed with PBS and incubated for five minutes with 2.5 mg/100 ml 3,3'diaminobenzidine tetrahydrochloride dissolved in PBS plus 0.03% hydrogen peroxide at room temperature. These slides were rinsed in distilled water, counterstained with Harris' hematoxylin, dehydrated using graded ethanol solutions, cleared in xylene and mounted in DPX. Some sections were counterstained with the periodic acid-Schiff (PAS) method to identify proximal tubules, since cells in this region present a PAS-positive brush border. Other sections were counterstained with Alcian blue (pH 2.5) to identify the "mucus-secreting cells" of the distal portion of the nephron.

Negative controls consisted in primary incubation with: (1) PBS-BSA-NaAz, (2) anti-TGF-α (0.2 μg/ml) preabsorbed with rat TGF-α (10 μg, fragment 1 to 50, Bachem, #H-5545).

Histochemistry for carbonic anhydrase

Sections from frozen kidneys were stained for carbonic anhydrase activity according to the method of Hansson [27] with some modifications. Sections were floated for five minutes on the surface of the incubation medium, which contained 1.75 mM $CoSO_4$, 53 mM H_2SO_4 and 11.7 mM KH_2PO_4 and 157 mM $NaHCO_3$ (freshly prepared). As a rinsing solution, 6.7×10^{-4} M PBS, pH 5.9 was used. Blackening solution was a freshly prepared 0.5% solution of $(NH_4)_2S$ in distilled water. Sections were treated with this solution for three minutes and washed in water before mounting in glycerol:PBS (1:9) with p-phenylenediamine.

Immunoelectron microscopy

Small pieces of the chicken kidneys were fixed in a mixture of 2% paraformaldehyde and 1% glutaraldehyde in PBS for two hours at 4°C. After washing in PBS the specimens were incubated in 50 mM NH_4Cl in PBS for one hour to block aldehyde residues. The specimens were dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Ultrathin sections were cut on a Reichert-Imy ultracut ultramicrotome using a diamond knife, and mounted on a nickel grid coated with formvar and carbon.

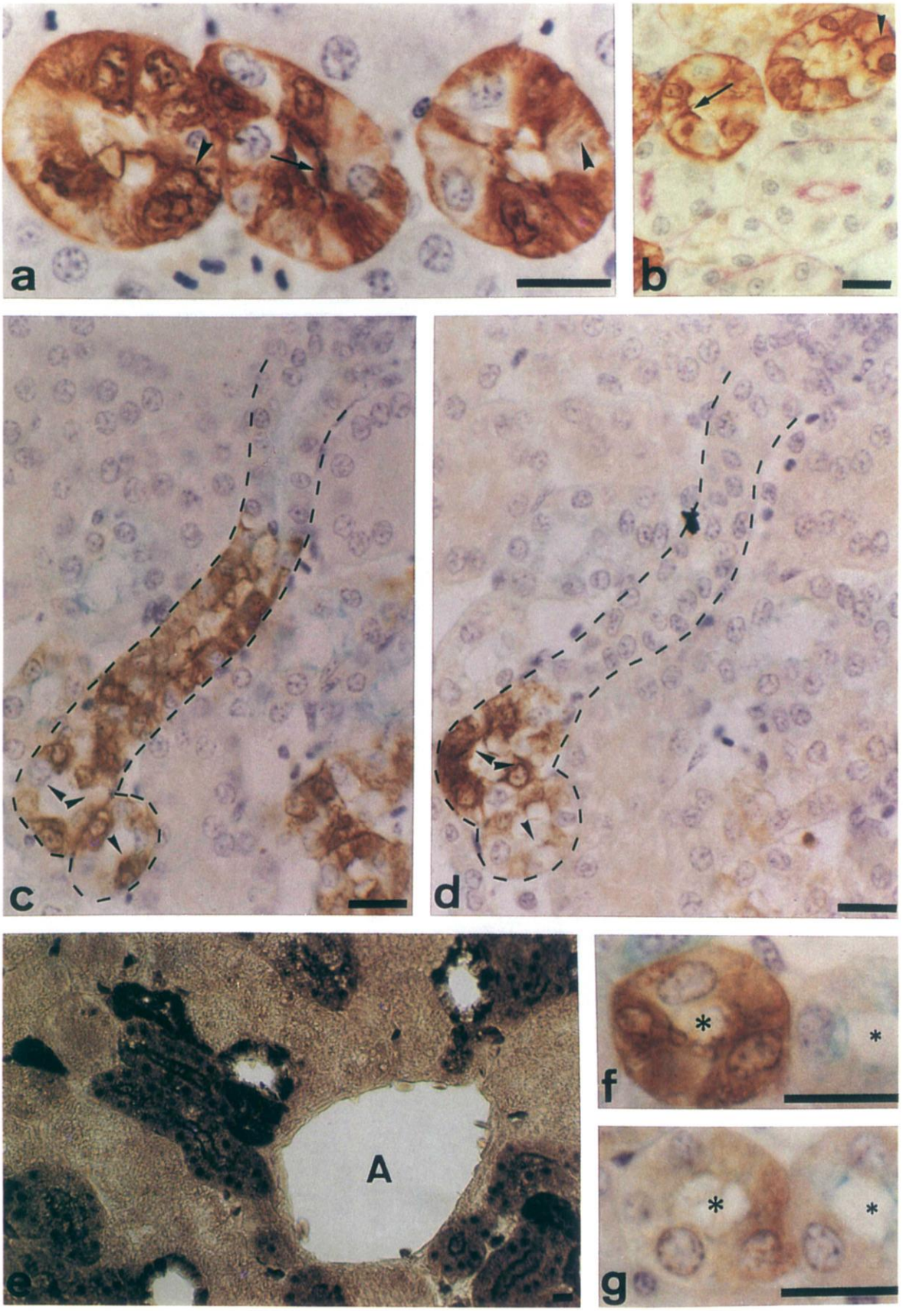
Immunoreactive sites were labeled by indirect immunogold staining as previously described [28]. The sections were incubated on a drop of normal goat serum (1:30 in PBS, pH 7.2, 0.1% BSA, 0.01% NaAz, 0.05% Tween-20), for 30 minutes to block the background, and for two hours at room temperature in mouse monoclonal anti-TGF-α (1 μg/ml, in PBS-BSA-NaAz-Tween-20; Oncogene Science, ref. GF10). The sections were washed in 5 drops of PBS and the primary antibodies were localized using colloidal gold (10 nm diameter)-labeled goat anti-mouse IgG (1:50 in PBS-BSA-NaAz-Tween 20; Sigma, ref. G7652) for one hour at room temperature. The sections were washed on drops of PBS and distilled water and stained with 2% uranyl acetate and lead citrate. Electron micrographs were taken using a Philips 301 electron microscope.

Negative controls consisted in primary incubation with PBS-BSA-NaAz-Tween 20.

Electrophoresis and Western blotting analysis

For immunoblotting analysis kidneys from adult chicks were homogenized, sonicated and left to stand for one hour at 4°C in lysis buffer containing 50 mM NaCl, 25 mM Tris-HCl (pH 8.1), 0.5% Nonidet P40, 0.5% Na-deoxicolat, 1 mM EDTA, and 0.02% NaAz, with or without 1 mM PMSF and 5 mg/ml Aprotinin. The samples were then centrifuged at 13000 rpm for 10 minutes. After determination of protein concentration in the supernatant by the Bradford method [29] samples were electrophoresed on Tricine-SDS-polyacrylamide gels [30] using sample buffer with or without beta-mercapto-ethanol. Two-dimensional gel analysis was carried out with the same samples, following the protocol of O'Farrel, Goodman and O'Farrel [31], except that the pH gradient contained 1.6% ampholytes (pH 3 to 10, purchased from Serva). Proteins were transferred to Immobilon P membranes for three hours at 60 V. Immunoblotting was performed as follows: blots were blocked in 5% dry milk in PBS for one hour and then incubated overnight at 4°C in primary antibodies diluted in 2% dry milk-PBS. The antibody used was monoclonal anti-TGF-α (0.2 μg/ml, Oncogene Science, ref. GF10). Blots were washed in 2% dry milk-PBS and bound antibodies were detected using peroxidase- or alkaline phosphatase-conjugated rabbit anti-mouse IgG. Blots were developed to yield the reaction products. We used TGF-α (Bachem, ref. H-5545) as a positive control for mAb. Molecular weights of the immunoreactive bands were estimated by running aside a mixture of molecular standards from Bio Rad (ref. 161-0304) and Sigma Chemial Co. (ref. MWM-SDS-17S). The standard for pI determination in two-dimensional gels was from Bio Rad (ref. 161-0320).

Fig. 1. *Light microscopy of adult chicken kidney.* (**a** and **b**) Indirect immunoperoxidase technique for TGF-α. Immunostaining appeared to be stronger in areas which resembled infoldings of the basolateral membranes of cells (arrowheads) and in the apical surface of cells (arrows). (**c** and **d**) Serial sections immunostained for TGF-α and calbindin, respectively. In both cases, Alcian blue negative tubules (identified as distal tubules) were stained, frequently showing an alternating pattern of immunostaining. Sometimes TGF-α was present in portions of the distal tubules where calbindin was absent. Sometimes both antibodies colocalized in the same portion of the distal tubules and showed alternative stainig of complementary cells (arrowheads). (**e**) Histochemistry for carbonic anhydrase. Note positive reaction on the brush border and nuclei of the proximal tubules. A: intralobular artery. (**f** and **g**) Serial sections incubated with anti-TGF-α and anti-TGF-α preabsorbed with TGF-α, respectively. Immunostaining almost disappeared in "g" (bars: 10 μm).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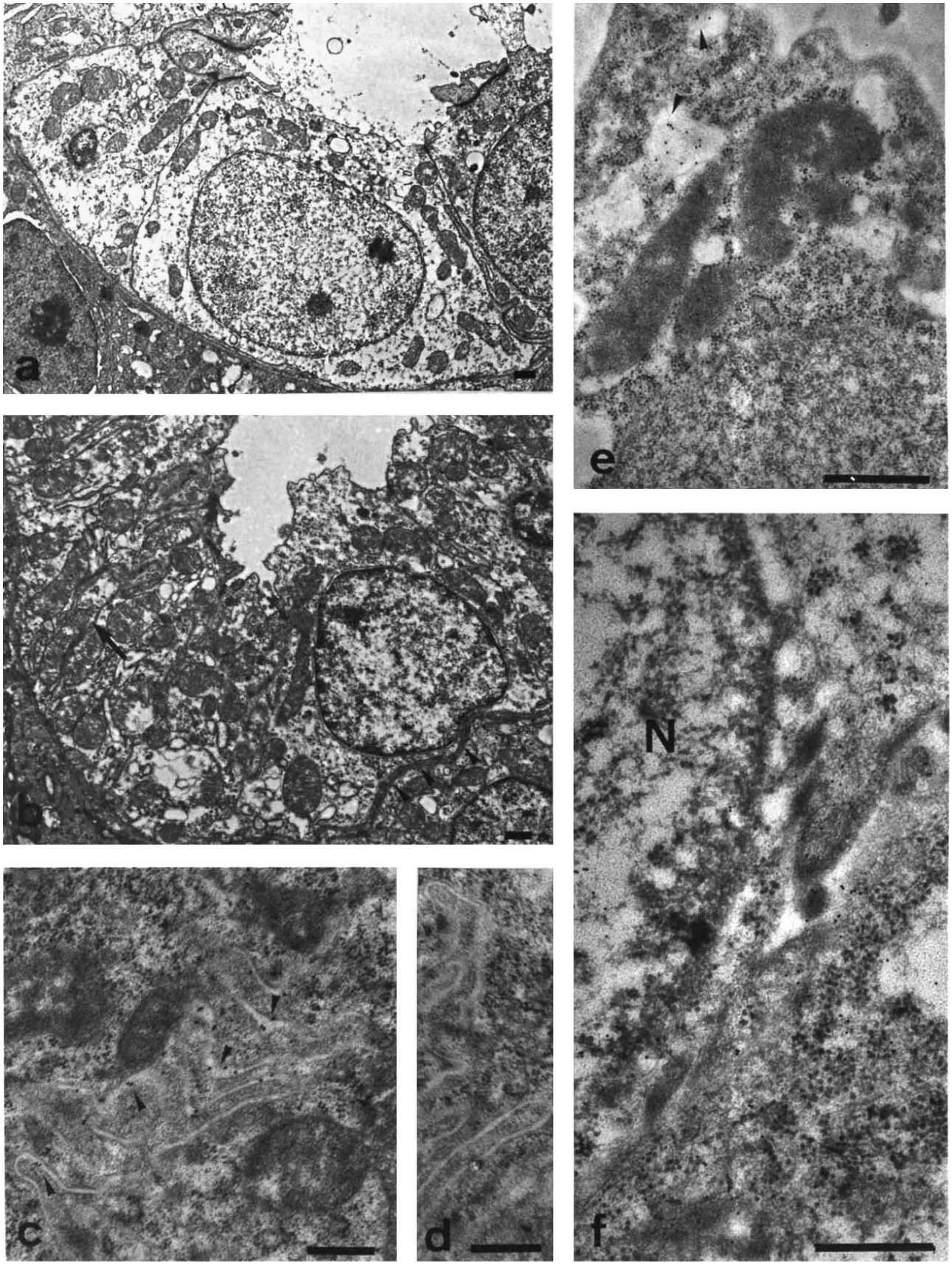
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Analysis of TGF-α immunoreactivity specificity

By using the Extended Genetic Computer Group (EGCG) software package we searched for similarities of the 50 amino acid TGF-α and the epitope recognized by mAb (residues 34 to 50 of soluble TGF-α, according to Sorvillo et al [26]), to known proteins in the database. In addition, we searched for sequence-known kidney-proteins and compared them to the TGF-α immunoreactive proteins described in the present report.

On the one hand, TGF-α was found to present significant homology only to the proteins of the EGF family. The antibody used does not cross react with EGF, as shown by Sorvillo et al [26] and corroborated here by testing mAb against human EGF on blots (PeproTech Inc., ref. 100-15). Laminin has EGF-like domains and it is also present in kidney. Through GCG data we found laminin B1 is 60% homologous to rat TGF-α in a 15 amino acid overlap, which corresponds to the most conserved domain in the EGF-like family, and it is included in the epitope recognized by mAb. Therefore, we also tested anti-TGF-α for cross reactivity against mouse laminin. In addition, we used a mouse monoclonal anti-human laminin (Boehringer Manheim, ref. 1087 746) and a rabbit polyclonal anti-laminin on the kidney blots.

On the other hand, calbindin-D28K and carbonic anhydrase are known to present very similar patterns of immunstaining to that shown for TGF-α in the chicken kidney by mAb. Moreover, they have the same Mr in gels as the 29 kDa TGF-α imunoreactive protein that mAb detected on kidney blots. Carbonic anhydrase also has very similar pI to the same protein. Therefore, mouse monoclonal IgG1 anti-calbindin-D28K (1:800, Sigma, ref. C8666) was used in two dimensional blots to demonstrate they are indeed distinct proteins, and anti-TGF-α antibodies were tested against carbonic anhydrase.

Elastase treatment

Elastase is known to mimic TGF-α release *in vitro* [19]. Therefore samples from the adult chicken kidney homogenates (obtained as described above) were incubated in 0.2 M Tris-HCl (pH 8.8) for 0.5, 1, 2 or 4 hours at 37°C with the following concentrations of elastase (Sigma Chemicals Co., ref. E-1250): 50 mg/ml, 100 mg/ml, or 200 mg/ml. Elastase activity was arrested by addition of 300 mM N-acetyl-trialanyl methylester (NATM) or 50 mM PMSF. The products were electrophoresed on Tricine-SDS-PAGE gels, electroblotted to immobilon P and immunostained with mAb anti-TGF-α.

Glycosilation study of the TGF-α-immunoreactive proteins

To study the glycosylation of the TGF-α-immunoreactive proteins, the following lectins were used on two dimensional kidney blots: concanavalin A (ConA), Sambucus nigra agglutinin (SNA), Ricinus comunis agglutinin (RCA), Datura stramonium agglutinin (DSA), and Maackia amurensis agglutinin (MAA). Blots were first blocked with 2% polyvinylpirrolidone in TBS-Tween 20 (TBS-T) for 30 minutes and then incubated with biotin-labeled lectins (10 μg/ml diluted in 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.5) for one hour. After rinsing with TBS-T, bound lectins were detected with streptavidin-peroxidase conjugate in TBS-T developed with DAB. Blots were double stained with mAb anti-TGF-α, detected with alkaline-phosphatase conjugated anti-mouse IgG, developed with 160 μg/ml bromo chloro indolyl phosphate and 0.8 mg/ml nitro blue tetrazolium.

Furthermore, kidney samples were incubated with N-glycosidase-F, O-glycosidase, and neuraminidase in 20 mM sodium phosphate, pH 7.2, for 18 hours at 37°C, electrophoresed in SDS-PAGE gels, electroblotted and tested with anti-TGF-α for products of digestion.

Primary and secondary culture of chicken kidney cells and immunocytochemistry

Primary cultures of kidney epithelial cells were prepared from kidneys of two- to three-week-old chicks. The cells were prepared either by the procedure described by Craviso, Garrett and Clemens [32] or according to the method of Henry [33]. Briefly, tissue was finely minced with scalpel blades on Ca^{2+} and Mg^{2+}-free buffer and incubated for 12 minutes at 37°C with 1 mg/ml collagenase and 0.8 mg/ml hyaluronidase. The tissue was gently pipetted and cells and tubules were collected by centrifugation at 700 g for five minutes and washed free of red blood cells and other debris by several additional centrifugation and resuspension steps in minimum essential medium (Gibco, ref. 410-1100, referred to as MEM) or serum-free medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle's medium, nutrient mixture F-12 Ham (DMEM/F-12 1:1, Sigma, ref. D8900, referred to as DMEM). The cells thus obtained were plated at density of 8×10^5 cells/ml in plastic culture dishes and grown in DMEM supplemented with 2 mM glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 50 ng/ml prostaglandin E1, 10 nM sodium selenite and 1% antibiotic-antimycotic mixture (Gibco, ref. 600-5240 AG), according to Craviso et al [32]. Following Henry's procedure [33], cells were diluted to 7.5×10^5 cells/ml in MEM containing 5% fetal calf serum (FCS) and 1% antibiotic-antimycotic mixture, and grown in plastic culture dishes. Kidney cells prepared by either of these procedures were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C. After a 24 hours incubation, old medium containing unattached dead cells was removed and replaced with fresh medium. Confluent monolayers were obtained in four days in MEM and five days in DMEM. After reaching confluency, cells were trypsinized, replated and cultured for further 24 hours in DMEM or MEM.

Cultures in MEM supplemented with serum have a major proportion of proximal cells and in cultures grown in serum-free hormonally-defined DMEM, distal cells are predominant (A.

Fig. 2. *Ultrathin sections of chicken kidney embedded in either Araldite (***a** *and* **b***) or Lowicryl (***c-f***) resins.* (a) Cuboidal, principal cells of distal tubule. No apical microvilli or processes were visible. Cytoplasm was electroluscent and had few organelles. (b) Dark cells of distal tubule had electrodense cytoplasm due to the existence of large numbers of ribosomes, glycogen-like particles and large mitochondria. Short apical microprocesses. Long projections of basal plasma membrane (arrows). Thick basal membrane (arrowheads). (c) Interdigitations of the lateral membranes of dark cells showing positive immunoreaction for TGF-α (10 nm gold-particles) (arrowheads). Note the abundance of ribosomes giving a dark appearance to the cytoplasm of both cells. (d) Negative control of the immunocytochemistry in an equivalent region to the previous photomicrograph. Almost no gold-particle could be observed when first antibody was omitted. (e) Immunogold particles in apical vacuoles (arrowheads). (f) Sometimes colloidal gold-particles were also seen in the periphery of the nucleus (N), and seemingly were related to ribosomes. (Bars: 100 nm)

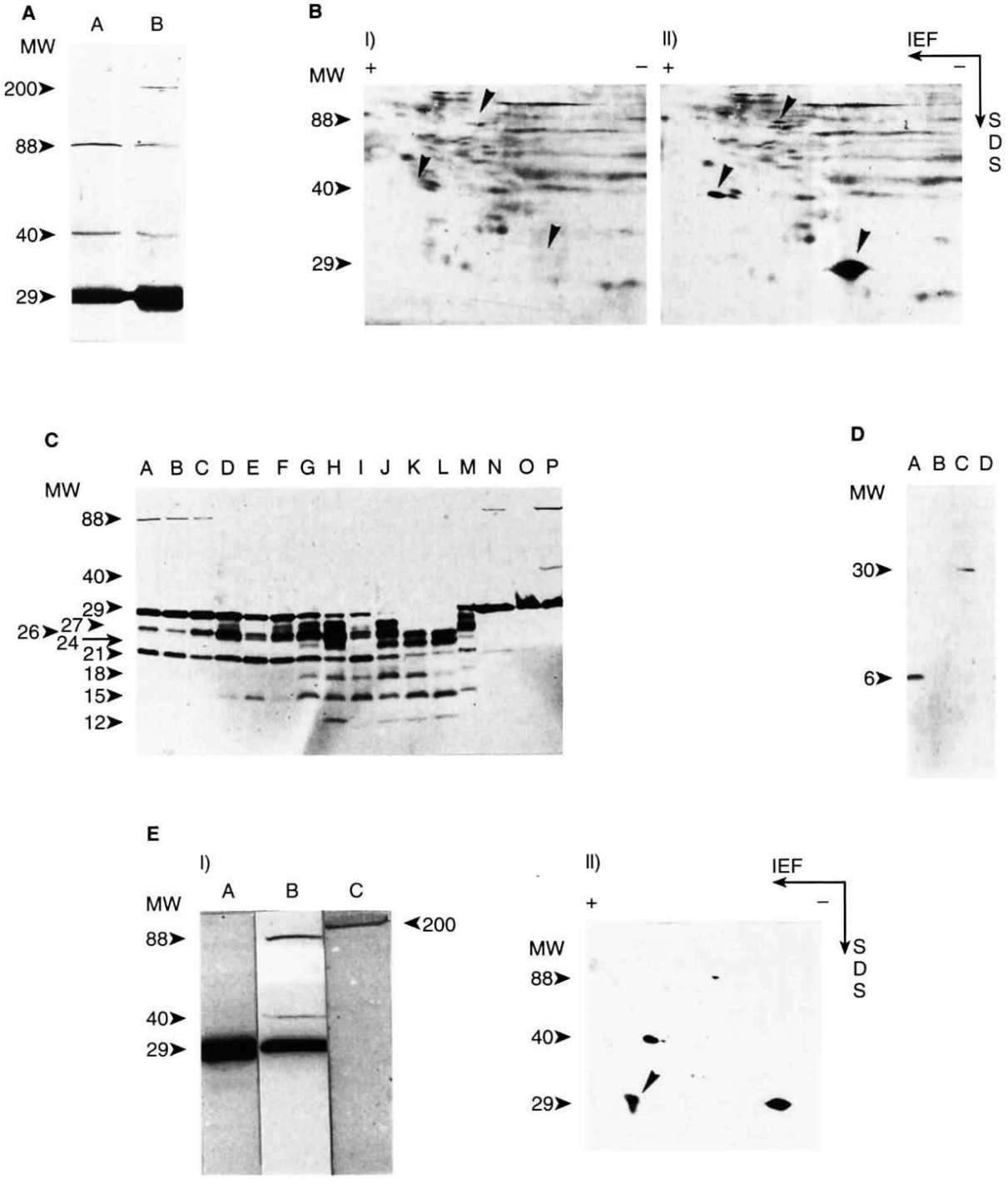

Fig. 3. *(A) 12% SDS-PAGE.* Western analysis of TGF-α-immunoreactive proteins on chicken kidney blots, with mAb. Lane A, with beta-mercapto-ethanol (BME) in loading sample buffer. Lane B, with no BME. Note the high Mr-immunoreactive protein which appeared with no BME and decrease in immunoreactivity for the 40 and 88 kDa TGF-α-forms. Molecular weight of the immunoreactive proteins are indicated by arrows. **(B)** 12% SDS-PAGE. Lectin blotting analysis for detection of glycosylated proteins. **(I)** Glycoproteins of chicken kidney detected by Con A. **(II)** Double staining of chicken kidney proteins with Con A and mAb. None of the TGF-α-immunoreactive proteins coincided with lectin stained proteins (arrowheads point to the localization of the TGF-α-immunoreactive proteins). **(C)** Elastase treatment of chicken kidney samples and analysis of products for TGF-α-immunoreactivity. The samples were incubated at 37°C, for A, E and I, 30 minutes; B, F, and J, one hour; C, G, and K, two hours; D, H, and L, four hours; lanes M-O, two hours; with the following concentrations of elastase: lanes A-D, 50 mg/ml; lanes E-H, 100 mg/ml; lanes I-L, 200 mg/ml; lanes M-O, 100 mg/ml. Lane M, plus 0.3% BSA; lane N, plus 300 mM N-acetyl-trialanyl methylester; lane O, plus 50 mM PMSF; lane P, negative control incubated without elastase, for four hours at 37°C. Note the rapid disappearance of the 40 kDa protein (lane A), followed by the 88 kDa protein (lane D). The 29 kDa protein was less sensitive to the action of elastase, and needed high concentration of enzyme and long time to be cleaved. Samples were run in 16% Tricine SDS-PAGE. **(D)** 16% Tricine SDS-PAGE. Western controls of mAb. Lane A, TGF-α; lane B, EGF; lane C, carbonic anydrase; lane D, laminin. **(E, I)** 16% Tricine SDS-PAGE. Chicken kidney blots tested with the following antibodies: lane A, monoclonal anti-calbindin; lane B, mAb; lane C, polyclonal anti-laminin. **(E, II)** 12% SDS-PAGE-2D-gel. Two dimensional blots of chicken kidney proteins stained with mAb and monoclonal anti-calbindin. Calbindin showed a clearly different pI (arrowhead) from the 29 kDa TGF-α-immunoreactive protein, despite of having the same Mr.

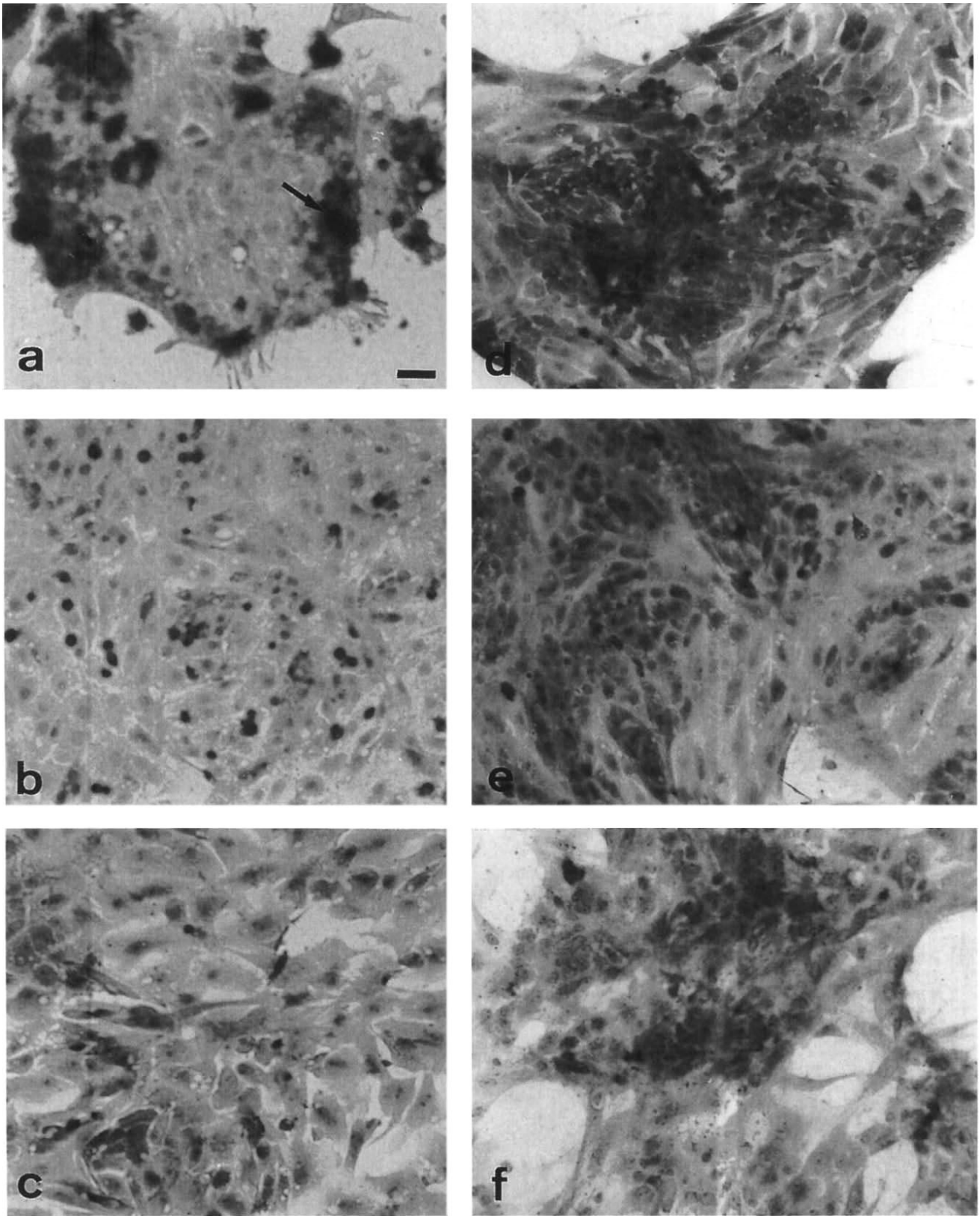

Fig. 4. *Immunocytochemistry for TGF-α on primary and secondary culture of chicken kidney cells.* (**a-c**) MEM plus fetal calf serum. (**d-f**) DMEM-F12 supplemented with insulin, transferrin, prostaglandin E1 and sodium selenite. (a and d) After 48 hours in culture, cells were immunoreactive under both serum conditions but the immunostaining was stronger for DMEM cells. MEM cells were mainly positive when still proliferating (round cells, not attached to plates-arrow). (b and e) After four days in culture, when reaching confluency, DMEM cells were still positive. MEM cells were almost negative. (c and f) Twenty-four-hour secondary culture from primary kidney cells grown in MEM and DMEM, respectively. TGF-α-immunoreactivity was hardly detectable in most of MEM cells; it decreased, but did not disappear in DMEM cells (Bar: 10 μm).

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Cells from each day of primary culture and the first day after replating were fixed in 1% glutaraldehyde in PBS for 30 minutes, washed in 70% ethanol for 15 minutes (4°C) and air dried at room temperature. Immunohistochemistry was performed on these cells as described above, using anti-TGF-α (mAb).

Results

Light microscopy

Nephrons in the chicken kidney mainly consist of proximal, distal and collecting tubules. Some nephrons also have the intermediate segment or limb of Henle.

TGF-α immunoreaction was found in Alcian blue- and PAS-negative tubules, which are the distal tubules of the nephrons. Positive cells frequently appeared in an alternating pattern of immunostaining. The intensity of immunoreaction was stronger in the regions corresponding to the basolateral infoldings of the plasma membranes and the apical surface of cells (Fig. 1 a, b). Serial sections incubated with anti-TGF-α and anti-calbindin showed some portions of the distal tubules positive for TGF-α and negative for calbindin (Fig. 1 c, d) and *vice versa*. Other portions of the distal tubules were positive for both anti-TGF-α and anti-calbindin and alternative staining of complementary cells was observed (Fig. 1 c, d). This result was corroborated by incubating serial sections with anti-TGF-α plus anti-calbindin antibodies, and either anti-TGF-α or anti-calbindin, respectively. In double-immunostained sections, some tubules showed positive staining in all the cells that presented alternating pattern of immunostaining when incubated with either anti-TGF-α or anti-calbindin alone (data not shown).

Glomeruli, proximal tubules and limb of Henle were always negative for both antibodies.

Histochemistry for carbonic anhydrase showed intense staining in the glomeruli, brush border, nuclei and sometimes lateral cell borders of the proximal tubule, were blackened by the precipitate product of the enzymatic reaction (Fig. 1e). The cytoplasm was also lightly stained. Distal tubules showed an alternating pattern of immunostaining.

Preabsortion of the mAb with TGF-α arrested the immunostaining almost completely (Fig. 1 f, g).

Immunoelectron microscopy

Distal tubules are composed of pincipal cells (Fig. 2a) and dark or intercalated cells (Fig. 2b).

TGF-α immunostaining was frequently seen in the lateral infoldings of the membrane between cells of the distal tubules (Fig. 2c). Gold particles were absent in negative controls (Fig. 2 d). Additionally, immunostaining was sometimes visualized in apical vacuoles (Fig. 2e), and the cytoplasm, colocalizing with ribosome-rich areas around the nucleus (Fig. 2f). Positive cells showed extreme electron density related to their high cytoplasmic ribosome and glycogen-like granular content and to the presence of numerous mitochondria. Adjacent basal processes interdigitated with others, arching into the space from out of the plane of section. Most had prominent apical microprocesses. The cytoplasm around mitochondria contained a variety of organelles: lysosomes, short strands of rough endoplasmic reticulum, a small amount of smooth endoplasmic reticulum, and Golgi bodies. Sections of these cells frequently did not cut the nucleus, suggesting that the cytoplasm was quite extensive (Fig. 2b). We identified those cells as the dark cells, according to previous description of the fine structure of cells from the distal tubules [34, 35].

Principal cells, proximal tubular cells, limb of Henle's cells, glomeruli and endothelial cells were negative.

Immunoblotting analysis

By using a monoclonal anti-TGF-α antibody, we identified three proteins of the following apparent molecular weights (Mr) and pI: 29 kDa (pI 7), 40 kDa (pI 5.5), and 88 kDa (pI 6.7) (Fig. 3 A, B). No difference was observed in the Mr or immunoreactivity of TGF-α immunoreactive proteins when protease inhibitors were not added to the homogenates.

In non-reducing conditions (when running samples without beta-mercapto-ethanol) a larger TGF-α immunoreactive protein of Mr higher than 200 kDa appeared, with a decrease but not the disappearance of the 40 and 88 kDa proteins. The large TGF-α immunoreactive protein was also less intensely immunostained, indicating lower sensitivity to antibodies (Fig. 3A).

None of the lectin tested was able to detect the TGF-α immunoreactive proteins in two dimensional blots (Fig. 3B). Incubation of the samples with N- and O-glycosidases and neuraminidase did not change Mr of any of the TGF-α immunoreactive proteins (data not shown), corroborating lectin results.

When the samples were incubated in different concentrations of elastase TGF-α imunoreactive products of 12, 15, 18, 21, 24, 26, and 27 kDa (Fig. 3C) were observed. The first TGF-α immunoreactive protein to disappear was the 40 kDa one, followed by the 88 kDa protein. The 29 kDa TGF-α immunoreactive protein was digested only when incubated at a high concentration of elastase and long period of time.

mAb detected rat TGF-α used on blots as positive control (Fig. 3D).

Analysis of the TGF-α immunoreactivity specificity

We searched GCG software package for TGF-α homologous proteins. We screened for homology to the 50 amino acid mature TGF-α, and to the epitope 34 to 50, recognized by the mAb antibody. The 40 best scores obtained corresponded to proteins in the EGF family. The highest homologies were to EGF (45.5% identity in 44 aa overlap), and laminin B1 (60% identity in 15 aa overlap). mAb did not cross react with EGF (Fig. 3D). Thus, we tested the anti-TGF-α antibodies against laminin, with negative results (Fig. 3D). In addition, anti-laminin antibodies were used on the blots of kidney samples and they recognized only a 200 kDa protein in the kidney samples, corresponding to the laminin B1 subunit (Fig. 3 EI).

Furthermore, we searched for kidney proteins of known sequence in GCG and found calbindin D-28K, and carbonic anhydrase as proteins with similar pattern of immunostaining on kidney sections, Mr and/or pI to the TGF-α-immunoreactive proteins that we described.

Therefore, we used an anti-calbindin D-28K antibody on kidney blots to demonstrate that it recognizes another, different 28 kDa protein of pI 4.8 (Fig. 3 EI, EII), and showed an alternating, complementary pattern of immunostaining on serial kidney sections (Fig. 1 c, d).

We tested the anti-TGF-α antibodies against carbonic anhydrase on blots, with positive results (Fig. 3D). However, histochemistry for carbonic anhydrase showed differential staining for carbonic anhydrase and TGF-α on kidney sections (Fig. 1e).

Immunocytochemistry of primary and secondary culture of chicken cells

Culture of kidney cells gave rise to cells of an epithelial morphology with little fibroblast contamination. Confluent cultures displayed domes characteristic of kidney and other epithelial cells in culture, which denote the active transcellular transport of water and solutes.

In MEM cells we observed positive immunoreaction while still proliferating (Fig. 4a), in the first 48 hours in culture. Positive, round, mitotic cells were mainly localized in the periphery of the growing colonies. After that point, immunostaining disappeared almost completely (Fig. 4b) and did not appear again in replated cells, where only weak staining was seen (Fig. 4c).

Cells grown in serum-free DMEM/F-12 1:1 medium supplemented with insulin, transferrin, PGE1 and sodium selenite, showed strong immunostaining for TGF-α (Fig. 4 d, f), especially during the first 48 hours in culture (Fig. 4d). They retained the immunostaining throughout the study, even in confluency (Fig. 4e) and after replating (Fig. 4f), although the intensity of immunostaining decreased a little in confluent and replated cells.

Immunostaining was localized mainly in the cytoplasm of proliferating cells in the first stages in culture and epithelial cells of domes when they differentiated to polarized epithelial cells in DMEM medium.

Discussion

In this report we show the immunolocalization of a TGF-α-related protein (TGF-α-RP) in the basolateral membrane of cells of the distal portion of the chicken nephrons. Additionally, TGF-α immunoreactive proteins present in homogenates of chicken kidney were characterized. The appearance of a higher Mr form in nonreducing conditions, and decrease but not disappearance of the 40 and 88 kDa forms indicate a protein complex formed by at least those two subunits, and referred to as "TGF-α-RP." The 40 and 88 kDa TGF-α immunoreactive proteins identified by the monoclonal anti-TGF-α are sensitive to cleavage by elastase. Furthermore, strong immunoreactivity was found in primary and secondary culture of chicken kidney cells grown in serum-free, hormonally defined DMEM, known to be predominantly selected as distal cells by medium conditions.

Initially, we demonstrated the specificity of the immunostaining by showing that these TGF-α immunoreactive proteins were not other kidney proteins with similar characteristics of Mr, pI, immunolocalization or sequence. Thus, the 29 kDa TGF-α form had same Mr as calbindin-D28K, as well as a very similar pattern of immunostaining. However, their pI in two dimensional gels were completely different and the pattern of immunostaining on kidney sections was complementary when both stainings coincided on the same tubule. Sometimes, anti-TGF-α and anti-calbindin stained different portions of the distal tubules. The 29 kDa TGF-α immunoreactive protein also had same Mr and pI as carbonic anhydrase. The anti-TGF-α that we used did recognize the mentioned enzyme on blots. However, immunostaining with anti-TGF-α on kidney sections did not coincide with the distribution of carbonic anhydrase activity, which appeared in other portions of the nephron where immunoreaction for TGF-α was absolutely absent.

Laminin is a protein of the EGF family with high homology to the epitope 34 to 50 recognized by mAb; however, it was not recognized by the anti-TGF-α. Moreover, polyclonal and monoclonal anti-laminin antibodies did not recognize any of the TGF-α immunoreactive proteins. It is also important to point out that the epitope between Cys 34 and 43 of the TGF-α-soluble sequence is the most highly conserved in the EGF family of proteins [36]. This decapeptide is included in the epitope recognized by mAb, but nonetheless, this antibody did not cross react with EGF or laminin (60% identical in the 15 aa overlap in the 34 to 50 epitope). These data strongly support the specificity of mAb for the TGF-α epitope and no other peptide in the EGF-family.

Next, the precise localization of TGF-α-RP was studied. Comparing serial sections immunostained for calbindin and TGF-α, respectively, and by immunoelectron microscopy, the nature of the TGF-α immunoreactive cells was elucidated. Calbindin is known to be present in the principal cells of distal tubules of chicken kidney [37]. Distal tubules, after turning at the central veins of the lobules, are composed of principal and dark or intercalated cells in an alternating pattern of distribution, which was apparent in some tubules of both calbindin and TGF-α immunostained sections. The TGF-α immunoreactive cells were complementary to calbindin-containing cells in portions of the distal tubules on serial kidney sections where they coincided. Sometimes, distal tubules were only calbindin positive. This portion of the nephron corresponds to the proximal stretch of the distal tubule, where the principal cells are predominant. Other times, only TGF-α-RP positive cells were found in the distal tubules, defining the distal stretch of the distal tubule, where dark cells are more abundant.

Therefore, TGF-α immunoreactive cells may be dark cells, and that was further demonstrated by immunoelectron microscopy. The ultrastructural features of the positive cells resembled the dark cells of the distal portion of the nephrons [34, 35].

Additionally, Western analysis of the TGF-α-RP in kidney samples was performed. Under reducing conditions the TGF-α immunoreactive proteins of 29, 40 and 88 kDa were identified.

A high Mr TGF-α protein appeared in non-reducing conditions suggesting the existence of a complex consisting of at least one subunit of the 40 kDa and one subunit of the 88 kDa TGF-α immunoreactive proteins, probably together with some other binding proteins, as described for EGF [38] and laminin-nidogen complex [39]. EGF, laminin and nidogen contain EGF-like repeats in their sequences. The complex has to be formed after the synthesis of the subunits, and therefore all forms coexisted in non-reducing conditions. The complex may act as a receptor or adhesion molecule, as described for laminin itself (chains A and B1 and B2).

Furthermore, we tested the response of the solubilized TGF-α immunoreactive proteins from the kidney samples to pancreatic elastase. On the one hand, we found specific cleavage of the 40 and 88 kDa TGF-α forms by elastase, since we had previously observed they are protease resistant when preparing the samples with no protease inhibitors. The 40 kDa protein was the most sensitive to the action of elastase. On the other hand, the products obtained were also immunoreactive for TGF-α and therefore, they still had the TGF-α epitope. The 40 and 88 kDa proteins

could be subunits of a larger protein, both including one or more TGF-α epitopes, which were progressively released during the digestion reaction, due to the existence of several sites sensitive to the cleavage by elastase. Differences in the Mr of the proteins and their elastase products compared to those described by other authors for pro- and soluble forms may be due to species differences in sequence, or the fact that these TGF-α forms come from organs *in vivo* and not from a source *in vitro*. Also, elastase products can contain one or more TGF-α-like epitopes and have different Mr.

The fact that we did not find 6 kDa TGF-α or mature TGF-α of low Mr may be due to several reasons. First, depending on cell status or conditions either pro-TGF-α was not accessible to cleavage or the enzyme responsible for the cleavage was not available. In support of this, no enzyme seemed to be active in the kidney samples, since no product appeared either after four hours of incubation at 37°C with no elastase added and no protease inhibitors, or when preparing samples with no protease inhibitors. Second, if excreted to the extracellular medium, the concentration of the growth factor is extremely low to be detectable by immunostaining techniques. TGF-α was still detectable while it remained in the cell, where it appeared to be more concentrated. Third, there may not be any reason for the existence of a soluble TGF-α if the TGF-α-related protein complex functions as a strict juxtacrine factor, receptor or adhesion molecule in these cells. The finding of EGF-R in the proximal tubules of the chicken kidney [24] (upstream to the portion of the nephron where we identified TGF-α, but in anatomical contact with them) and the localization of TGF-α in the basolateral membrane (as shown by electron microscopic studies) support this possibility.

Furthermore, we found strong immunostaining in primary culture of kidney cells grown in serum-free, hormonally-defined-DMEM/F-12 1:1 medium. In these conditions, cells are predominantly selected as distal cells (personal communication, Dr. Henry). Cells grown in MEM (selective for proximal cells) displayed lighter immunoreaction during the first two days in culture and very weak or no TGF-α immunoreaction when reaching confluency or after replating. TGF-α immunostaining results in cultured cells support Henry's evidence (paper in preparation) for the selective role of the two different growth conditions: cells grown in DMEM/F-12 hormonally defined medium mainly select for cells with characteristics of distal cells, and were TGF-α-immunoreactive as they were in tissues, and cells cultured in MEM plus serum contain a greater proportion of proximal tubule cells and were negative for TGF-α. This evidence points to a role for TGF-α in the differentiation and cell fate of renal cells. The existence of stronger TGF-α immunoreactivity during the first two days in culture under both medium conditions, when cells are proliferating more actively, suggests a role for TGF-α in cell proliferation. Reappearance of immunostaining in replated DMEM cells, but not in MEM cells, supports the implication of TGF-α in both proliferation and differentiation mechamisms.

Interestingly, we did not find EGF (data not shown) in the chicken kidney, when it was identified in the mammalian kidney [25, 40] together with TGF-α [25]. That can be correlated to the finding by Lax et al [41] of more affinity of human TGF-α for the chicken EGF receptor (CER) than human EGF. Recently, Kramer et al [42] showed structural differences of CER from the human EGF-R (HER) that explain distinct binding affinities of human TGF-α and human EGF for CER, whereas they bind equally well to HER. Therefore, it may not be just a coincidence that EGF has not been found in the avian kidney thus far. Perhaps TGF-α (or TGF-RP) is the effective ligand for CER.

All these results and considerations complete previous findings on TGF-α requirement in tubulogenesis *in vitro* [16, 8] and EGF-R in differentiation of structures derived from the ureteric bud, as observed in knockout mice [43]. Consequently, correct function of TGF-α through its receptor is important for chicken kidney growth and development.

Taken together, these results suggest that the TGF-α-related protein complex described here may be very important both as specific protein for dark cells of distal tubules of the kidney and as growth factor involved in proliferation and/or differentiation of cells of that portion of the nephrons or surrounding tubules by endocrine, paracrine, autocrine, or juxtacrine mechanisms.

Other experiments are being performed in our laboratory in order to elucidate not only the nature but also the physiology of the TGF-α-related protein found in the kidney.

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Appendix

Nonstandard abbreviations are: TGF-α-RP, transforming growth factor-alpha-related protein; CER, chicken epidermal growth factor-receptor.

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