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Renin in Blood Vessels in Human Pulmonary Tumors

An Immunohistochemical and Biochemical Study

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The authors have used a sensitive alkaline phosphatase–anti-alkaline-phosphatase immunohistochemical method to examine 28 human pulmonary carcinomas for the presence of renin. Immunoreactive renin was found in 23 (82%) cases. Specific staining was always associated with small vessels in the stroma of the tumor or in adjacent areas of inflamed fibrous tissue. Within vessels, renin was localized in the cytoplasm of medial cells. Adenocarcinoma exhibited the most consistent staining (11/12 cases), and this appeared to be independent of the degree of tumor differentiation. Immunoreactive renin was also detected in squamous cell (7/8 cases), undifferentiated large cell (4/4 cases), and small cell undifferentiated carcinoma (1/1 cases), but the number of vessels and intensity of staining were usually less than seen in adenocarcinoma. Staining was not found in the bronchioloalveolar variant of adenocarcinoma (0/3 cases). By means of immunoaffinity chromatography with monoclonal antibodies (MAbs) raised to kidney renin, both active and inactive renin were extracted from homogenates of surgical specimens. The molecular weight of both forms of renin was approximately 59,000 daltons. (Am J Pathol 1988, 130:543–551)

THE ENZYME RENIN is a highly specific endopeptidase which acts on a plasma substrate, angiotensinogen, to form the decapeptide angiotensin I (ANG I). ANG I is in turn rapidly converted to the vasoactive octapeptide angiotensin II (ANG II) by angiotensin converting enzyme (ACE), a dipeptidyl peptidase present in the vascular endothelium of many tissues and plasma.[1] In addition to active renin, normal human plasma also contains an inactive, high-molecular-weight (HMW) form, which is thought to be prorenin. Up to 90% of circulating renin may be in an inactive form in man.[2] Although the juxtaglomerular (JG) cells, situated in the walls of afferent arterioles in the kidney, are acknowledged as the main source of circulating renin,[3] the presence of inactive renin in the plasma of anephric patients[4,5] indicates extrarenal synthesis, at least in this group of patients.

In addition to the kidney, a number of extrarenal tissues contain renin activity[6,7] or immunoreactive renin,[8] and the enzyme may be expressed by cultured bovine vascular endothelial cells[9] and in smooth muscle cell cultures from dog[10] and human arteries.[11]

Because the JG cells are themselves believed to be modified smooth muscle cells, some authors have proposed that blood vessels are possible sites of renin production in anephric patients[11] and that local renin–angiotensin systems may be capable of modulating vascular tone via autocrine or paracrine mechanisms.[12,13]

Production of renin has also been reported in a number of renal[14,15] and extrarenal tumors, notably of the ovary,[16] pancreas,[17] and lung.[18] These neoplasms may be associated with hypertension and hypokalemia, the symptoms of which can be alleviated by surgical removal of the tumor.[14,16] Because inactive renin is often the predominant form produced,[17,19] inappropriate or ectopic production need not necessarily be clinically apparent.

We have examined human pulmonary tumors obtained at operation and report that immunoreactive renin is present in the small vessels associated with a large proportion of these and have extracted and studied some properties of this form of renin.

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Materials and Methods

Renin Antisera

Two monoclonal antibodies (MAbs) to human kidney renin, R-3-36-16 and R-3-27-6, raised by immunization of BALB/c mice with highly purified renin were kindly donated by Professor K. G. Hofbauer of Ciba Geigy, Basel, Switzerland. The apparent IC_{50} values for these antibodies are 1.3×10^{-11} and 1×10^{-10}, respectively. Each antibody recognizes an epitope at or around the active site of renin. A review detailing the properties and specificities of these and other monoclonal renin antibodies has been published.[20] A polyclonal renin antibody, R-15, raised in rabbits after immunization with purified juxtaglomerular tumor renin,[21] was a generous gift from Professor J. Menard, INSERM, Paris. The specificity and use of this antiserum in the immunohistochemical localization of renin has been previously documented.[22] All three antibodies recognize both active and inactive renin.

Localization of Renin in Paraffin Sections

Specimens

Formalin-fixed and routinely processed paraffin-embedded sections from a total of 28 human lung tumors were examined for the presence of immunoreactive renin. Of these, 12 were adenocarcinoma (excluding those showing a bronchioloalveolar pattern of growth), 8 were squamous cell, 1 was a small cell undifferentiated carcinoma, 3 were bronchioloalveolar, and 4 were undifferentiated large cell tumors. The specimens were studied retrospectively on tissue embedded within the previous 3 years. Surgical specimens were also obtained from patients having resection of adenocarcinoma of the lung to permit extraction and measurement of biologically active renin.

Immunostaining

Sections were dewaxed, rehydrated, and washed in tap water for 5 minutes. Undiluted human plasma was applied to the slides for 20 minutes at room temperature to block nonspecific binding sites. Sections were then incubated at 4 C overnight with primary polyclonal antiserum R-15 at 1 : 10,000 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Slides were then washed in three changes of PBS over 10 minutes, and the primary antibody localized by successive exposure of the sections at 24C to the following antisera, all of which were obtained from DAKO Ltd. (High Wycombe, Bucks, UK), 1) mouse anti-rabbit, (code MR 12/53, 1:5 for 30 minutes); 2) affinity-purified alkaline-phosphatase-conjugated rabbit anti-mouse (code D 314, 1:20 for 1 hour), and finally 3) soluble complexes of mouse alkaline-phosphatase–anti-alkaline phosphatase (APAAP, code D 651, 1 : 40, for 2 hours). All antibody dilutions were made in PBS/BSA, pH 7.4, and slides were extensively washed between antisera applications in PBS. After incubation with the substrate (napthol AS-MX phosphate/Fast Red TR salt) for appropriate times (15–30 minutes), sections were counterstained with Mayer's hematoxylin and mounted in glycerol gelatin. Control slides received PBS/BSA in place of primary antibody. On selected sections staining strongly for renin, additional experiments were performed to check the specificity of the procedure. These included preabsorption of R-15 antiserum with purified renin, the use of renin monoclonal antibody R-3-27-6 as primary in place of the rabbit polyclonal antiserum, substitution of either rabbit serum or a rabbit anti-human IgG antiserum (DAKO, code A 090) in place of primary antibody, and the purification of polyclonal antiserum R-15 by immunoaffinity chromatography on a column of renin coupled to Sepharose beads.

Extraction of Pulmonary Tumor Tissue

Pulmonary tumors obtained at operation were transported to the laboratory on ice and frozen in liquid nitrogen within 30 minutes of removal. Samples of pulmonary adenocarcinoma tissue were finely minced with a scalpel blade and homogenized in 0.1 M citrate buffer, pH 6.0, containing 0.01M EDTA, benzamidine, and ethylmaleimide (NEM), and 0.06 M CHAPS detergent (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfate, Sigma Chemical Co., St. Louis, Mo) with 3 × 15-second pulses in a Polytron homogenizer (Northern Media Supply Ltd., North Humberside, UK). The extracts were frozen and thawed three times and centrifuged at 80,000*g* in an MSE high-speed 50 ultracentrifuge at 4 C. The resulting supernatant was dialyzed in 1 liter of 0.1 M Tris, pH 7.4, containing the same proteolytic enzyme inhibitors, less CHAPS, for 16 hours at 4 C, concentrated to 5 ml, and applied to an immunoaffinity column prepared with monoclonal renin antibodies R-3-36-16 and R-3-27-6 coupled to activated Sepharose.

Immunoaffinity Chromatography

Anti-Renin MAb-Conjugated Sepharose

A column of monoclonal renin antibodies conjugated to Sepharose was prepared and used in the purification of human kidney renin and in the extraction

of active and inactive renin from pulmonary tumor homogenates. The preparation of the column was as follows. Activated Sepharose-4B (2.5 g, Sigma A-9019) was washed in 1 liter 0.1 M HCl, followed by 400 ml 0.1 M MOPS buffer, pH 7.4 (Sigma M-1254) and resuspended from a glass sinter in 30 ml of MOPS. To this was added 2 mg of each monoclonal anti-renin antibody, R-3-36-16 and R-3-27-6, in a total of 400 μl PBS/BSA. After continuous mixing overnight at 4 C, any active sites remaining on the Sepharose were blocked by treatment with 1 M ethanolamine, pH 9, at room temperature for 1 hour. The prepared beads were then packed into a column 1 × 10 cm and washed in 300 ml 0.1 M Tris, pH 7.4, and then with 50 ml of Tris containing 1 M NaCl, and finally with 50 ml 0.1 M glycine HCl buffer, pH 2.8. Before use the column was reequilibrated in start buffer (250 ml 0.1 M Tris).

Purification of Human Kidney Renin

Human renal renin (approximately 5 ml, made to 1 mg/ml protein with BSA) which had been partially purified by DEAE–Sephacel and carboxymethyl Sepharose affinity chromatography and by gel filtration on Sephadex G-100[5] was dialyzed overnight against 0.1 M Tris, pH 7.4, and applied to the combined MAb column. The column was eluted with 15 ml each of Tris, pH 7.4, Tris containing 1 M NaCl, and finally glycine HCl, pH 2.8. Thirty 2-ml fractions were collected at a flow rate of approximately 2 ml/min.

Preabsorption of Polyclonal Antibody R-15 With Purified Renin

Two milliliters of stock purified renin solution, having a total renin activity of 32.2 nmol ANG-I/hr was evaporated to dryness at 40 C under a continuous stream of nitrogen. The residue was taken up in 1 ml of stock polyclonal antibody R-15, 1 mg/ml diluted 1/10,000 in PBS/BSA. The antibody/antigen mixture was allowed to equilibrate for 48 hours at 4 C before use.

Measurement of Active and Inactive Renin

Renin in tumor extracts and column fractions was measured as the rate of formation of ANG I when incubated with partially purified human angiotensinogen.[23] Inactive renin was similarly determined after the pretreatment of fractions before incubation with trypsin at a final concentration of 250 ng/ml for 30 minutes at 4 C. Incubation conditions were otherwise as previously described.[5]

Purification of Polyclonal Antibody R-15

Renin-containing fractions eluting from the monoclonal anti-renin column after applying partially purified human kidney renin were pooled, concentrated to 4 ml by pressure ultrafiltration with an Amicon PM 10 membrane in an ultrafiltration cell (Amicon, Model 12) and coupled onto activated Sepharose by the same procedure described above for the preparation of the MAb column.

An aliquot (40 μl) of stock R-15 polyclonal antibody containing 1 mg/ml protein was made to a volume of 2 ml with PBS and dialyzed overnight against 1 liter column start buffer (0.1 M Tris, pH 7.4) and applied to the renin–Sepharose column. This was washed with 50 ml of start buffer and a similar volume of buffer containing IM NaCl. Antiserum specifically bound to the renin–Sepharose column was eluted with glycine HCl, pH 2.8. The pH of this latter fraction was adjusted to neutrality by overnight dialysis in PBS and concentrated to 4 ml by pressure ultrafiltration. Antiserum purified as described was diluted appropriately in PBS/BSA to yield theoretic titers of 1 : 5000 and 1 : 10,000 (assuming 100% recovery from the column) and used to test tumor sections for the presence of immunoreactive renin.

Gel Filtration

Molecular weight (MW) determination of renin and inactive renin was performed using Sephadex G 100 Superfine (Pharmacia, Uppsala, Sweden) as described.[5]

Area Under the Curve

Area under the curve (AUC) for renin activity eluted from immunoaffinity and gel filtration columns was calculated with a modification of Simpson's integration rule with a specific program on a Transam S 100 microcomputer (Transam Microsystems, Ltd., London, UK).

Results

Immunohistochemical Localization of Renin

Sections from 28 pulmonary tumors were examined for renin, and positive staining was present in association with 23 (Table 1). Immunoreactive renin was found in the walls of blood vessels. Figures 1 and 2 show typical findings in a moderately well differentiated adenocarcinoma with the use of antibody R-15. The vessels in which staining was seen were predominantly arterioles and small muscular arteries, which

Table 1—Details of Human Pulmonary Carcinomas Examined and Numbers Showing Presence of Immunoreactive Renin

Type of tumor	Number stained	Number with positive staining for vascular renin
Adenocarcinoma (excluding bronchioloalveolar cell carcinoma)	12	11
Bronchioloalveolar cell carcinoma	3	0
Squamous cell carcinoma	8	7
Small cell carcinoma	1	1
Large cell carcinoma	4	4
Total	28	23 (82%)

ranged in diameter from approximately 50 to 250 μ. In a few cases, vessels that appeared to be venules were also positive. Staining was within the media. It appeared cytoplasmic, and in those vessels that were positive it was present in most of the medial cells. In some vessels the staining appeared as a double ring of positive cells (Figure 2A) with a layer of cells immediately beneath the endothelium and another ring at the outer edge of the media; this appearance was associated with marked elastosis in the stroma. Occasional vessels with staining in the media showed obliteration of the lumen by inflamed connective tissue, possibly an organized thrombi. No endothelial staining was seen.

The strongest staining was seen in vessels in the stroma of adenocarcinomas, with other tumors showing weaker staining within the stroma. However, in some cases, the predominant staining was not in the body of the tumor itself, but within the area of inflamed fibrous tissue and fibrotic lung at its border. The appearance of the positive vessels at this site was similar to that within the tumors. In one adenocarcinoma, positive staining was present in arterioles in a lymph node metastasis. In general, staining within tumors was most prominent in those cases with a high volume of stroma, compared with epithelium.

No positive staining was seen when either normal rabbit serum or an irrelevant rabbit anti-human IgG antiserum was substituted for the primary antibody. Preabsorption of the primary polyclonal antibody (R-15) with purified human kidney either abolished or markedly reduced the intensity of staining, as previously reported.[22] This is illustrated in Figure 3. Results obtained with the MAb R-3-27-6 were generally disappointing; only faint vascular staining was seen in 2 of 4 cases of adenocarcinoma, which stained strongly with the polyclonal antibody. We now believe that this may have been due to poor preservation of the epitope recognized by the MAb in formalin-fixed tissue and the lower sensitivity of the technique. We therefore further purified the polyclonal antibody using immunoaffinity chromatography as detailed in Materials and Methods. The use of this purified antibody on selected sections revealed identical positive staining confirming the specificity of the technique.

Biologic Activity of Renin in Tumor Homogenates

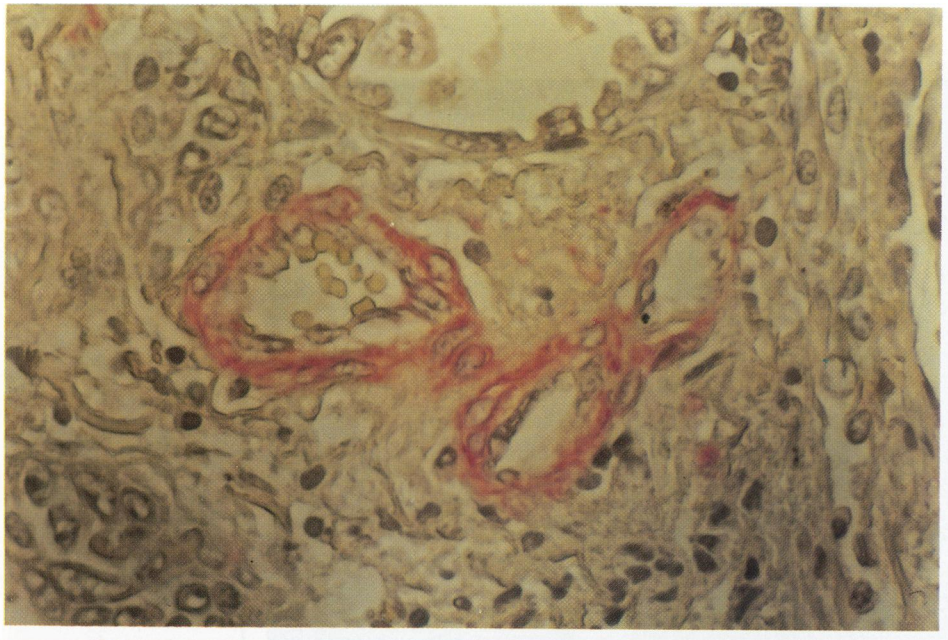
Initial experiments showed that tumor extracts contained only low levels of reninlike activity and that another, nonspecific protease(s) capable of generating ANG-I from renin substrate at pH 6.0 was also present. Therefore, immunoaffinity chromatography with the MAbs R-3-27-6 and R-3-36-16 coupled to Sepharose was used to concentrate and purify renin prior to molecular sizing with gel filtration. The results of one such experiment are shown in Figure 4. A total of 10 g of tumor, pooled from 4 adenocarcinomas, was extracted as described in Materials and Methods and applied to the column, which was washed with 30 ml of 0.1 M Tris HCl buffer, followed by 30 ml of 0.1 M Tris HCl containing 1 M NaCl. Stepwise reduction of the pH of the column buffer to 2.8 eluted both active and inactive renin specifically bound to the MAbs. These renin-containing fractions were pooled, concentrated by ultrafiltration to 2 ml, and applied to the Sephadex G100 column. Both renin and inactive renin were detected at an elution volume of 86 ml, which suggests an MW of approximately 59,000 daltons for both forms (Figure 5).

Discussion

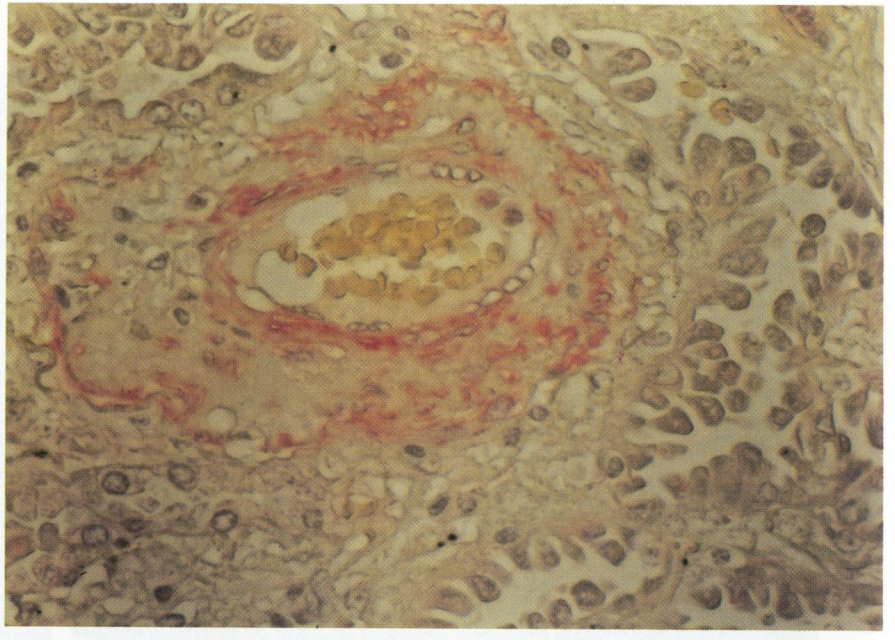
We have demonstrated that immunoreactive renin is present in the vasculature in a high percentage (82%) of common human pulmonary carcinomas. The specificity of the staining was confirmed by preabsorption of the primary antibody with human renin and after immunoaffinity chromatographic purification of the polyclonal antibody on a column containing renin purified with two renin-specific MAbs. In addition, extracts of surgical specimens of

Figures 1-3—Localization of immunoreactive renin in vessels in a moderately well differentiated adenocarcinoma with primary antibody R-15 localized by the APAAP technique (see Materials and Methods). The patient was a 65-year-old man. **Figure 1A**—Typical appearance of immunoreactive renin in a group of arterioles showing circumferential staining. (Hematoxylin counterstain, ×512) **B**—A single arteriole showing staining of a layer of medial cells with surrounding amorphous hyaline material. (Hematoxylin counterstain, ×640). **Figure 2A**—Localization of renin forming an apparent double ring within the media of an arteriole. (Hematoxylin counterstain, ×460) **B**—Appearance of vascular renin staining in an arteriole cut longitudinally. (Hematoxylin counterstain, ×288)

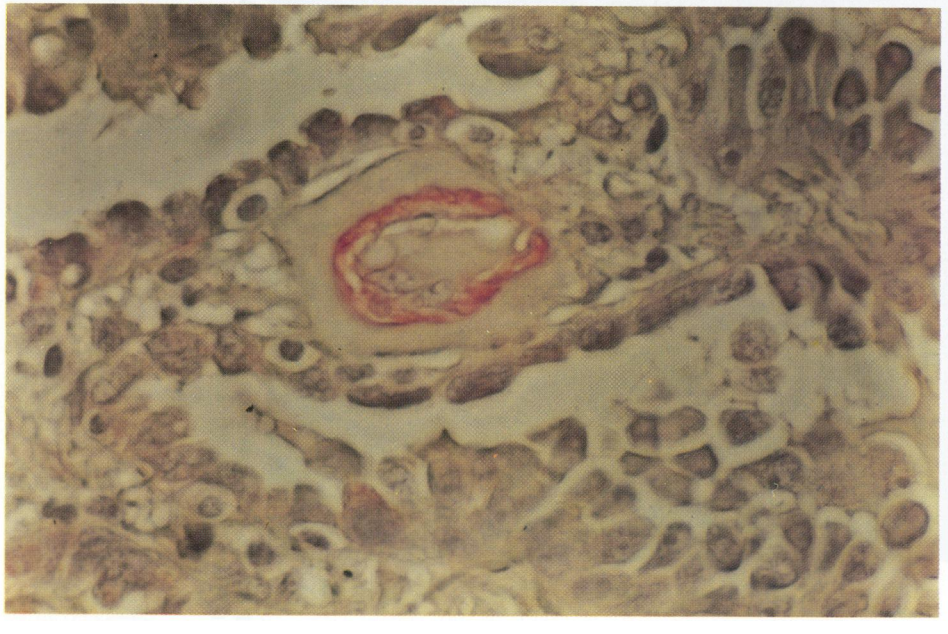
1A



2A



1B



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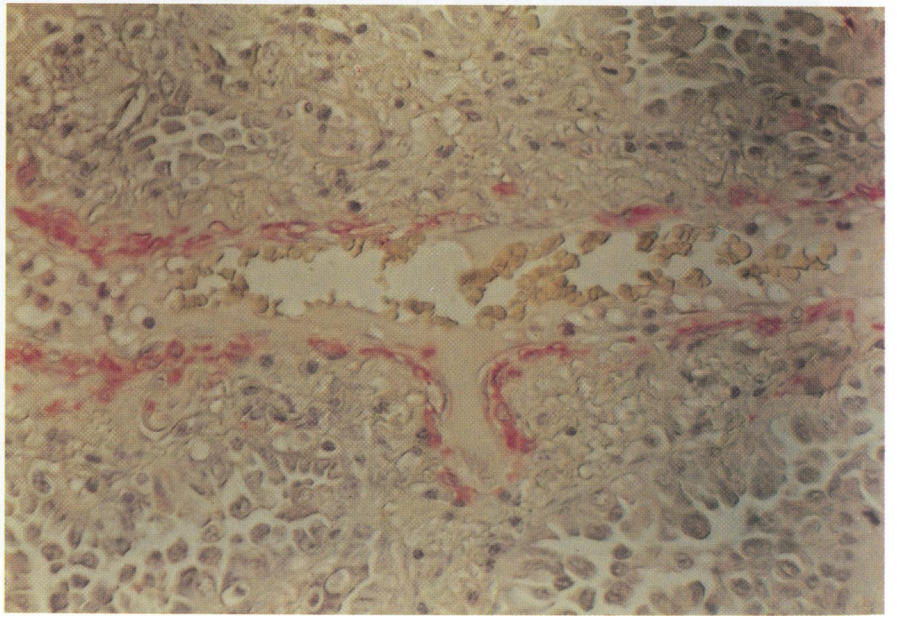

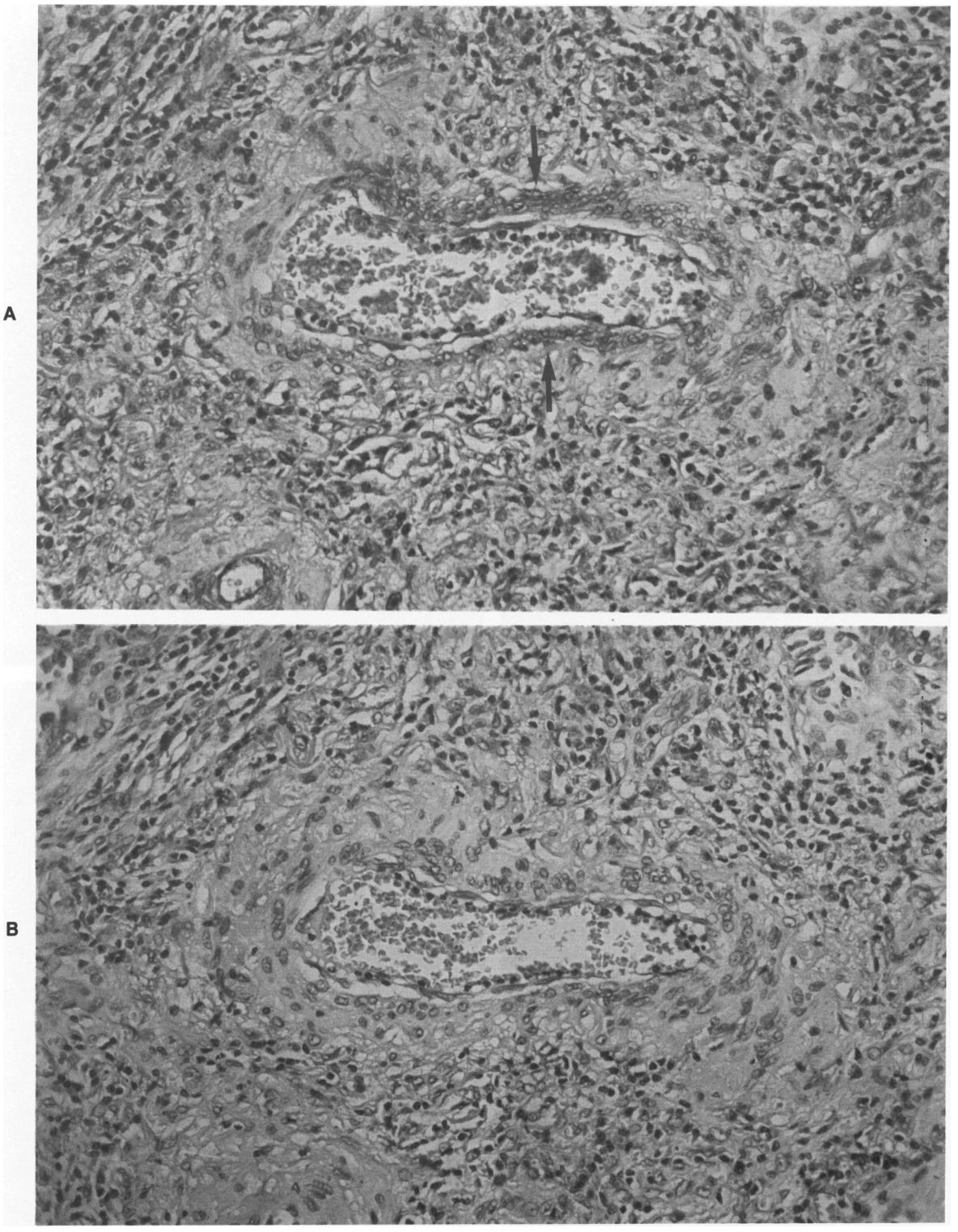

Figure 3A—Vascular renin staining in an inflamed region of the tumor. **B**—Abolishment of staining following preabsorption of the primary antibody with purified human renin. (Hematoxylin counterstain, ×224)

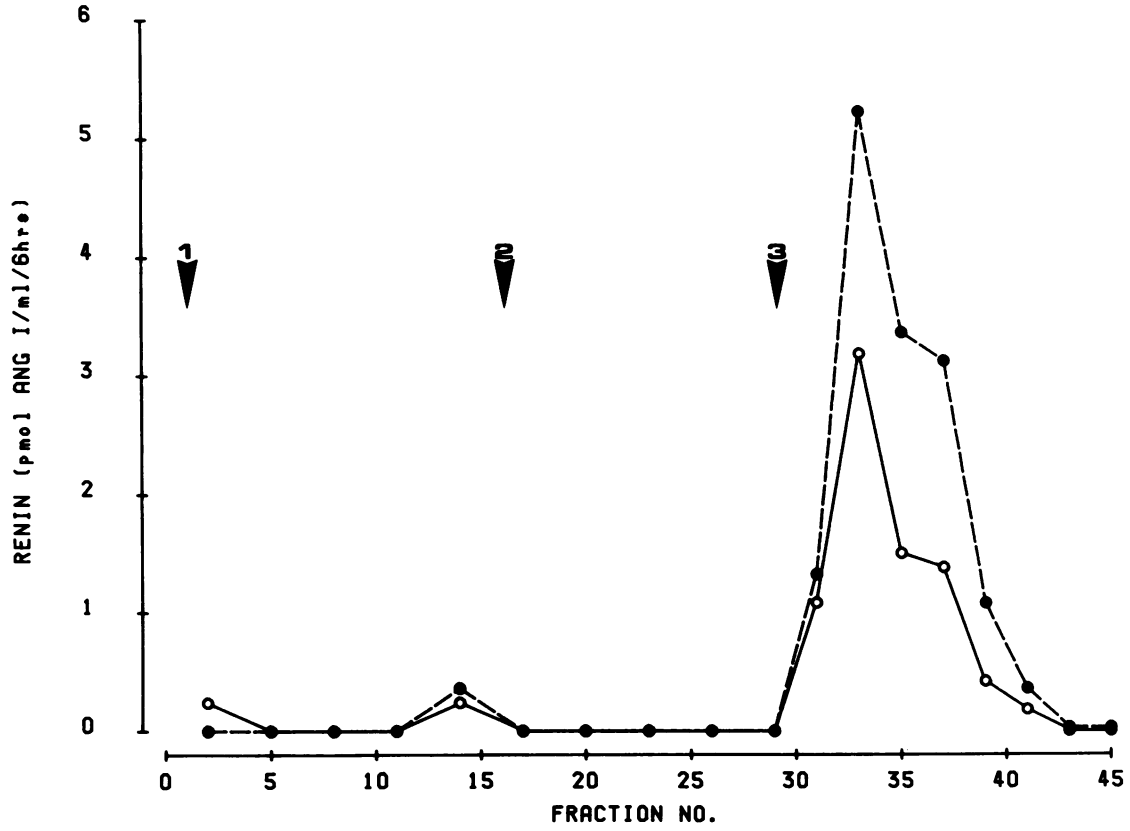

Figure 4—Immunoaffinity chromatography of pulmonary adenocarcinoma extract prepared as described in Materials and Methods. Approximately 5 ml homogenate, equilibrated in Tris-HCl (0.1 M, pH 7.4) was applied to the monoclonal renin column. *Arrows 1–3* indicate elution with 1) 0.1 M Tris-HCl, pH 7.4; 2) Tris-HCl containing 1 M NaCl, and 3) stepwise elution with 0.1 M glycine HCl buffer, pH 2.8. Renin activity in control (○—○) and trypsin-treated (●—●) eluates. AUC for active and total renin peaks were 28.6 and 53.9 units, respectively (arbitrary units). Fractions 30–40 were pooled, concentrated to 2 ml by ultrafiltration, and applied to the Sephadex column.

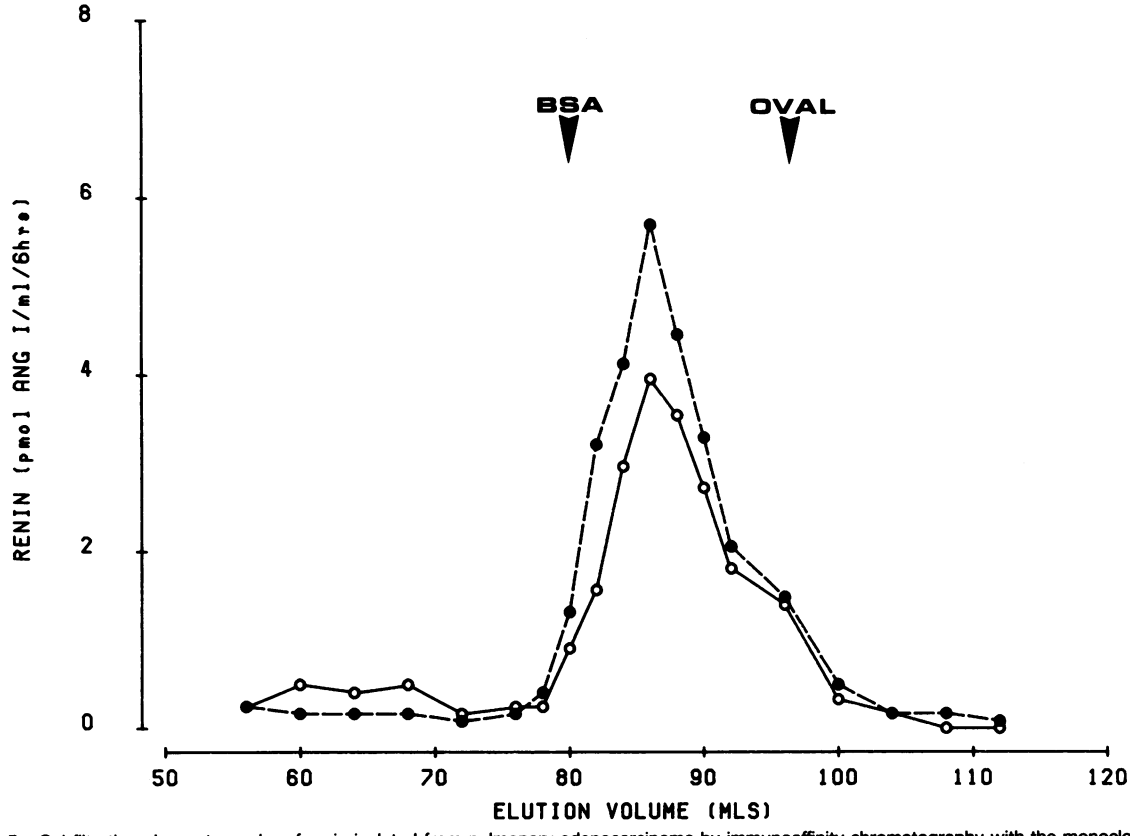

Figure 5—Gel filtration chromatography of renin isolated from pulmonary adenocarcinoma by immunoaffinity chromatography with the monoclonal renin column shown in Figure 3. Renin activity in control (○—○) and trypsin-treated (●—●) eluates. AUC for active and total renin peaks were 42.7 and 56.4 units, respectively (arbitrary units). *OVAL*, ovalbumin.

adenocarcinomas were found to contain both active and inactive renin, which were recognized by the MAbs. The molecular size of 59,000 daltons for both forms is not significantly different, either from the values we have previously found for inactive and activated renin purified from anephric and normal plasma[5] or from those reported by others who have studied plasma[2] and is significantly larger than found for renins purified from kidney (40,000–50,000).[2] This suggests that at least part of the inactive renin in plasma in anephric and normal man may be of vascular origin.

Renin was found within arterioles both in the stroma of tumors and within fibrous tissue at the borders of tumors. The appearances are consistent with renin localization within medial smooth muscle cells. The presence of renin in areas of scarring at the borders of the tumor indicates that this is not a feature unique to tumor vessels; we have not examined lungs involved by other inflammatory disease. In 1 case staining was present in vessels in a lymph node metastasis and thus is not specifically a property of lung vessels.

Our results do not distinguish between production of renin by these cells and selective uptake. Uptake of circulating renin into the vasculature has been described by Swales and colleagues in the rat.[24] However, the observation of strongly positive cells at the outer edge of the media (Figure 2A) suggested to us synthesis, rather than uptake. Renin production has been demonstrated in cultures of vascular endothelial and smooth muscle cell lines obtained from arterial tissue in various species.[9–11]

In the present investigation, vascular immunoreactive staining was not associated with hypertension in any of the cases. This is consistent with the observation of low levels of both active and inactive renin in tumor homogenates. Therefore, the cases we have described should not be confused with true renin-secreting tumors.

Two cases of renin-secreting tumors of the lung have been described. A giant cell variant of pulmonary adenocarcinoma was reported by Genest et al,[18] and elevated plasma renin with hypokalaemic alkalosis has also been reported in a patient with a pulmonary oat cell carcinoma.[25] Immunohistochemical localization of renin was not performed in either of these earlier studies.

The classic example of a renin-secreting JG cell tumor was that first described by Robertson and coworkers.[26] Typically, these renal cortical tumors are small and encapsulated and contain smooth muscle and tubular components. Hypertension may also be associated with other renal tumors, such as nephroblastoma.[15] The hypertension may be secondary to obstruction of renal vessels, or may result from inappropriate renin secretion. A similar situation may occur in patients with congenital mesoblastic nephromas,[27] hypernephromas,[28] and even extrarenal parovarian[16] and pancreatic neoplasms.[17] Immunohistochemical studies have shown that while renin may be present in cytoplasm of the tumor cells themselves,[22] localization is often perivascular.[28] The cases we describe herein of tumor-associated vascular renin are clearly different from the true renin-secreting tumors of both renal and extra renal origin which are invariably associated with hypertension.

It is worth noting that none of the bronchioloalveolar cell carcinomas showed positive vascular staining for renin. This may be significant, because the vessels seen within this variant of adenocarcinoma represent preexisting pulmonary vessels and appear not to be induced by the tumor. The tumor grows along the alveolar septa, preserving the underlying pulmonary architecture. It may therefore be that renin is associated with new vessel formation in tumors.

As the formation of new vessels occurs in other pathologic and physiologic conditions, particularly in inflammatory states such as arthritis, diabetes, and wound healing, these may also be worthy of study. Fernandez and colleagues have recently described a case of primary reninism with hypertension which was associated with angiolymphoid hyperplasia with eosinophilia, a reactive inflammatory condition affecting the skin, where there is marked vascular proliferation.[29] These authors also noted the presence of immunoreactive renin at perivascular sites in a total of 6 of 8 additional cases.

In conclusion, this study demonstrates that renin and inactive renin are present in arterioles in the stroma of human pulmonary tumors. Speculation as to the role that locally produced ANG II may play in the modulation of blood flow in these tumor-associated vessels or as a growth factor[30,31] must await demonstration of other components of the renin–angiotensin system, namely, ACE activity and ANG II receptors.

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