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Research Article

Renin biosynthesis was studied in a juxtaglomerular cell tumor. The tumoral tissue had a high renin content (180 Goldblatt Units/g of tissue), was heavily stained by immunofluorescence using human renin antiserum, and exhibited numerous characteristic secretory granules by electron microscopy. In one series of experiments, renin biosynthesis was studied in tissue slices, by following the incorporation of radiolabeled amino acids into specific immunoprecipitable renin. Time course studies showed that renin was first synthesized in a high molecular weight form, 55,000 mol wt, i.e., 10,000 mol wt higher than that of active renin, and was then converted into a 44,000-mol wt form. In a second series of experiments renin tumoral cells were cultured. Small, round, birefringent cells obtained after collagenase digestion produced renin in both primary culture and subculture media. After 5 d most of the renin found in the culture medium was inactive, but could be activated by trypsin treatment. The tumoral tissue exhibited a strong renin immunofluorescence and numerous secretory granules were observed by electron microscopy. In contrast, the renin-producing cells isolated from this tumor and grown in culture showed little renin immunofluorescence and no secretory granule could be observed. The renin-producing cells in primary culture and subculture were pulsed with radiolabeled amino acids, and immunoprecipitable radiolabeled renin was found in the culture media, thus demonstrating the actual biosynthesis […]

Renin Biosynthesis by Human Tumoral Juxtaglomerular Cells

Evidences for a Renin Precursor

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Abstract. Renin biosynthesis was studied in a juxtaglomerular cell tumor. The tumoral tissue had a high renin content (180 Goldblatt Units/g of tissue), was heavily stained by immunofluorescence using human renin antiserum, and exhibited numerous characteristic secretory granules by electron microscopy. In one series of experiments, renin biosynthesis was studied in tissue slices, by following the incorporation of radiolabeled amino acids into specific immunoprecipitable renin. Time course studies showed that renin was first synthesized in a high molecular weight form, 55,000 mol wt, i.e., 10,000 mol wt higher than that of active renin, and was then converted into a 44,000-mol wt form. In a second series of experiments renin tumoral cells were cultured. Small, round, birefringent cells obtained after collagenase digestion produced renin in both primary culture and subculture media. After 5 d most of the renin found in the culture medium was inactive, but could be activated by trypsin treatment. The tumoral tissue exhibited a strong renin immunofluorescence and numerous secretory granules were observed by electron microscopy. In contrast, the renin-producing cells isolated from this tumor and grown in culture showed little renin immunofluorescence and no secretory granule could be observed. The renin-producing cells in primary culture and subculture were pulsed with radiolabeled amino acids, and immunoprecipitable radiolabeled renin was found in the culture media, thus demonstrating the actual biosynthesis of the enzyme. This renin was not stored inside cultured cells but was rapidly released into the medium and had a molecular weight of 55,000. No conversion of this inactive high molecular weight renin into the active, 44,000 mol wt form of renin was observed. We postulate the existence of two pathways for the processing, packaging, and secretion of renin in the tumoral cells: in juxtaglomerular cells of tumoral tissue renin is synthesized as a preprorenin and rapidly converted into prorenin (55,000 mol wt), which is in turn packaged in secretory granules where it is processed into active renin (44,000 mol wt) and finally secreted; in the cultured tumoral cells renin is still biosynthesized as a preprorenin molecule and then converted into prorenin, but is neither stored as granules nor processed into active renin. In this case the renin is released in an inactive form.

Introduction

Although the syndrome of primary reninism is an exceptional cause of hypertension it is interesting to study for many reasons. It provides the best example of a purely renin-dependent hypertension, as removal of the renin-producing tumor cures the hypertension (for review, see reference 1). It also permits extensive biochemical and morphological studies that would otherwise be extremely difficult to perform due to the scarcity of renin-producing cells in the normal kidney. For this reason, juxtaglomerular cell (JGC)[1] cultures have only been recently

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1. *Abbreviations used in this paper:* AI, angiotensin I; EM, electron microscopy; FCS, fetal calf serum; GU, Goldblatt Unit, JGC, juxtaglomerular cell; MEM, minimum essential medium; PRA, plasma renin activity; RPMI, Roswell Park Memorial Institute Medium; SDS, sodium dodecyl sulfate.

described by Rightsel et al. (2) in the rat. We observed a patient with hypertension due to a JGC tumor and we have previously described the complete purification of human renin (3), which was extracted from this tumor. The biochemical, enzymatic, and immunological characteristics of tumoral renin were indistinguishable from those of cadaver kidney renin. Polyclonal (3) and monoclonal (4) antibodies were raised to this renin.

Endocrine tumors also permit the study of hormone biosynthesis, and in a pioneer experiment, Steiner et al. (5) demonstrated the existence of an insulin precursor in a pancreatic insulinoma. In the case of human kidney renin no biosynthetic studies have yet been performed, due to the lack of both a sufficient number of renin-producing cells and specific renin antibodies. However, from previous renin biosynthesis studies performed in mouse submaxillary gland tissue (6) and in canine aortic smooth muscle cells, (7) renin appeared to be synthesized as a large precursor that was then converted into an active enzyme of ~40,000 mol wt. The finding of another patient with a renin-producing JGC tumor offered us a unique opportunity to study renin biosynthesis in tumor slices as well as in cell culture. In the present experiments, human renin was biosynthesized as a larger molecular weight inactive precursor and then processed into a smaller active molecule.

Methods

Case report

A 25-yr-old male patient suffered from severe hypertension (240/154 mmHg) discovered during a systemic examination a few months before. There was no history of cardiovascular events. Analysis of plasma electrolytes showed an hypokalemia of 2.9 mmol/l associated with hyponatremia of 131 mmol/l and a metabolic alkalosis with a bicarbonate level of 32 mmol/l. Both plasma renin activity (PRA) in upright position (18 ng angiotensin I (AI)/ml per h, normal values < 3 ng/ml per h on a daily 60-meq sodium intake), and plasma aldosterone (800 pg/ml, normal values < 300 pg/ml) were significantly elevated. Initially no tumor was found on renal arteriography, and treatment with a combination of captopril (300 mg/d) and hydrochlorothiazide (50 mg/d) effectively normalized blood pressure for 21 mo. Subsequently the patient's hypertension became refractory to this therapy and a second renal arteriogram was performed, which demonstrated a poorly vascularized tumor in the lower pole of the right kidney. During the last hospital admission before surgery, his recumbent blood pressure was 178/126 mmHg on treatment with captopril (300 mg/d), furosemide (80 mg/d), and guanfacine (2 mg/d). On the fifth hospital day, on a low sodium diet without diuretics, and on the same antihypertensive drugs, the patient's blood pressure was 130/80 mmHg. Recumbent PRA was 30.8 ng AI/ml per h and plasma aldosterone was 153 pg/ml. After acid activation at pH 3.3 (8), total renin was 435 ng AI/ml per h. The percentage of inactive renin was therefore 93%. The level of renin measured by direct radioimmunoassay (RIA) (8) was 4 ng/ml. Tumorectomy was then performed, which resulted in an immediate normalization of the patient's blood pressure (130/85 mmHg) and was associated with a return to normal of PRA (1.5 ng/ml per h) and plasma aldosterone (30 pg/ml). The percentage of inactive renin was 90%, and no renin was detectable by direct RIA. At the present time, 6 mo after surgery, blood pressure is normal without treatment.

Method of culture

PRIMARY CULTURES. A 3 × 2 × 2-cm fragment of tumor was used for cell culture. Two methods were used to dissociate the cells: mechanical dispersion and enzymic digestion. The mechanical dispersion was performed by gently pressing the tissue while in a Petri dish. From the cell suspension obtained, samples of 3×10^5-cells were layered in 35 × 10-mm Petri dishes containing 2 ml of the incubation medium described below. The tumor that remained after the mechanical dispersion was cut into small pieces and enzymic digestion was carried out with 0.05% clostridium collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in Gey's balanced salt solution, containing 0.1% pure bovine serum albumin. Five successive pools of cells, referred to as C_1–C_5, were released from the tumor by five successive 15-min periods of incubation with collagenase, at 37°C. Each pool of cells was collected separately and distributed to two different types of culture dishes: 25-cm^2 flasks with 8×10^5 cells/flask and 75-cm^2 flasks containing $1.2–3 \times 10^6$ cells, corresponding to a mean of $1.6–4 \times 10^4$ cells/cm^2. Cell were cultured in Roswell Park Memorial Institute medium (RPMI), containing a final concentration of 0.6 mM aspartic acid, 1 mM glycine, and 3 mM glutamine. The medium was supplemented with 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin; 100 μg/ml streptomycin). Cultures flasks were gassed with 10% CO_2 in air and placed at 37°C. Petri dishes were incubated at 37°C under 5% CO_2 in air. In all cases the medium was changed three times per week and the used medium was stored at −20°C before biochemical studies.

SUBCULTURES. Flasks C_2 and C_5 were maintained in primary culture for 16 and 27 d, respectively. They were then trypsinized using 0.05% trypsin (Gibco Laboratories, St. Lawrence, MA) and 0.02% EDTA in Mg^{++}- and Ca^{++}-free Gey's balanced salt solution. The cells obtained from flasks C_2 and C_5 were then incubated in the culture medium described above and grown for a period of 15–20 d. Some flasks from this first passage were then similarly trypsinized and maintained in culture for a further three successive passages.

CELL COUNTING. Since at day zero of the culture the same number of cells per square centimeter were inoculated in each flask, it was possible to calculate the number of cells remaining after the first change of medium (day 3) by substracting the number of cells in the discarded medium in the initial cell count. At different stages of the primary culture or subculture, one flask from each group was used for cell counting to express renin production per 10^6 cells.

Morphological studies

TUMOR STUDY. For light microscopy each specimen of tissue was fixed in Bouin's fluid and embedded in paraffin. Serial sections (3 μm thick) were prepared for staining with either hematoxylin-eosin-safran or Masson's trichrome and silver impregnation. Renin immunofluorescence was performed on paraffin-embedded material. After removal of paraffin with xylene, sections were passed through successive alcohol solutions and washed in phosphate-buffered saline (PBS). Indirect immunofluorescence was carried out as described previously (9) using R_{15} rabbit renin antiserum. The specificity of R_{15} rabbit antiserum was first described by Galen et al. (3) and further documented in studies dealing with the direct RIA of renin (8, 10) and with the immunohistochemistry of renin in human kidney (9). These data show that our antiserum crossreacts exclusively with renin both in plasma and kidney. Sections were incubated for 1 h with a 1:50 dilution of the antiserum. After repeated washing the tissue sections were exposed to fluoresceinated goat anti-rabbit IgG (Hyland Laboratories, Costa Mesa, CA) for 30 min. The slides were mounted in buffered gelatin and examined under ultraviolet light microscopy (Leitz Orthoplan-Ortomat, E. Leitz, Inc., Rockleigh, NJ).

For electron microscopy (EM) the tissue was cut into 1-mm^3 fragments and fixed in 2.5% glutaraldehyde with phosphate buffer (pH 7.4). After postfixing in 1% osmium tetroxyde and embedding in Epon, 1-μm tissue sections were prepared and stained with toluidine Blue. Ultrathin sections of selected areas were cut and stained with uranyl acetate and lead citrate.

CELL CULTURE STUDY. Periodically, throughout the culture period, morphology of the cells was observed in reverse microscopy, and photomicrographs were made. Immunofluorescent and EM studies were performed between days 15 and 27 of culture. Cultured cells grown on coverslip or plastic flasks were briefly rinsed with PBS and then fixed in methanol −20°C for 15 min. After hydration in PBS they were incubated with R_{15} antiserum, diluted 1:50, in a moist chamber at room temperature for 1 h. They were then washed twice for 15 min with PBS and incubated for 30 min with fluorescinated goat antirabbit IgG (Behringwerke AG, Marburg, Federal Republic of Germany) diluted 1:4. After being rinsed again in the PBS the preparations were mounted in buffered gelatin and viewed in a Leitz-photomicroscope using epifluorescence illumination. For EM studies the cultured cells were fixed in situ with 2% glutaraldehyde in 0.1 M cacodylate buffer at 4°C for 2 h, rinsed in buffer and postfixed 1 h in 1% osmic acid. After three rinses with distilled water, cells were stained in situ with 2% uranyl acetate for 30 min, then rinsed in distilled water. They were dehydrated with ethanol and then with hydroxypropyl methacrylate, and embedded in Epikote 812 resin. Sections were cut, parallel to the plane of the cell layers, with a diamond knife in a ultrocut ultramicrotome (Reichert, Austria). They were stained with lead citrate and examined with an Elmiskop 101 electron microscope (Siemens, Federal Republic of Germany).

Assay of components of the renin-angiotensin system in the tumor and in cell cultures

250 mg of tumor was homogenized with a Teflon glass homogenizer in 1 ml of 0.1 M sodium phosphate buffer, pH 7.5, containing the mixture of protease inhibitors described by Murakami et al. (11) (buffer A) and centrifuged at 20,000 *g* for 30 min. Aliquots of the supernatant were stored at −20°C before assay.

RENIN ASSAYS. *Enzymatic assay.* The concentration of active renin was assayed by its enzymatic activity on 0.5 μM renin-free human angiotensinogen prepared as already described (8). AI produced during the 30-min incubation was measured by RIA (12). Inactive renin was activated by trypsin treatment according to Soubrier et al. (13) as follows: 25 μl culture medium (up to 1/5,000 dilution) was incubated at 4°C for 10 min with 1 μg trypsin (Sigma Chemical Co., St. Louis, MO) in 25 μl 0.1 M phosphate buffer, pH 7.5. The reaction was stopped by adding 100 μg soybean trypsin inhibitor in 0.1 M citrate-phosphate buffer, pH 5.7. This concentration of trypsin resulted in maximal renin activation. Thus measurement of renin concentration after trypsinization provides an estimate of total renin that includes both active and inactive renin.

Direct renin RIA. A rapid direct renin RIA was established in order to follow the daily renin production in cultures. The modification of the previously described method (10) consisted of a decrease in the incubation time of renin with antiserum (2 h instead of 48 h) and an increase in the amount of both renin and antiserum. The modification made it possible to obtain renin measurements within 6 h.

RENIN CHARACTERIZATION. Renin produced in cell cultures was characterized by the effect of pH on its reaction with human angiotensinogen, and by the effect of R_{15} antiserum. These effects were compared with those of standard human renal renin (Medical Research Council, London, England). The optimum pH of trypsin-activated renin was determined by incubation with 0.5 μM human angiotensinogen in citrate-phosphate buffer at various pH, from 4.5 to 7.0. The ability of renin antiserum to inhibit the enzymatic activity of 25 μ Goldblatt Units (GU) of trypsin-activated renin was tested by incubating the renin and antiserum together for 24 h at 4°C and then measuring the remaining activity. The high immunospecificity of this human renin antibody has already been demonstrated, by adsorption of R_{15} antiserum with pure human renin, in both immunofluorescence staining (9) and in renin biosynthetic experiments (14).

ANGIOTENSINOGEN ASSAY. Angiotensinogen was measured by direct RIA (15). The sensitivity of this method is 200 pg/assay tube.

CONVERTING ENZYME ASSAYS. Converting enzyme was measured by both the spectrophotometric assay of Cushman and Cheung (16) and by a direct RIA (17). The sensitivity of the RIA was 1.5 ng/assay tube.

Renin biosynthesis

LABELED AMINO ACIDS INCORPORATION. Biosynthesis experiments were performed both on the tumor tissue directly after surgery and on cells in primary and secondary culture.

Tumor tissue. Tissue was finely chopped using scissors, and 200 mg of minced tissue was incubated with 2.8 ml methionine- and leucine-free Eagle minimal essential medium (MEM) (Gibco Laboratories) supplemented with 110 μCi [^{35}S]methionine (Commisariat a l'Energie Atomique, Saclax, France [CFA]) and 200 μCi [^{3}H]leucine (CEA) with specific activity of 1,095 and 50 Ci/mmol, respectively. Incubation was performed at 37°C with horizontal agitation under 5% CO_2 in O_2. The pulse experiments lasted for 20 min or 4 h, followed by a chase of 4 or 6 h, respectively. The chase was realized by addition of 2 μmol of nonlabeled methionine and leucine. In a separate flask the pulse experiment was extended up to 10 h. The reaction was stopped by removing the medium. Tissue was homogenized with a Teflon glass homogenizer using 1 ml buffer A/0.1 g tissue. The extracts were centrifuged 30 min at 20,000 *g*. The supernatant was dialyzed overnight against buffer A and stored at −20°C.

Cell cultures. On day 12 of the primary culture of flask C_5, and day 17 of the secondary culture obtained from C_5 cells, the medium was replaced by serum-free RPMI culture medium. 20 h later the medium was changed and cells were incubated in methionine- and leucine-free Eagle MEM (Gibco Laboratories), supplemented with 75 μCi of [^{35}S]methionine/ml (CEA) and 75 μCi of [^{3}H]leucine/ml (CEA) with a specific activity of 1,070 and 105 Ci/mmol, respectively. Incubations were continued for 48 h and an aliquot of each supernatant was removed at various times. In parallel experiments a 1,000-fold excess of nonradioactive methionine and leucine was added after 1, 3, 6, 10, 24, and 48 h. The medium was then dialyzed overnight against buffer A, centrifuged, and stored at −20°C. The cells were scrapped off the flask with a rubber policeman and washed three times with 2 ml buffer A. Cells were then suspended in 0.5 ml of the same buffer, sonicated for 15 s and stored at −20°C. Simultaneous measurements of radioactive-labeled proteins and renin were performed both in the medium and in the cell sonicate. In a separate flask C_5, the incorporation of labeled amino acids was performed in the presence of 10^{-5} M cycloheximide. After a 24-h incubation the reaction was stopped as described above.

DETERMINATION OF THE RADIOACTIVE-LABELED PROTEINS. The incorporation of [^{35}S]methionine and [^{3}H]leucine into proteins in incubation media or cell extracts was measured in 5-μl samples that were precipitated with 100 μl 10% trichloracetic acid in the presence of 100 μl albumin (1 mg/ml) as a carrier protein. The precipitate was washed four times with 1 ml 5% trichloracetic acid, dissolved in 500 μl 0.1 M NaOH, and counted in a Packard tricarb liquid scintillation

spectrometer using Picofluor 15 (Packard Instrument Co., Inc., Downers Grove, IL) as scintillation cocktail. Correction was made for the overlap of ^{35}S in the ^{3}H-channel.

PURIFICATION OF RADIOACTIVELY LABELED RENIN. Renin present in the incubation medium and in cell and tissue extracts was purified by two different methods.

Method 1. Immunoprecipitation with the renin antiserum R_{15}. 5 μl of tissue extract or 25 μl of culture medium or cell extract were incubated for 24 h at 4°C, with 5 μl R_{15} antiserum in 0.1 M phosphate buffer, pH 7.5, containing 0.2% EDTA, 1% Triton X-100, and 1 mg/ml albumin, in a final volume of 200 μl. The immune complex was then adsorbed on 30 μl Pansorbin (Calbiochem-Behring Corp., San Diego, CA) and washed four times with the same buffer. Incorporation of labeled amino acids into renin was calculated as the difference between the radioactivity incorporated into the immune complex formed with R_{15} antiserum and the radioactivity incorporated into the complex formed with preimmune serum. Using iodinated pure human renin (10) as a probe, it was verified that 5 μl R_{15} bound 80% of the renin contained in 5 μl of tissue extract and 90% of that contained in 25 μl dialyzed culture medium or cell extract. Radioactivity was quantitated as described above.

Method 2. Affinity chromatography on antihuman renin monoclonal antibody-Sepharose was performed using a new monoclonal antibody directed against human renin, 1A12 F37, obtained using the method already described (4). 1A12 F37 differs from the first monoclonal antibody, F15, by its higher affinity for human renin (association constant 10^9 M^{-1}). Half-maximal binding to iodinated renin is obtained at a concentration of 5×10^{-10} M. The antibody is specific of primate renin and does not bind renin of other species nor other acid proteases: the binding of iodinated renin to the antibody is displaced by 5 ng human renin whereas up to 5 μg mouse or hog renin, hog pepsin, or human cathepsin D do not modify this binding. The specificity of 1A12 F37 for human renin was further documented by its ability to exclusively stain JGC of human kidney, both in indirect fluorescence and peroxydase-antiperoxydase technique. The purified IgG fraction of the antibody was coupled to Sepharose 4-B according to March et al. (18) using 10 mg IgG/ml gel. The immunoadsorbant obtained was characterized by its ability to bind human active and inactive renin at pH 7.5. Active renin was obtained from a kidney extract. The source of inactive renin was the supernatant of chorionic cell culture that contains renin under a 95% inactive form (14). Both forms of renin were eluted in 6 M guanidine. Three different types of extracts were purified on 0.5 ml immunoadsorbant: (*a*) extracts from tumor slices (450 μl); (*b*) culture medium corresponding to 48 h of biosynthesis (10 ml); and (*c*) 500 μl of cold medium from C_5 flask, supplemented with ~50,000 cpm iodinated pure human renin. This preparation was run on the gel as a control for the elution pattern of both active (iodinated) and inactive (culture medium) renin. Renin containing fractions were dialyzed and lyophylized, and aliquots of the fractions were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis according to Laemmli (19) with 10% acrylamide. Protein standards for molecular weight determination were obtained from Bio-rad Laboratories (Richmond, CA). Labeled proteins were detected by fluorography (EN^3 HANCE, New England Nuclear, Boston, MA) using Kodak X-ray film NS2T (Eastman Kodak Co., Rochester, NY). Autoradiograms were either revealed or scanned using cellomatic equipment (Sebia, Paris, France).

Results

Characterization of the tumor

MORPHOLOGY. The tumor weighed 14 g and was delimited by a fibrous capsule.

Light microscopy. The tumor consisted of polygonal cells arranged in cords from one to several cells thick. Tumor cells showed large vesicular nuclei with occasional nuclear polymorphism and finely granular cytoplasm. On impregnation with silver stain, cells appeared to be surrounded by a basement membrane, which was particularly evident in close proximity to small muscular arteries. In all areas the tumor was richly vascularized, with ectasic vascular profiles in some areas. Small arteries showed intimal thickening and hyalinosis.

Renin immunofluorescence. The cytoplasm of most tumor cells was fluorescent (Fig. 1 *B*). Some groups of cells showed intense homogenous fluorescence, while other cells were negative or faintly positive. Some cells had positive cytoplasms in the media of intratumoral muscular arteries. Controls performed with preimmune serum were negative.

Electron microscopy. Tumor cells showed large Golgi complexes, and well developed rough endoplasmic reticulum. Microfilaments and attachment bodies were present in the cytoplasm of many tumor cells. Two types of electron opaque secretory granules were evident (Fig. 1 *A*): Rhomboid cristalloids within membrane-bound vesicles adjacent to Golgi (protogranules) and spherical or irregular shaped uniformly dense granules (mature granules). In some cells the smooth endoplasmic reticulum was markedly dilated.

BIOCHEMISTRY. Active and total renin were measured in the tumoral tissue extract by enzymatic assay: 130 and 180 GU were, respectively, obtained per gram tissue. The same concentration of total renin was obtained by direct RIA, i.e., 180 GU (=220 μg) per gram. This signifies that 72% of renin in the tumor is present as an active form. Active and total renin were reassayed after 1 mo storage of the tissue extract at −20°C. Identical results were obtained.

Characterization of cells in culture

MORPHOLOGY. About 30–40% of cells were lost at the first change of medium on day 3. Most of the discarded cells consisted of blood cells. Some of the remaining cells started to divide and different morphological aspects were observed, depending on the original technique of cell dissociation.

The mechanically dispersed cells started to divide after 6 d in culture forming large monolayer colonies of regular polygonal cells. These "epithelial-like" cells were confluent by day 10–15. After trypsinization, the same morphology was observed in secondary culture. No significant immunofluorescence staining was detected with renin antiserum.

The collagenase digestion produced a mixed population of cells: (*a*) A very low amount of "epithelial-like" cells (about one colony per flask) was observed in flasks containing the cells extracted during the first two collagenase incubations (C_1 and C_2); (*b*) A few elongated cells resembling smooth muscle cells were growing as multilayered colonies (Fig. 2 *A*) and were evenly distributed in the cultures flasks (2–3 colonies per 75-cm^2 flask), except in flasks C_4 and C_5. Immunofluorescent study was performed on days 14 and 19 and showed a granular fixation of renin antiserum in some cells of the colonies; and (*c*) Round and birefringent cells aggregated in small clusters were the pre-

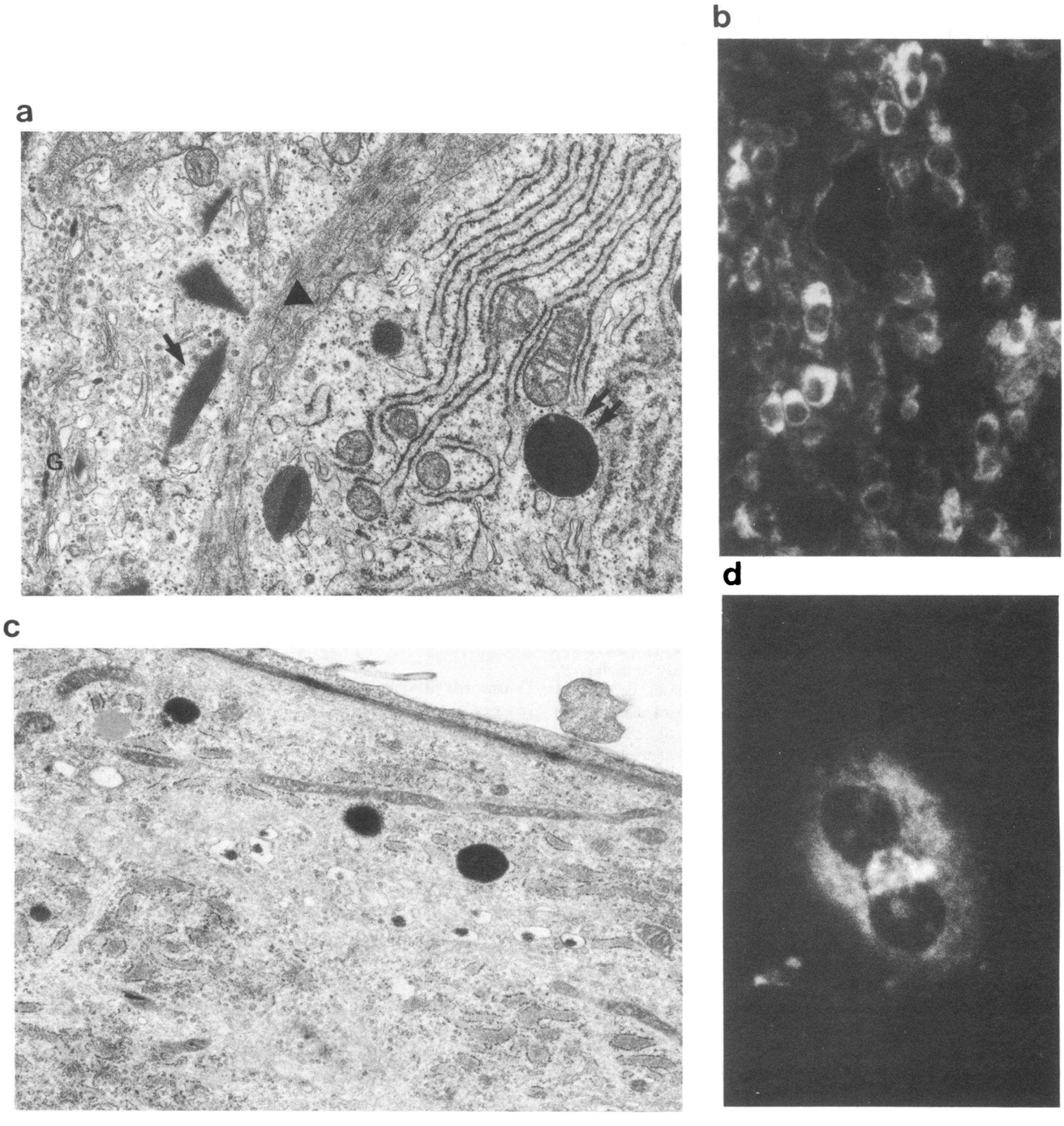
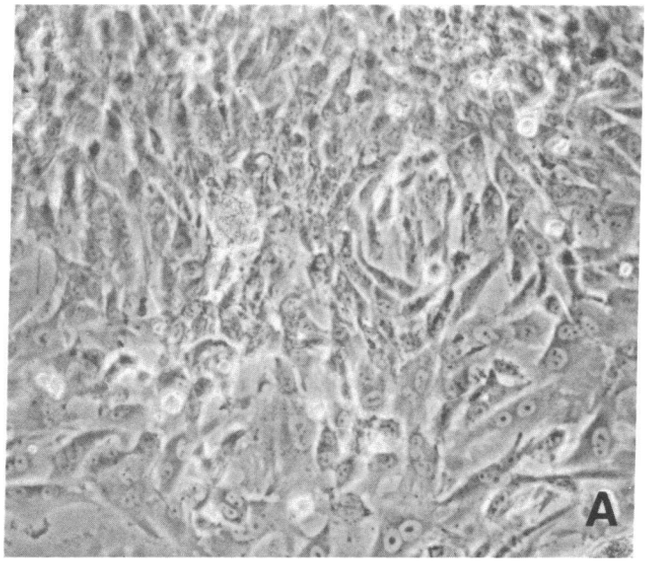


Figure 1. Immunofluorescence and electron microscope photographs of tumoral tissue and cultured cells obtained from the tumor. (*A*) Electron microscope of the tumor. Portion of two cells including protogranules (single arrow), homogenous mature granules (double arrow), and the Golgi (*G*). Also shown are intracytoplasmic myofilaments (▲). × 14,000. (*B*) Immunofluorescence of the tumoral tissue (× 300). Numerous cells exhibited a cytoplasmic immunofluorescence. (*C*) EM of a cell in culture. Detail of a large elongated cell showing peripheral bundle of myofilaments with dense bodies, proeminent rough endoplasmic reticulum, and vacuoles containing amorphorm electron-dense material (× 24,000). (*D*) Immunofluorescence of the cells in culture. With renin antiserum a granular cytoplasmic fluorescence is observed within one binuclear cell. Adjacent cells are negative.



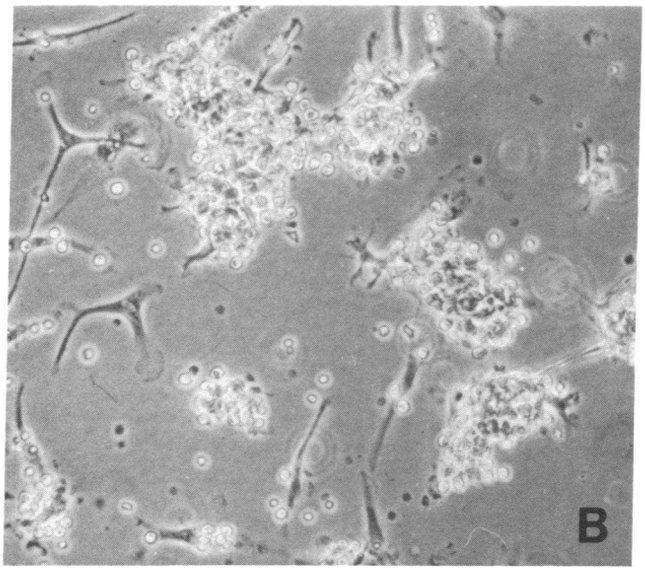


Figure 2. Phase-contrast microscopy of tumoral cells in culture (× 140). (*A*) Elongated cells forming mono- or multilayers. (*B*) Round and birefringent cells aggregated in small clusters.

dominant cells obtained in all five series of flasks (Fig. 2 *B*) and were the only cell types observed in flasks C_4 and C_5. These cells showed a uniform morphology. In flasks C_4 and C_5 mitosis was observed only between days 8 and 12 of primary culture. During the following days, the cells appeared to be in a resting phase. By immunofluorescence, carried out after 20 d of primary culture, some of these small round cells were diffusely stained with renin antiserum; in some cells, the specific immunofluorescence was more marked (Fig. 1 *D*). By electron microscopy, also carried out at the end of the primary culture, no granules similar to those observed in the tumoral tissue were found in the cultured small round cells. Most cells had an extremely abundant rough endoplasmic reticulum containing frozen material. A few bundles of microfilaments presenting fusiform, electron-dense bodies were present in their periphery (Fig. 1 *C*). Subcultures were performed on the collagenase-dispersed cells. After the first trypsinization of flasks C_5, the same morphology of the small round cell clusters was observed with the presence of mitosis between days 5–8. Isolated cells seemed to degenerate very rapidly. After the second or third passage, cells were elongated and proliferated very quickly.

BIOCHEMISTRY. No renin could be detected in mechanically dispersed cells grown as monolayer of "epithelial-like" cells. However, renin was repeatedly detected in the culture medium of all primary cultures obtained by collagenase treatment of the tumoral tissue (C_1 to C_5). During the first days of culture, a very large amount of immunoreactive renin was found in the culture medium. The renin level decreased during the first days and remained fairly stable from day 6 to day 24 (Fig. 3). Enzymatic and immunoreactive renin was found in the medium of primary cultures up to day 27 and in secondary and tertiary culture media. After three passages, no renin was detected, whichever method was used (Table I).

On the first day of culture, 45% of renin was present in an active form but this percentage rapidly decreased with increased duration of culture (Fig. 4). By day 10, <5% of renin was active. As for the tissue, measurement of total renin by direct RIA or by assay of the amount of AI produced after trypsin activation gave similar results when expressed in GU ($r = 0.96$ for $n = 24$).

Renin present in the culture medium showed enzymatic and immunological properties similar to those of MRC human renin: trypsin-activated renin from the culture medium had the same optimum pH curve with human angiotensinogen (pH 5.7) as MRC renin (results not shown); the enzymatic activity of both MRC human renal renin and renin from cell culture were fully inhibited by renin antiserum at a 1/10,000 dilution and 50% inhibition was obtained using a 120,000-fold dilution of the antiserum. It was verified that the culture medium containing FCS itself did not generate AI during the 30-min incubation and did not displace renin in the direct RIA. Finally, pure human renin and renin from the cultures exhibited parallel curves when assayed in serial dilutions in the direct renin RIA (Fig. 5).

Angiotensinogen and converting enzyme were measured in the media of flask C_5 at days 2, 6, 19, 22, and 23 of primary culture and in control incubation medium containing 10% FCS. Assay of converting enzyme by the enzymatic method revealed the presence of a low concentration of converting enzyme (1.00 to 1.44 mU/ml) in the culture medium, with similar levels being obtained in control medium (1.69 mU/ml). Converting enzyme measured by the direct RIA specific for the human enzyme was undetectable throughout the culture as well as in the control medium. It is therefore assumed that converting enzyme activity detected by the enzymatic assay was due to the presence of the enzyme in FCS, and that no human converting enzyme was secreted by the cells.

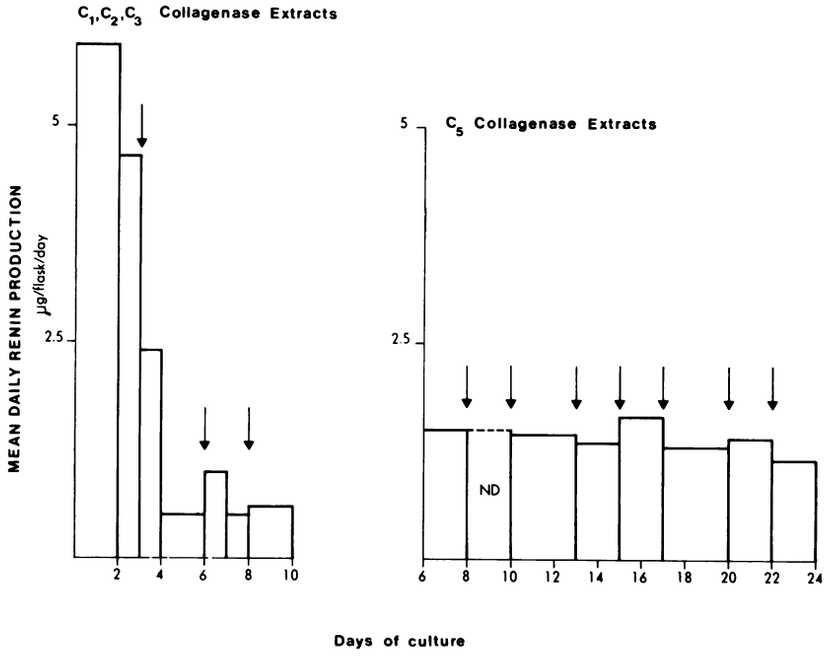


Figure 3. Mean daily renin production in the medium of primary cell cultures. Immunoreactive renin was detected by rapid direct RIA from day 2 to day 24. Results are expressed in microgram renin in the medium per day of culture. Determinations were performed on three separate flasks (C_1, C_2, C_3) for the first 10 d of culture and on three separate C_5 flasks from day 6 to day 24. The arrows indicate the change of medium. Renin determination was not performed between day 8 and 10 in C_5 flasks. ND, not determined.

By direct RIA specific for human substrate, angiotensinogen was undetectable in the medium throughout the culture as well as in the control medium. Therefore, it is assumed that these renin producing cells do not release angiotensinogen into the medium.

Biosynthesis of radiolabeled renin

BIOSYNTHESIS EXPERIMENTS ON CELL CULTURES. Radioactive amino-acid incorporation into total protein and renin was studied in flask C_5 at day 13 of primary culture and at day 18 of subculture. Fig. 6 shows the time course of the distribution of radiolabeled proteins and renin between the cell extracts and the culture medium. Renin, like most newly biosynthesized proteins, was not stored in the cells but was rapidly secreted into the medium. Cycloheximide abolished renin biosynthesis during the 24 h of the study. Since renin was not stored inside the cell, further studies were carried out on renin secreted into the medium for up to 48 h. Renin biosynthesis was linear from

Table I. Immunoreactive Renin Production by Tumor Cells in Culture (ng/10^6 cells/h)

	C_2 flasks			C_5 flasks	
Primary culture	10			38	
First passage	11	ND	10	22	ND
Second passage	0	ND	5	ND	3
Third passage	0			—	0
Fourth passage	—			—	0

ND, not determined.

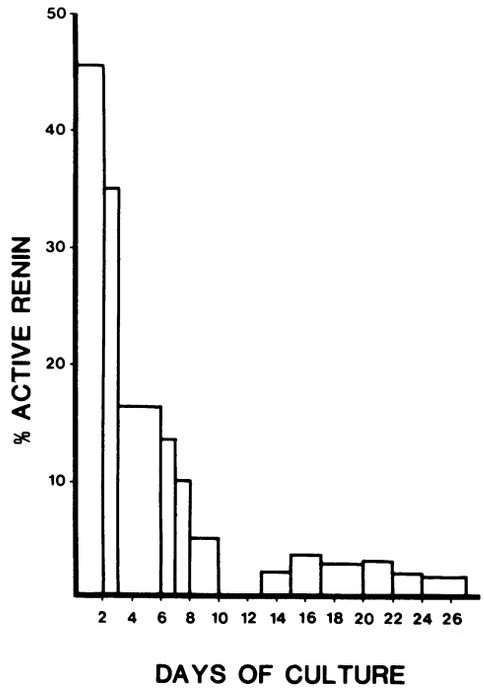


Figure 4. Variation of percentage of active renin in the medium during primary culture. The percentage of active renin is the percentage ratio of active renin (measured before trypsinization) to total renin (measured after trypsinization). No determinations were performed between day 10 and 13. The total renin production was 6, and 2×4 ng/flask per 10^6 cells per day on days 2 and 4, respectively, and was stable at ~1.5 ng between day 4 and 24.

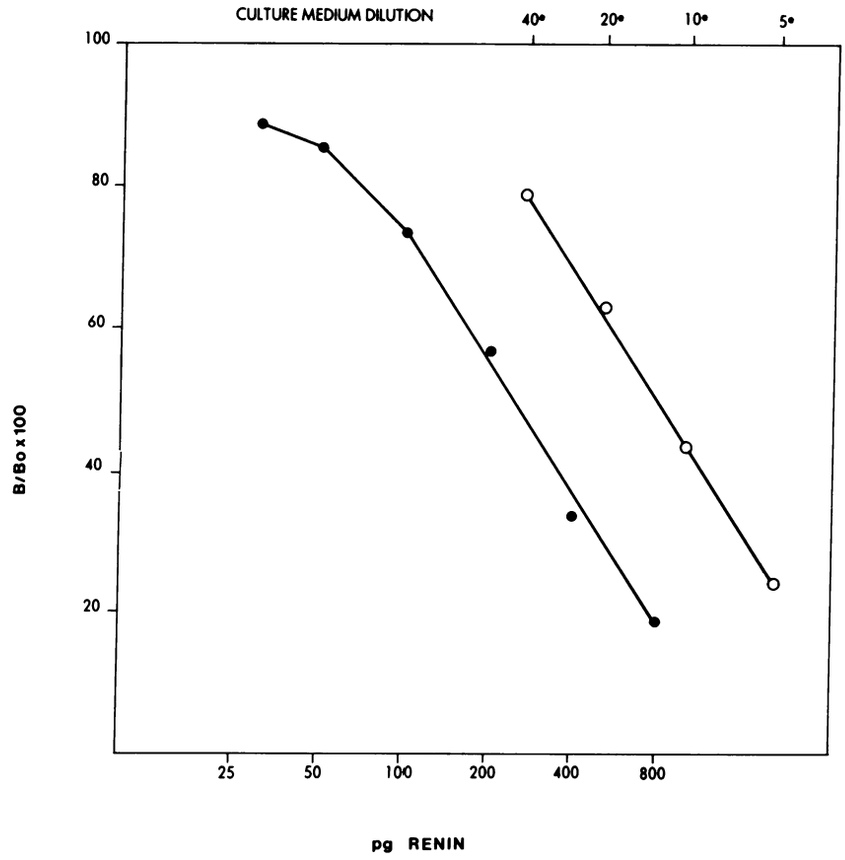


Figure 5. Direct RIA of renin in the culture medium. (Standard curve obtained with pure human renin (25–800 pg/assay) (●). Serial dilution of culture medium (5–40-fold dilution) (○). Abscissa are plotted in a logarithmic scale.

6 to 48 h. From these experiments it was calculated that renin represented 3–10% of the total biosynthesized proteins secreted into the culture medium. When biosynthesis studies were performed again, on day 18 of secondary culture, renin was still radiolabeled but represented only 1–2% of the biosynthesized proteins in the medium.

The radioactive labeled renin was characterized in SDS-gel electrophoresis after purification by immunoaffinity chromatography on an antirenin 1A12 F37 antibody-Sepharose column (Fig. 7 *A*). Renin exhibited a single band with an apparent molecular weight of 55,000. In the same conditions, pure active human renin had an apparent molecular weight of 44,000. No 44,000-mol wt renin could be detected in any of the cell culture media assayed. Using the same antibody-Sepharose column, both iodinated pure human renin and immunoreactive renin from the culture medium were eluted in the same guanidine fraction (Fig. 7 *B*).

BIOSYNTHESIS EXPERIMENTS ON TUMOR SLICES. Labeled renin was also obtained in the experiment performed on the tumor slices and was purified by the same affinity gel, specific for human renin. Purified fractions were analyzed on SDS-gel electrophoresis. The patterns of the gels after autoradiography and scanning are given in Fig. 8. No radioactive material could be detected on the extract corresponding to 20-min incorporation with [^{35}S]methionine and [^{3}H]leucine. In contrast, the extract corresponding to a 4-h pulse was characterized by the presence of a major band of 55,000 and a minor band of 44,000 mol wt, while the extract corresponding to a 4-h pulse + 6-h chase exhibited a minor band of 55,000 and a major band of 44,000 mol wt.

Discussion

This new case of JGC tumor shows a number of similarities with most of the previously described cases. The renin content of the tumor was ~1,000 times higher than that found in a normal human kidney (20). By light microscopy and immunofluorescent staining, these tumoral cells possessed the structural characteristics and the immunochemical properties of epitheloid cells from the JGC apparatus. Almost no immunofluorescence was found in the cytoplasm of the myocytelike cells surrounding the vessels, whereas granular and diffuse immunofluorescence was observed in the renin-producing cells. Ultrastructural studies of these cells showed the presence of two types of granule, specific rhomboidal protogranules with a paracrystalline structure and spherical dense renin granules.

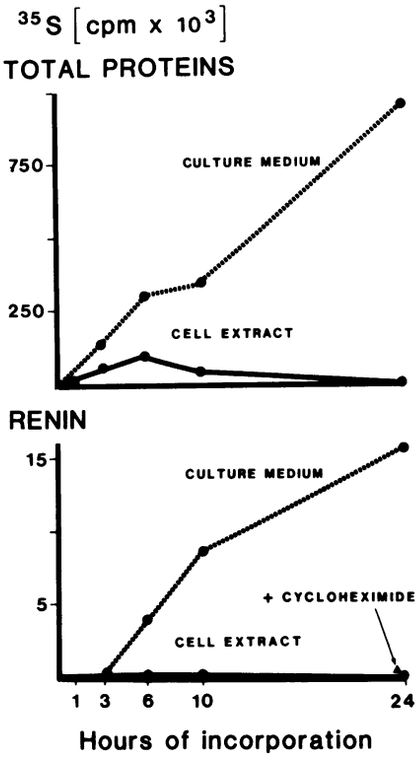


Figure 6. [^{35}S]methionine incorporation into total protein and renin. Counts per minute of ^{35}S-labeled total proteins (*top*) and ^{35}S-labeled renin (*bottom*) were measured in culture medium (● - - - ●) and cell extracts (● —— ●) after 1 to 24 h of incorporation. ▲ represents the cpm of ^{35}S-labeled renin in the culture medium containing 10^{-5} M cycloheximide.

This renin tumor offered a unique opportunity to culture and subculture the human JGC. Such studies are extremely difficult to perform in normal kidney due to the scarcity of these cells. The availability of a rapid direct renin RIA greatly facilitated the detection of renin production in the culture medium; results could be obtained within 6 h. The high specificity of the polyclonal antibody raised to human renin prevented any interference with renin in FCS. Since the increase in the enzymatic activity of renin after trypsin treatment in vitro, in plasma, amniotic fluid (8), and chorionic cells (14) is not accompanied by a change in immunodetectable renin, it is concluded that our antibody, as others (21) recognizes both the active and inactive forms of the enzyme. The results of the enzymatic assay of trypsin-activated renin are identical to those with the RIA (r = 0.96). This identity has also been confirmed by Craven et al. (22) with chorionic renin, using our antibody.

Conn et al. (23) have previously cultured renin cells from a JGC tumor and were able to maintain a renin production up to 500 h. In the present study, two types of cell preparations were tried and only collagenase treatment produced cells that synthesized renin. The small round cells observed after long exposure to collagenase are most likely to be the renin-producing cells since biosynthesis of radiolabeled renin was observed in the flasks that contained only this cell type. No firm conclusion can be drawn on the ability of the smooth musclelike cells to produce renin, since no culture flask contained exclusively this type of cells. Although it is difficult to compare the morphology of cells in culture with that observed in tissue, the morphology of the cultured "smooth musclelike" cells was close to that of the myocytelike cells of the tumor that failed to exhibit immunofluorescence with renin antiserum. Finally, the round renin-producing cells became elongated after 2–3 passages and at the same time lost their ability to produce renin. This dedifferentiation of renin-producing cells is expected as JGC tumors are nonmalignant tumors.

The AI-producing enzyme released by the cells in culture was characterized as renin by its ability to hydrolyze human angiotensinogen, its pH optimum, the inhibition of its enzymatic activity by renin antiserum at the same dilution as standard human renin, and its immunological identity with standard renal and pure tumoral renin (10) demonstrated by the parallelism of the dilution curves in our direct RIA. During the first days of culture, 45% of renin released was in active form, but the percentage of active renin in the medium decreased with the duration of culture and by day 10 essentially all renin in the medium was inactive. The relatively high levels of active renin in the medium initially may correspond, at least in part, to the release of the enzyme previously stored within the secretory granules of the tumor, as the tumor itself contains predominantly active renin. Similarly, an initial large release of active renin into the culture medium was found by Conn et al. (23). After this sharp decrease in renin release, a stable renin concentration was found in the culture medium, both in this study and in the report of Conn. However, Conn may have underestimated renin concentration since only active renin was measured. This fairly stable renin concentration in the culture medium suggests that the cultured cells are actually synthesizing renin. Definitive proof of this synthesis was provided by the incorporation of labeled amino acids into the renin molecule in both primary and secondary culture. The fact that newly synthesized renin is rapidly released in the culture medium and not stored in the cell is consistent with the low level of immunofluorescence staining in the cultured cells and with the absence of granules by electron microscope.

The inactive renin found in the primary and secondary culture media was activated by trypsin, as with plasma or amniotic inactive renin. After activation, renin had the same properties as fully active renin. Inactive renin (after 24 and 48 h of incorporation) was biosynthesized as a single molecule of 55,000 mol wt, larger than the fully active pure human renin molecule (44,000 mol wt) (14). By contrast, in the biosynthesis experiments performed on the tumor slices two forms of labeled renin were obtained, 55,000 and 44,000 mol wt, and the pulse chase experiment indicated that the 55,000-mol wt renin was converted into 44,000-mol wt renin. Using human chorionic cell cultures, which produced almost exclusively inactive renin, we also found

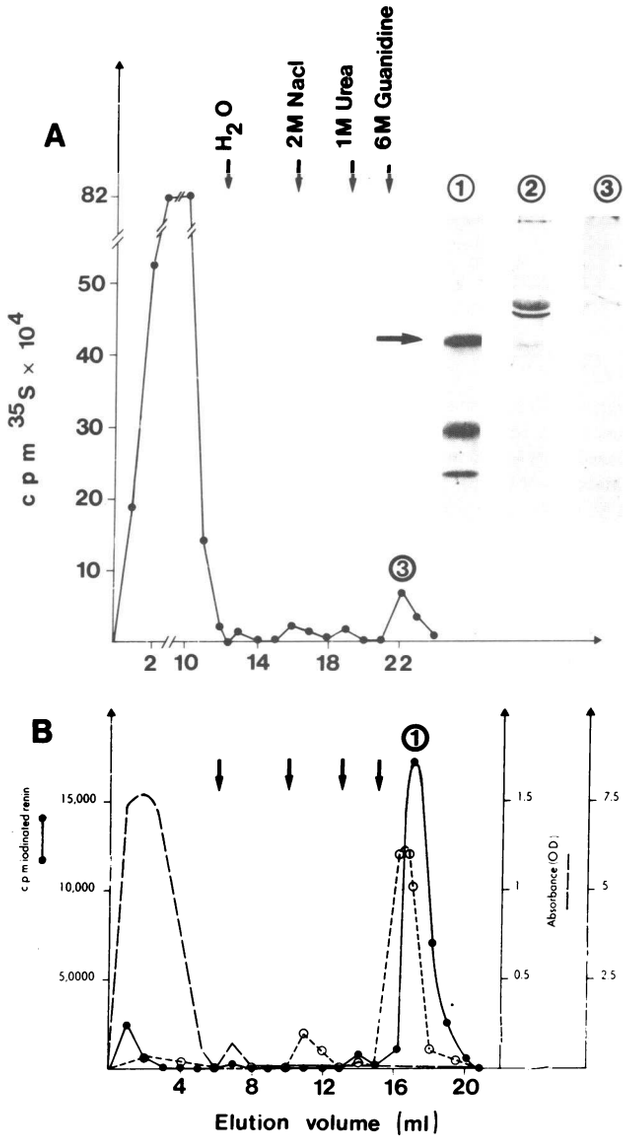


Figure 7. Purification of the labeled culture medium by immunoaffinity and determination of the molecular weight of purified fractions on SDS-gel electrophoresis. (*A*) (*right*) SDS-gel electrophoresis is revealed by fluorography: (①) Iodinated pure human renin submitted to affinity chromatography (*B*) and eluted in 6 M guanidine. This consists of 44,000-mol wt normal renin (arrow) and two "small" renins, 23,000 and 17,000 mol wt, found only in dissociating conditions (3). ② Labeled total culture medium. ③ Labeled culture medium purified on affinity chromatography (6 M guanidine fraction). (*A*) (*left*) Represents the elution pattern of ^{35}S-labeled culture medium (•). 10 ml of this medium (②) was applied to 0.5 ml immunoadsorbant obtained by coupling the antirenin monoclonal antibody to Sepharose. ③ Renin-containing fraction eluted with 6 M guanidine. (*B*) Control elution pattern on 0.5 ml of the same immunoadsorbant. (①) Peak of iodinated pure human renin (•). Renin purification from 0.5 ml of unlabeled C_5 cell culture medium is represented by absorbance peak (– – –) and immunoreactive peak (o – – – o). Arrows indicate changes of eluting conditions similar to *A*.

that renin was biosynthesized as a 54,000-mol wt protein that was converted into a 44,000-mol wt molecule after activation by trypsin (14). Taken together, these experiments suggest that renin is biosynthesized as an inactive precursor (prorenin) with an apparent molecular weight 10,000 higher than active renin. Such results are supported by the recent model for the processing of submaxillary gland mouse renin, deduced from the cloned cDNA of renin. Mouse renin is synthesized as a precursor (44,000 mol wt) and processed into active renin (38,000 mol wt) after release of a prosequence of 46-amino acid length (24). Murakami (25) has recently shown that in man renal preprorenin synthetized in a cell-free reticulocyte system has a molecular weight of 45,000. The apparent increase in molecular weight of the presently described 55,000-mol wt prorenin can be explained by the fact that human renin, but contrast with mouse renin, is glycosylated (3, 26). Glycosylation is well known to be responsible for an apparent increase of protein molecular weight on SDS-gel electrophoresis. 55,000-mol wt glycosylated prorenin is further processed into 44,000-mol wt glycosylated renin. A comparative schema for the maturation of mouse and human renin is proposed in Fig. 9. Molecular cloning and study of the structure of human renin cDNA will most likely confirm this model of processing.

In this study, the cultured cells did not release angiotensinogen or converting enzyme into the medium: these components of the renin-angiotensin system were not detected by enzymatic or direct RIA. This is in contrast with the report of Rightsel et al. (2) who found angiotensin converting enzyme activity and (AI and angiotensin II/III) in a culture of rat JGC. In this study, no determination of angiotensinogen or converting enzyme were performed on the cell extracts and therefore it cannot be assessed whether tumoral cells either fail to produce or only fail to release these proteins.

Based on the data obtained during the culture experiments of renin-producing cells (from JGC tumor and chorion) and renin production by tumoral tissue slices, a model for renin biosynthesis, packaging, processing, and secretion can be proposed. We hypothesize that renin is synthesized in a preproform, and that the prefragment is rapidly removed in the rough endoplasmic reticulum. Renin could then be released via one of two different pathways (Fig. 10). Pathway 1 would be as follows: (*a*) accumulation of renin in the Golgi apparatus; (*b*) packaging of renin in the rhomboidal protogranules and then in the spherical renin granules; (*c*) processing of prorenin into active renin (associated with the packaging); and (*d*) secretion of active renin by exocytosis. An alternative pathway (pathway 2) involves secretion of inactive prorenin without formation of renin granules. Several observations are consistent with this two-pathway hypothesis: the presence of both active and inactive renin in the tumoral tissue has been demonstrated by biochemical mea-

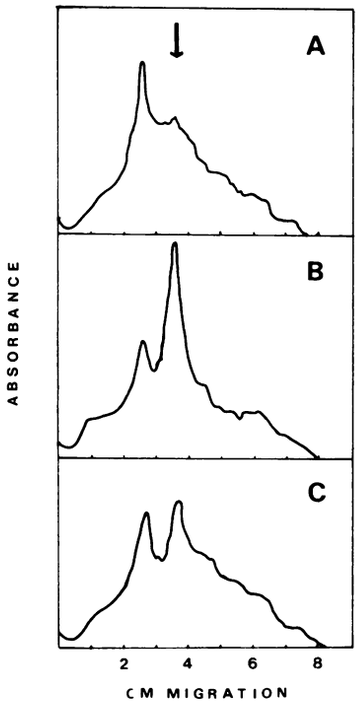


Figure 8. Scanning of SDS-gel pattern of radiolabeled renin obtained from tumor slices. Tissue was pulsed with [^{35}S]methionine and [^{3}H]leucine. (*A*) 4-h pulse; (*B*) 4-h pulse + 6-hr chase; and (*C*) 10-h pulse. Arrow indicates position of iodinated renin run on the same gel.

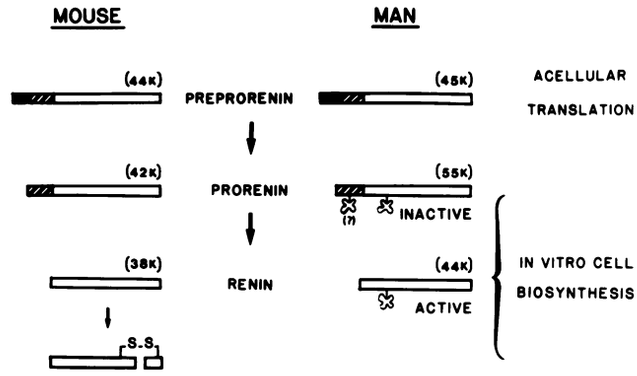


Figure 9. Comparative schema for the maturation of mouse and human renin. Maturation of mouse renin was deduced from the renin-cloned cDNA (24) and from the biosynthetic pathway described by Catanzaro et al. (6). Human preprorenin was obtained by Murakami in an acellular translation system (25). 55,000- and 44,000-mol wt renin were obtained in the JGC tumor. ▬, Prefragment; ▭, profragment; □, active renin; and ⊠, site of glycosylation.

surements performed on the tumor homogenate. The biosynthesis experiments have also shown the presence of two forms of renin (55,000 and 44,000 mol wt) in the tumor and the conversion of the 55,000-mol wt form (prorenin) into the 44,000-mol wt form (active renin). In this tissue, renin is found in secretory granules and morphological data suggest that the mature granules were derived from the protogranules. In the kidney, also, the same two types of renin granules have been described: sharply angulated granules (identical to our protogranules) indicative of a high rate of renin synthesis and lobulated granules (identical to our mature granules) (27). By contrast, no renin granules could be detected by EM in cultured cells between days 15 and 27. It was not possible, because of the limited number of culture flasks, to make serial EM studies throughout the culture, and no correlation could be made between renin granularity and the decreased active per inactive renin ratio in the supernatant. However, renin was not stored or processed in the cells at a time when the cells were still able to synthesize renin and to release it at a constant rate. We speculate that cultured cells from the JGC tumor progressively lose the capacity to package and process renin (pathway 1) and that these cells then secrete inactive renin via pathway 2. Pathway 2 might also exist in vivo in chorionic cells that are characterized by the presence of predominantly inactive renin both in tissue homogenates (28) and in cell culture (14). This two-pathway model, which implies that mature granules contain mainly active renin, involves several assumptions that will have to be proven. The exact nature of renin in the renin granules is not known, since some authors found inactive renin in purified rat renin granules (29) whereas others found mainly active renin in granules from dog (30) and human (31) JGC. It will also be important to further document the fact that prorenin is not stored in any other type of granule in cultured cells, and is indeed immediately secreted.

Finally, renin secreting cells can be compared with other endocrine cells. According to our hypothesis, renin packaging associated with renin processing in JGC is similar to the anterior pituitary cell line that contains only mature forms of corticotropin and beta lipotropin in their secretory granules (32). In these cells the presence of two distinct pathways have been described for the transport of glycoproteins leading to the se-

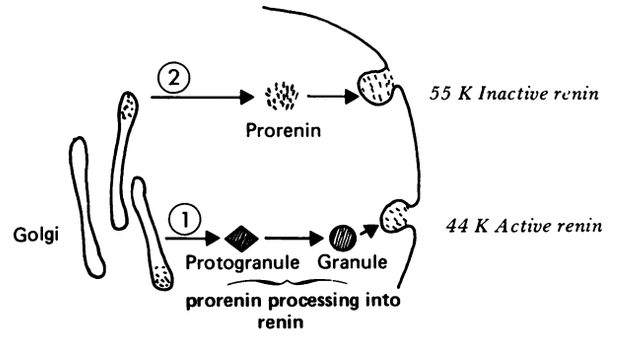


Figure 10. Proposed model of two pathways for the release of renin. Pathway ① would be predominant in JGC of the tumoral tissue (55,000-mol wt renin). Pathway ② would be predominant in the renin-producing cells in culture (44,000-mol wt renin).

cretion in the medium of either ACTH precursor or mature ACTH (33).

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