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AMINOPEPTIDASE A IS A CONSTITUENT OF ACTIVATED PERICYTES IN ANGIOGENESIS

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SUMMARY

Monoclonal antibody (MAb) RC38 recognizes a human renal antigen of 160 kD recently identified as human aminopeptidase A (APA; EC 3.4.11.7). This ectoenzyme is able to hydrolyse selectively N-terminal glutamyl and aspartyl residues from oligopeptides. By enzyme histochemistry, APA activity has also been localized in the microvessels of all organs in animals and man. The purpose of this study was to investigate the distribution of human APA as recognized by MAb RC38 in the microvasculature of normal human tissues and pathological conditions associated with neovascularization. Unexpectedly, in normal tissues vascular staining with MAb RC38 was generally weak and often absent, while in tumours, granulation tissue, and chronic synovitis, marked microvascular staining was demonstrated. By immuno-electron microscopy, the antigen was found on the cell membrane of activated pericytes and their processes in the tumour vasculature. RC38 expression could not be detected on cultured human endothelial cells or pericytes. These observations suggest that pericyte expression of a subtype of APA (as recognized by MAb RC38) is markedly enhanced in the vasculature of tumours and wound healing tissue as compared with normal resting tissues. This provides further evidence of the altered state of pericytes in these conditions. Pericyte APA may be involved in the metabolism of biologically active oligopeptides during neovascularization, supporting a regulatory role of pericytes in this process. In addition, MAb RC38 may be useful as a marker of pericyte activation in tissue sections.

KEY WORDS—aminopeptidase A; angiogenesis; pericytes; MAb RC38; tumours; wound healing

INTRODUCTION

The role of pericytes in the process of angiogenesis remains controversial.¹⁻³ Based on *in vitro* studies, some authors attribute to these cells an inhibiting influence on the proliferation of endothelial cells in the end stage of the angiogenic response.^{1,4,5} On the basis of morphological studies,⁶⁻¹⁰ others have suggested that pericytes already play an active role in the initial stages of neovascularization.

Early ultrastructural reports on angiogenesis *in situ* demonstrated pericytes with plump cell bodies and an increased number of cell organelles near angiogenic stimuli, signs that were attributed to cellular 'activation'.^{11–14} In recent reports, we have demonstrated that such activated pericytes occur in increased numbers in microvessels in tumours and granulation tissue.^{7,8,15} Furthermore, these cells were found to have enhanced expression of a chondroitin sulphate proteoglycan (HMW-MAA) that was previously implicated in the metastatic potential and migration of melanoma cells.^{7,15} From these studies we concluded that activated pericytes are already present at the onset of angiogenesis and that their altered phenotype may enable them to migrate or perform other specialized functions.

In the present study we have focused on the expression in pericytes of the ectoenzyme aminopeptidase A (glutamyl aminopeptidase, APA; EC 3.4.11.7) as recognized by the monoclonal antibody (MAb) RC38. 16 APA is able to hydrolyse selectively N-terminal glutamyl and

aspartyl residues from oligopeptides.^{17–20} Angiotensin II, a potent constrictor of blood vessels, is an important substrate of the enzyme. APA activity has been demonstrated by enzyme histochemistry in epithelial brush borders in the kidney and small intestine and in the microvasculature of all organs in the rat, guinea pig, and man.^{17,21} The latter was interpreted as endothelial reactivity. However, a recent immunohistochemical study of APA in the rat brain suggested that vascular staining is solely based on expression by pericytes.²⁰

The RC38 antibody was previously described as recognizing a renal antigen. 16 Its distribution in the kidney and other characteristics suggested similarity to MAb S4,²² which recognizes a renal 160 kD glycoprotein that was recently identified as human APA.²³ In this report we demonstrate by immunoprecipitation studies that MAb RC38 also recognizes human APA and we report on the tissue distribution of human APA as recognized by MAb RC38 in normal tissues and in pathological conditions associated with angiogenesis. We found marked staining of microvessels in tumours and granulation tissue, compared with weak or absent staining in vessels of normal organs. This appeared to be based on staining of activated pericytes, as evidenced by immunoelectron microscopic studies. We conclude that activated pericytes express a form of APA that may be involved in the metabolism of biologically active peptides during angiogenesis.

MATERIALS AND METHODS

Tissue samples

Samples from normal and pathological human tissues were obtained from fresh surgical specimens and from

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Table I—Tissues used for immunohistochemistry

Normal tissues	Number	Pathological tissues	Number
Skin	6	Vasoformative tumours	
Oesophagus	1	Haemangioma	2
Jejunum	1	Angiosarcoma	1
Colon	4	Pyogenic granuloma	2
Pancreas	4		
Liver	2	Other tumours	
Spleen	5	Breast neoplasms	16
Lymph node	4	Colonic neoplasms	12
Tonsil	2	Neuroblastoma	1
Thymus	4	Sarcoma	4
Thyroid	2	Melanoma	4
Lung	1	Meningioma	2
Kidney	4	Glioblastoma multiforme	8
Ovary	1	Haemangiopericytoma	i
Testis	1		
Cerebral cortex	5	Wound healing	
Cerebellum	2	Decubitus lesion	6
Skeletal muscle	2	Burn wound	4
Breast	3	Chronic synovitis	4
Placenta	4		

patients who were autopsied within 8 h after death. Included in the study were normal tissues, neoplasms, granulation tissues, and vasoformative tumours (Table I). All tissues were snap-frozen and stored at -70° C. For immuno-electron microscopy, selected tissue samples were promptly fixed in 2 per cent paraformal-dehyde in Sörensen phosphate buffer after surgical removal.

Cultured cells

Microvascular endothelial cells were isolated from human foreskin and cultured in Dulbecco's minimal essential medium supplemented with 40 per cent normal human serum.²⁴ Human umbilical vein endothelium, fibroblasts, human brain pericytes, and a renal carcinoma cell line (SK-RC-1) were isolated and grown in culture as described elsewhere.^{7,24} Different passages were used for immunoperoxidase staining of cytospin preparations.

Immunoperoxidase staining

Air-dried frozen tissue sections and culture coverslips were fixed with acetone for 10 min and stained by a sensitive indirect immunoperoxidase procedure, using MAb RC38 and anti-endothelial MAb PAL-E²⁵ at appropriate dilutions and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, TAGO Inc., Burlingame, CA, U.S.A.) as secondary antibody. 3,3-Diaminobenzidine (DAB) containing 1 mM imidazole and 0.01 per cent H₂O₂ was used as substrate for 10 min.⁷ In a double staining experiment, sections of a lung carcinoma metastasis in the brain DAB stained with RC38 (brown precipitate) were washed three times with 0.1 M glycine-HCl, pH 3.0 to remove bound primary antibody and conjugate and then stained with anti-

endothelial MAb PAL-E, using 4-chloro-1-naphthol as substrate (blue-grey precipitate). Sections were counterstained with haematoxylin. The sections were examined by two independent observers (ROS and DJR). In the occasional case of discrepancy, the slides were jointly re-evaluated until consensus was reached. Staining intensity was graded as follows: no staining (-), weak staining (+/-), moderate staining (+), or intense staining (++).

Immuno-electron microscopy

A pre-embedding immunoperoxidase technique described in detail elsewhere^{7,15} was used to demonstrate the ultrastructural distribution of the staining with MAb RC38. Briefly, surgical material with a maximum size of $4 \times 6 \times 2$ mm was promptly fixed for 4 h in 2 per cent paraformaldehyde in Sörensen buffer (pH 7·4). After washing in 2.3 m sucrose solution, the tissue was immediately frozen in liquid nitrogen and stored at - 196°C. On a cryomicrotome (Micron, Heidelberg, Germany), $30 \,\mu \text{m}$ sections were cut at $-20 \,^{\circ}\text{C}$. These sections were incubated overnight with the first antibody at 4°C. After washing, the sections were incubated at room temperature for 1.5 h with a peroxidaseconjugated rabbit anti-mouse and/or swine anti-rabbit antibody (Dakopatts/ITK) at appropriate dilutions in PBG. Control sections were incubated with the second antibodies alone. After extensive washing, the sections were incubated in a 5 mg/ml DAB solution in PBS 3 mg/ml ammonium-nickel-sulphatecontaining hexahydrate (FLUKA 09885, Buchs, Switzerland). The sections were then developed for 8–10 min in the same solution containing 0.01 per cent H_2O_2 (30 per cent). The reaction was terminated by rinsing with tap water. The sections were post-fixed in 1 per cent osmium tetroxide for 5 min at room temperature, dehydrated, and embedded in Epon 812. Ultrathin sections were contrasted for 1 min with 3 per cent uranyl acetate and examined and photographed with a JEOL 1200 EX/II electron microscope (Tokyo, Japan) at 40 kV.

Characterization of the RC38 antigen

Sequential immunoprecipitation with MAb RC38 and MAb S4, an antibody known to recognize human aminopeptidase A,^{22,23} was performed as follows: SK-RC-1 cells were metabolically labelled overnight with [³H]glucosamine, washed, and lysed as described in ref. 23. Cell lysates were incubated with RC38 or S4 for 2 h and incubated with Sepharose-CL-4B protein A beads. After I h, the beads were washed and the immunoprecipitates were separated by polyacrylamide gel electrophoresis (PAAGE). Autoradiography was performed using Kodak diagnostic film. Films were developed in a Kodak RP X-OMAT processor (Eastman Kodak, Rochester, NY, U.S.A.).

Lysates depleted by RC38 or S4 were treated with the same MAb as described above, to ensure complete depletion of the lysate of their respective antigens. These immunoprecipitates were also investigated by PAAGE. Thereafter, RC38-treated lysates were incubated with

MAb S4 and vice versa. The remainder of the procedure was as described above.

RESULTS

Tissue distribution by MAb RC38

The distribution of the antigen recognized by MAb RC38 was studied using immunohistochemistry on frozen tissue sections. Variable but often intense staining of capillaries and small venules with MAb RC38 was observed in the stroma of most neoplasms (Figs 1E and 1F). In benign lesions of the breast, microvascular RC38 staining was strong in proliferative mastopathy but weaker in other lesions (Table II). In vasoformative tumours, profound staining of stromal capillaries was seen. The multilayered cell layer lining the vascular spaces of a pyogenic granuloma and some spindle cell areas in an angiosarcoma were also positive (results not shown). In chronically inflamed synovia and in the superficial parts of granulation tissue in decubitus lesions, vascular staining with MAb RC38 was also observed (Figs 1A and 1B). In the deeper fibrous areas of granulation tissues and in adjacent normal skin, RC38 staining was focally weak or absent.

Capillaries and small venules in normal tissues showed only focal and weak positivity in pancreas, lymphoid tissues, and intestinal mucosa (Figs IC and 1D). In the other normal tissues, no microvascular staining with MAb RC38 was observed. Smooth muscle cells in the walls of arterioles, arteries, and larger veins were negative in all tissues (Fig. 1D).

The epithelium of the proximal tubule and glomerulus of the normal kidney showed marked staining, while reactivity was also found in the luminal epithelial brush border of the small intestine and colon, the placental trophoblast, the red pulp of spleen, and periportal sinusoidal liver cells, a pattern suggestive of staining of Kupffer or Ito cells (results not shown).

In the RC38-stained microvessels in tumours and granulation tissue, unstained luminal cells were occasionally seen, suggesting that non-endothelial cells were recognized by the antibody (Figs 1B and 1F). To investigate which cells were recognized, a double-staining experiment was performed in sections of a lung carcinoma metastasis to the brain, with MAb RC38 and with MAb PAL-E, a general endothelial marker. The complementary staining pattern for PAL-E (luminal cells) and RC38 (abluminal cells) suggested staining of endothelial cells and pericytes, respectively (results not shown).

Immuno-electron microscopy

The distribution of the RC38 antigen at the ultrastructural level was studied using pre-embedding immuno-

peroxidase staining of thick cryomicrotome sections of a glioblastoma, a neuroblastoma, three colonic carcinomas, normal kidney, and prostate. In all tumours marked staining of pericyte cell membranes and pericyte processes was observed (Fig. 2). In the normal kidney, staining with RC38 was confined to the brush border of the epithelium of renal proximal tubules and glomeruli. Pericytes and their processes, observed in the walls of capillaries in normal kidney and prostate, did not stain (results not shown). In none of the examined tissues was endothelial staining observed.

Cultured cells

There was no staining of endothelial cells, fibroblasts, keratinocytes, or melanocytes in primary human foreskin cultures, or of cultured human brain pericytes and endothelial cells from umbilical vein. Weak staining was seen in SK-RC-1 renal carcinoma cells (results not shown).

Biochemical characterization of the RC38 antigen

The RC38 antigen showed a normal tissue distribution very similar to that of the antigen recognized by MAb S4.¹⁶ We therefore investigated whether the RC38 antigen might be identical to the S4 antigen. Radioimmunoprecipitation of SK-RC-1 cells with RC38 revealed the specific precipitation of a glycoprotein with a molecular weight of 160 kD (gp160), identical to the antigen recognized by MAb S4. However, where S4 precipitates revealed multiple gp160 species, probably due to different glycosylation patterns,²² the RC38 precipitates showed only one discrete gp160. The molecule recognized by MAb S4 has recently been identified as aminopeptidase A.²³ Sequential immunoprecipitation of gp160-positive cell lysates revealed that RC38 was unable to deplete any gp160 from S4-depleted material. In the reverse experiment, S4 precipitated gp160 material, with a clear defect of the RC38-related gp160, indicating that RC38 recognizes a subset of the S4-related gp160.

DISCUSSION

Pericytes are found in varying numbers in all organs,²⁶ but their function is poorly understood. Like vascular smooth muscle cells, they contain all the necessary elements for contraction, suggesting a role in the regulation of blood flow in the microvasculature.^{2,26}

Recently, these cells have received much attention as to their presumed role in the process of angiogenesis. Early reports suggested a function in mechanical support and basement membrane formation in newly

Fig. 1—Immunoperoxidase staining with anti-APA MAb RC38 (B, D, and F, right panels) and with anti-endothelial MAb PAL-E (A, C, and E, left panels) of adjacent frozen tissue sections of surgically removed granulation tissue (A and B, upper panels) and serial sections of normal colon (C and D, middle panels) and colonic carcinoma (E and F, lower panels). MAb RC38 stains microvessels in the granulation tissue (B) and tumour stroma (F); note the unstained endothelial nuclei along the lumina of vessels (indicated by arrows). In normal colonic mucosa and submucosa, microvessels are staining with MAb PAL-E (C, indicated by arrows), but not with MAb RC38 (D)




Table II—Staining of vascular structures with	MAb RC38 in normal	tissues and benign	and malignant
turnours of the breast and colon			

		Mumber	Microvascular staining
	Normal manmary gland		
	Non-proliferative mastopathy		
	Proliferative mastopathy		
	Carcinoma		
	Normal mucosa		Characteristics (1) 1 (1) (1) (1) (1) (1) (1) (1) (1) (
	Adenoma		Settlement Company Settlement Set
	Carcinoma		Security Control Contr

^{+/ -} sporadic weak staining.

formed vessels, 26 but more recently, pericytes have been proposed to act as controllers of endothelial proliferation in resting tissues which, during angiogenesis, appear only in the end stage of angiogenesis. 1,4,27,28 Indeed, in vitro, pericytes are able to inhibit endothelial proliferation in co-cultures.^{4,5} However, morphological studies of angiogenesis in vivo have demonstrated pericytes in the earliest stages of vascular sprout formation and support an active role of these cells in all the stages of neovascularization. 7-10 In tumours and other conditions with neovascularization, pericytes differ morphologically from their normal tissue counterparts. They are more plump, show signs of amoeboid movement, are mitotically active, and have an increased number of cell organelles. 11-13 Recently, we reported that such 'activated' pericytes have enhanced expression of a glycosaminoglycan known as the high molecular weight melanoma associated antigen (HMW-MAA).7 From this study we concluded that activated pericytes have an altered phenotype and are present in increased numbers in the microvasculature of tissues with neovascularization. In addition, activated pericytes were recently shown to express the receptor for epidermal growth factor in granulation tissue.29

Here we report on the microvascular distribution of human aminopeptidase A (APA, glutamyl transpeptidase; EC 3.4.11.7) as recognized by MAb RC38.¹⁶ Similarly to HMW-MAA, we noted strong staining for RC38-related APA of microvessels in tumours and granulation tissue. Much weaker or absent microvascular RC38 staining was found in normal tissues. By immuno-electron microscopy, the cells staining in these vessels were identified as activated pericytes. These observations suggest a quantitative up-regulation of RC38-related APA in activated pericytes.

Biochemical comparison of the antigens recognized by MAb RC38 and MAb S4, the latter identifying human APA,^{22,23} revealed that MAb RC38 recognized a subspecies of aminopeptidase A. Sequential radioimmuno-precipitations with MAb S4 and MAb RC38 showed that MAb RC38-depleted APA-containing lysates still contained S4-precipitable material, but not vice versa. As RC38 recognizes a peptide sequence¹⁶ and not a carbohydrate moiety, it is likely that the MAb RC38

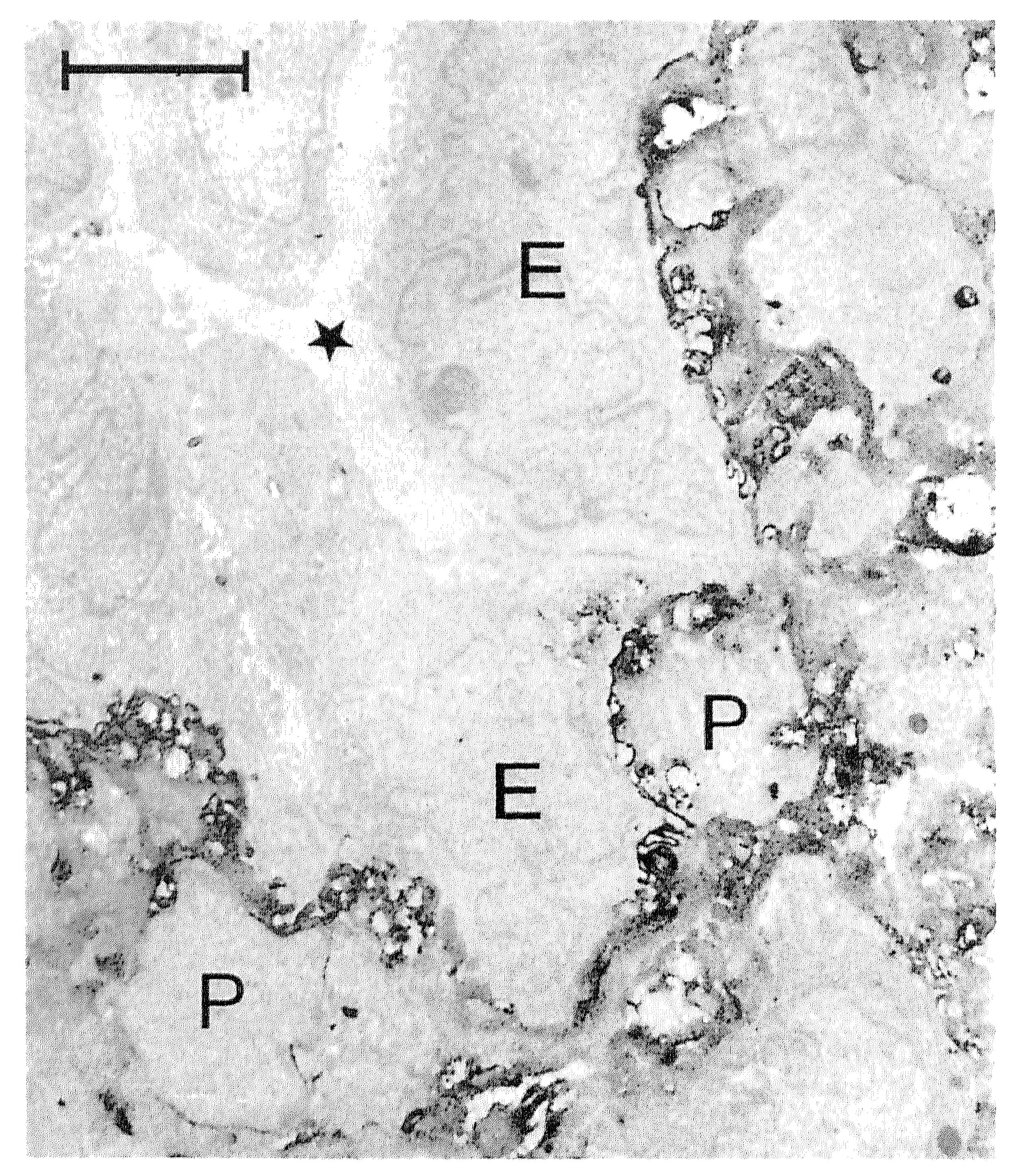


Fig. 2—Electron microscopy of immunoperoxidase staining with anti-APA MAb RC38 in an area with glomeroid microvascular proliferation in a surgically removed glioblastoma multiforme. Note the staining of pericytes (P) and their processes. Endothelial cells (E) are not stained. The asterisk indicates lumen; bar = $3 \mu m$

epitope is masked in the APA that is still precipitable by MAb S4.

Early distribution studies on APA in animal and human tissues by enzyme histochemistry demonstrated active APA in brush border epithelia in the kidney and small intestine and in the vasculature of all organs.^{17,19} The latter was interpreted as endothelial reactivity.¹⁷ Recently, however, APA in the rat brain was demonstrated in pericytes by immunohistochemical staining,²⁰ suggesting that pericytes may be the source of microvascular APA in other organs as well. MAb S4, shown to recognize human APA,²³ does stain microvessels in normal tissues (personal communication, Dr C. L. Finstad). It is therefore surprising that MAb RC38

shows only weak or absent microvascular staining in normal tissues. This suggests, like our biochemical findings, that RC38 recognizes a separate species of APA (probably underglycosylated) that has enhanced expression in activated pericytes and therefore may have a special function in these cells, possibly related to angiogenesis.

APA is not the first ectoenzyme demonstrated on pericytes. Previously, gamma-glutamyl transpeptidase and aminopeptidase M were localized in brain pericytes. ^{30,31} As these enzymes may regulate or neutralize neurotransmitters and other active oligopeptides, their expression by cerebral pericytes was considered as a sign of involvement of these cells in the blood-brain barrier function of brain vessels. ^{30,31}

Aminopeptidase A has not previously been implicated in pericyte activation or angiogenesis. The enzyme acts on peptides with an N-terminal acidic amino acid and thus selectively hydrolyses glutamyl and aspartyl residues.^{20,23,32} An important substrate of APA is angiotensin II, which is metabolized into angiotensin III. These are both potent vasoactive peptidases with systemic and local effects on vascular tone.²⁰ Interestingly, angiotensin II can also stimulate angiogenesis in the rabbit cornea and chorioallantoic membrane (CAM) angiogenesis assays.^{32–35} Recently, a novel receptor for angiotensin II was demonstrated in the CAM³⁴ and this receptor is probably located on the pericytes.³⁶ We suggest that APA is involved in the degradation by pericytes of angiotensin II and other important regulatory oligopeptides²³ involved in tissue regeneration and angiogenesis. The numerous activated pericytes in microvessels in angiogenesis^{7,8} are strategically localized to act, by their expression of APA, as regulators of the activity of such factors.

Vascular smooth muscle cells were not stained with MAb RC38. This observation is interesting, as pericytes and smooth muscle cells have many common features and are regarded as closely related cell types.^{4,26} Membrane determinants distinguishing these cell types could be useful in histopathology, or help to identify functional differences.^{2,3,31}

In conclusion, the special form of APA that is recognized by MAb RC38 in activated pericytes may provide these cells with a regulatory ectoenzyme involved in the process of angiogenesis. Our findings give further evidence for an altered phenotype of activated pericytes and identify MAb RC38 as another marker for these cells in conditions with neovascularization.

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