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Expression of the High Molecular Weight Melanoma-associated Antigen by Pericytes During Angiogenesis in Tumors and in Healing Wounds

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In the course of immunohistochemical characterization of murine monoclonal antibodies recognizing the human high molecular weight-melanoma associated antigen (HMW-MAA), a striking reactivity with blood vessels in the tumor stroma was noted. Immunocytochemical analysis by light- and electronmicroscopy of a panel of tissues and cell lines showed that the staining of microvessels by anti-HMW-MAA monoclonal antibodies was restricted to pericytes. Correspondingly, anti-HMW-MAA monoclonal antibodies were found to react with cultured pericytes from human brain, but not with endothelial cells in serologic assays, and to immunoprecipitate from biosynthetically labeled pericytes an antigen with the characteristic structural profile of HMW-MAA. At the subcellular level, the expression of HMW-MAA in cultured pericytes was mainly restricted to microspikes that are localized in clusters on the cellular membrane. Staining by anti-HMW-MAA monoclonal antibodies of pericytes was not only found in the tumor stroma, but also in other lesions associated with angiogenesis, such as granulation tissue of wound healing and synovitis. In these lesions, microvascular staining for another marker of pericytes, ie, alpha-smooth muscle actin, also was observed. These results suggest that, in conditions associated with vascular proliferation, 1) pericytes acquire HMW-MAA and 2) the number of pericytes may be increased as compared with normal tissues. (Am J Pathol 1990, 136:1393–1405)

The high molecular weight-melanoma associated antigen (HMW-MAA), a chondroitin sulfate proteoglycan, also designated the melanoma proteoglycan (Mel- CSPG or MP),[1–3] was described previously as a marker for melanoma cells.[4,5] Functional studies in cultured melanoma cells suggested a role of HMW-MAA in cell–substratum and cell–cell interactions[3,6] associated with metastasis and autonomous growth of neoplastic cells. In the course of immunohistochemical analysis of tumor lesions with anti-HMW-MAA monoclonal antibodies, we observed staining of microvascular structures in the tumor stroma. Therefore, in the present investigation we addressed ourselves to the following questions:

1) What vascular cell type is stained by anti-HMW-MAA monoclonal antibodies?
2) What is the nature of the antigen recognized?
3) Which mechanism(s) underlie(s) the higher staining of vessels in tumor stroma than in normal tissues?

Materials and Methods

Tissue Samples

Samples from normal and pathologic human tissues (Table 1) were obtained from fresh surgical specimens and from patients on whom autopsies were performed within 6 hours after death. Granulation tissue was obtained from burn wounds and decubitus lesions and inflamed synovia from patients with chronic rheumatoid arthritis (Table 1). Liver, spleen, skin, gut, lung, and eye from a 20-week-old human fetus and normal adult (gut, cerebral cortex, and

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Table 1. *List of Organs and Lesions Used for Immunohistochemistry*

Normal organs	No. of samples	Benign and malignant lesions	No. of samples
Pancreas	4	Endothelial Tumors	
Esophagus	1	Hemangioma	2
Jejunum	1	Pyogenic granuloma	2
Colon	4	Angiosarcoma	1
Spleen	4	Other tumors	
Lymph node	4	Colon carcinoma*	8
Tonsil	2	Breast carcinoma	4
Thymus	4	Lung carcinoma	4
Kidney*	4	Sarcoma*	4
Lung	1	Melanoma	4
Liver	4	Meningioma	2
Cerebral cortex	5	Glioma	8
Cerebellum	2	Hemangiopericytoma	1
Skin	6	Rabbit VX2-carcinoma	4
Skeletal muscle	2	Feline carcinoma	2
Thyroid*	2	Wound Healing	
Ovary	1	Decubitus lesion	6
Testis	1	Burn wounds	4
Placenta*	4	Synovitis	4

* Lesions included in immunoelectronmicroscopic study.

tonsil) and neoplastic tissues from rat, rabbit, cat, and cow also were included. All tissues were snap-frozen and stored at −70°C.

Cultured Cells

Microvascular endothelial cells were isolated from human foreskin as described by Davison et al,[7] and cultured in Dulbecco's minimal essential medium supplemented with 20% normal human serum. Human umbilical vein endothelium was isolated from umbilical cords and cultured as described by van Hinsbergh et al.[8] Human brain pericytes were isolated from cerebral cortex obtained from autopsy patients and were cultured following Vinter's method.[9] Briefly, capillary fragments were isolated via adherence to plastic surfaces, treated with collagenase (Boehringer Mannheim, FRG) (1 mg/ml for 15 minutes at 37°C), and seeded into fibronectin-coated culture dishes. After 5 to 10 days, outgrowth of pericytes was observed, and pure cultures of these cells were obtained by selective cloning. Cultured human fibroblasts, U937 hemopoietic cells, smooth muscle cells, and the melanoma cell line M14 were cultured in medium RPMI 1640 supplemented with 10% fetal calf serum (FCS).

Monoclonal Antibodies and Conventional Antisera

The monoclonal antibodies (MAb) AMF-6 (provided by Dr. C. Figdor), 225.28S, 763.74, and TP41.2 to distinct and spatially distant determinants of HMW-MAA, the MAb PAL-E and EN4 (Sanbio BV, Uden, The Netherlands) to endothelial markers, the MAb CLB-35 to von Willebrand factor (provided by Dr. J. van Mourik), the MAb RD301 to desmin (Organon Teknika, Boxtel, The Netherlands), the MAb anti-alpha-sm1 to alpha-smooth muscle actin (Biomakor, Rehovot, Israel), a marker for smooth muscle cells and pericytes, the MAb 3G5 (provided by Dr. C. Nayak) to a marker for pericytes in culture and the anti-collagen type IV MAb 1042 (provided by Dr. F. T. Bosman) were developed and characterized as described elsewhere.[10–15] Horseradish-peroxidase (HRP) conjugated goat anti-mouse gamma G immunoglobulin (IgG) antibodies, HRP-conjugated rabbit anti-mouse IgG antibodies, and 10-nm gold conjugated goat anti-mouse IgG antibodies were purchased from TAGO Inc, Burlingame, CA, from Dakopatts, Copenhagen, Denmark, and from Janssen Pharmaceutics, Beerse, Belgium, respectively.

Immunoperoxidase Staining

Staining was performed as previously described.[13] Briefly, cultured cells at different passages were seeded on plastic microscope coverslips coated with fibronectin and cultured for 24 hours. Air-dried 4-μ frozen tissue sections, culture coverslips, and cytospins of cultured cells were fixed with acetone for 10 minutes and sequentially incubated at room temperature with appropriate dilutions of monoclonal antibodies for 120 minutes and HRP conjugated anti-mouse Ig xenoantibodies for 60 minutes.[16,17] 3-3-di-amino-benzidine (DAB) 0.075% in TRIS-buffered saline (TBS) pH 6.0 containing 0.001 mol/l (molar) imidazole and 0.01% H_2O_2 was used as a substrate.[17] Sections were counterstained with hematoxylin. Staining was graded as −, when no staining was detectable; ±, when the staining intensity was weak; +, when it was moderate; and ++, when it was marked. When indicated, staining was quanti-

Table 2. *Staining with Anti-HMW-MAA Monoclonal Antibodies * of Frozen Sections of Lesions Associated with Neovascularization*

Blood vessel	Endothelial tumors	Other tumors	Granulation tissue	Rheumatoid synovitis
Arteries, arterioles				
Smooth muscle cells	++	++†	++	++
Endothelium	–	–	–	–
Capillaries	++	++	++	++

* Staining results obtained by anti-HMW-MAA MAbs 225.28S, 703.74, or TP 41.2 were similar.
† No staining; ++, marked staining.

tated by counting the percentage of stained capillary cross-sections, using MAb PAL-E stained capillaries in consecutive sections as a reference. Specificity of staining was monitored by incubating tissue substrates with HRP-conjugated anti-mouse Ig xenoantibodies. In some tumors, a double staining procedure by immunoperoxidase and immunofluorescence in consecutive steps was performed according to Lechago et al,[18] using the MAb anti-alpha-sml and the anti-collagen type IV MAb 1042.

Indirect Immunoprecipitation and Sodium Dodecylsulphate Polyacrilamide Gel Electrophoresis (SDS-PAGE)

Melanoma cells and pericytes were grown to 50% confluency in a 150-cm culture flask (5 × 10 cells/flask). After a 30-minute incubation in precursor-free medium, containing 10% dialyzed FBS, cells were labeled in 25 ml of the same medium, supplemented with 100 μCi of 14C-leucine or 1000 μCi of 35S-sulphate (Amersham International, Amersham, UK). After a 16-hour incubation at 37°C, cells were harvested by scraping, washed three times with Hank's balanced salt solution (HBSS), solubilized with NP40, precleared with Protein A-sepharose (Pharmacia, Uppsala, Sweden) coated with rabbit anti-mouse Ig xenoantibodies, and mixed with monoclonal antibodies bound to anti-mouse Ig xenoantibody-coated Protein A-sepharose. At the end of a 2-hour incubation at room temperature, immunocomplexes were washed extensively and analyzed by SDS-PAGE (7.5%) using the buffer system described by Laemmli.[19] Gels were fluorographed as described by Bonner and Laskey.[20]

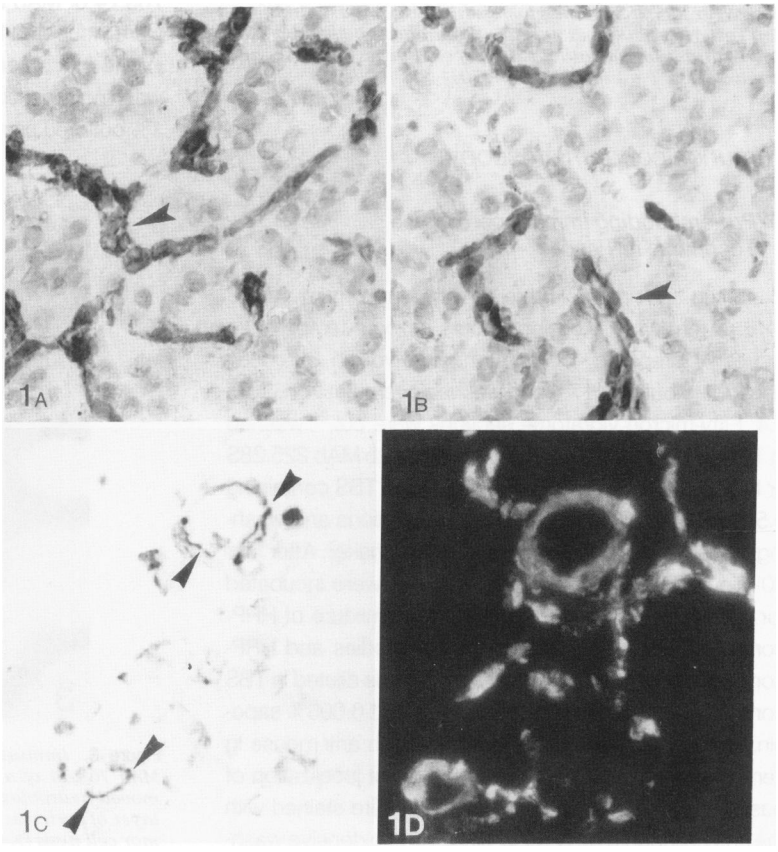

Figure 1. *Immunoperoxidase staining with anti-HMW-MAA MAb 225.28S* **(A)**, *with anti-endothelium MAb PAL-E* **(B)**, *and with MAb anti-alpha-sml* **(C)** *of frozen sections of a surgically removed seminoma lesion. Immunofluorescence staining with anti-collagen type IV Mab 1042 of the frozen section shown in* **C (D)**. *Mab 225.28S stains capillaries (arrowhead) in tumor stroma. MAb PAL-E stains endothelium in capillaries (arrowhead). T = tumor cells. MAb anti-alpha-sml stains pericytes (arrowhead) that can be recognized by their location in the microvascular wall, visualized by the staining of collagen type IV (×450).*

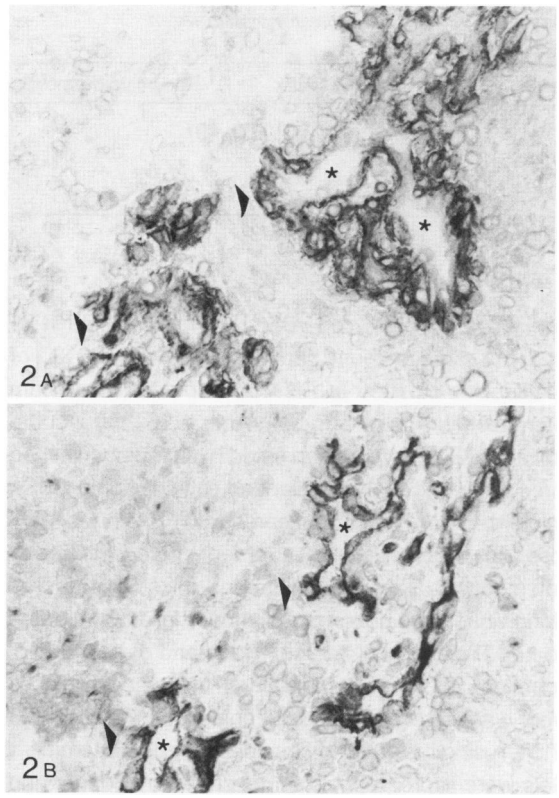

Figure 2. *Immunoperoxidase staining with anti-HMW-MAA MAb 225.28S* (A) *and with anti-endothelium MAb PAL-E* (B) *of serial frozen sections of a surgically removed astrocytoma lesion. MAb 225.28S stains glomeruluslike capillary proliferation (asterisks) but does not stain lumina (arrowheads). MAb PAL-E stains luminal endothelium of capillaries (asterisks and arrowheads). T = tumor cells (×450).*

Immunoelectronmicroscopy

Pre-embedding Immunoperoxidase Technique in Tissues

Tissue specimens (Table 1) with a maximum size of 4×6×2 mm were fixed for 4 hours at room temperature in freshly prepared 4% paraformaldehyde in Sorensen phosphate buffer pH 7.4 and washed overnight in TBS pH 7.4. Fifty-micron vibratome sections were cut,[21] washed in TBS, and incubated overnight at 4°C with MAb 225.28S or MAb 763.74 at appropriate dilutions in TBS containing 0.5% BSA and 0.005%. saponin. Incubations and washing steps were performed using a rotary shaker. After four 30-minute washings with TBS, sections were incubated for 3 hours at room temperature with a mixture of HRP-conjugated rabbit anti-mouse IgG antibodies and HRP-conjugated goat anti-mouse IgG antibodies diluted in TBS containing 20% normal human serum and 0.005% saponin. Control sections were incubated with anti-mouse Ig xenoantibodies alone. To define the exact localization of basement membranes, serial sections were stained with the anti-collagen type IV MAb 1042. After extensive washings in TBS, sections were preincubated for 10 minutes in TBS pH 5.7 supplemented with DAB 0.75 mg/ml and 0.001 mol/l (molar) imidazole. Sections then were developed for 10 minutes in the same solution supplemented with 0.01% H_2O_2; the reaction was terminated with TBS. Subsequently, sections were post-fixed for 40 minutes in 1% osmium tetroxide in Palade buffer, then dehydrated, embedded in epoxy resin (Epon-812, E. Merck, Darmstadt, West Germany) and processed routinely. One-micron sections were cut for light microscopy. Ultrathin sections were cut and not contrasted further. They were examined and micrographed on a Philips 300 electron microscope (Philips, Eindhoven, The Netherlands) at 40 kV.

Pre-embedding Immunogold Staining of Cultured Cells

Immunogold staining was performed according to Garrigues et al.[3] Briefly, human brain pericytes and human umbilical vein endothelial cells were cultured for 48 hours on 5- × 12-mm Melinex (ICI, Hertz, United Kingdom) coverslips.[20] Coverslips were fixed for 2 hours at 37°C in 0.65% gluteraldehyde and 2% paraformaldehyde in PBS, pH 7.7. After a 30-minute incubation with 20% human serum in TBS to reduce background staining, coverslips were incubated for 4 hours at room temperature with MAb 763.74 or MAb EN4. After washing with PBS, coverslips were incubated for 2 hours with colloidal 10-nm gold-conjugated goat anti-mouse IgG antibodies. After further washing, postfixation was performed for 40 minutes with 1% buffered osmiumtetroxide in Palade buffer, and cover-

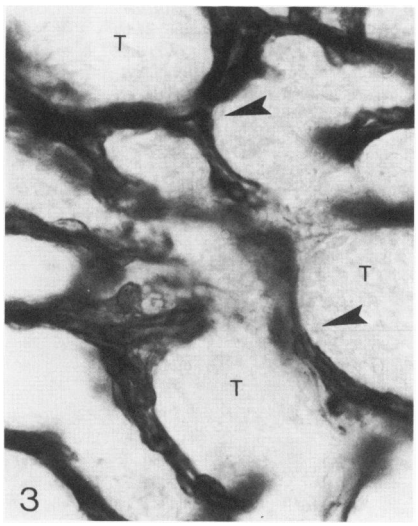

Figure 3. *Immunoperoxidase staining with anti-HMW-MAA MAb 763.74 of a thick vibratome section of a surgically removed neuroblastoma lesion. MAb 763.75 stains a continuous layer of pericytes in a network of capillaries (arrows). T = tumor cell nests (×450).*

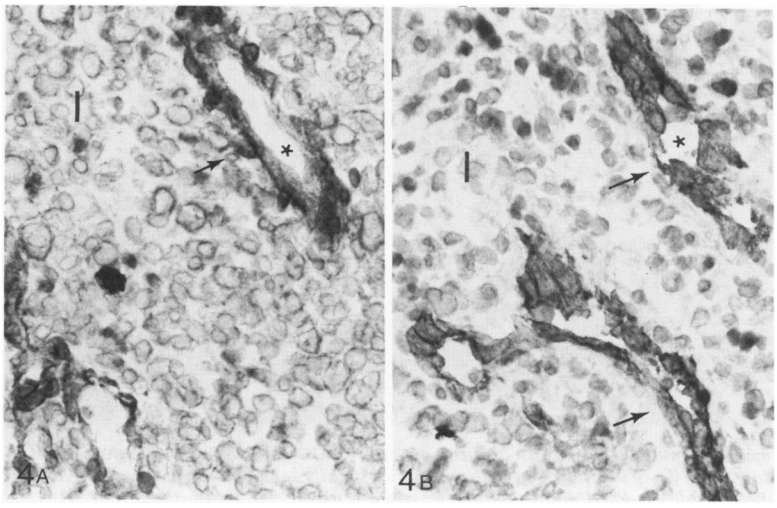

Figure 4. *Immunoperoxidase staining with anti-HMW-MAA MAb 763.74* **(A)** *and with anti-endothelium MoAb PAL-E* **(B)** *of serial frozen sections of a surgically removed granulation tissue of healing skin wound. MAb 763.74 stains vascular wall (arrow) but does not stain lumina (asterisk). MAb PAL-E stains vascular lumina (asterisk and arrows). I = cellular infiltrate (×450).*

slips were embedded in Epon-812. Ultrathin sections were cut in two planes, perpendicular and parallel to the Melinex surface. Sections were contrasted further with lead citrate and uranyl acetate. To quantitate differential labeling of the membrane of cellular processes *versus* underlying cell surface, micrographs of three complete immuno-gold stained cells were used. Gold particles were counted and the mean number of particles per micrometer of membrane was determined for the two categories of cell surface.

Results

Distribution of HMW-MAA in Malignant Lesions, in Benign Lesions, and in Normal Tissues

The distribution of HMW-MAA in the microvasculature of pathologic conditions and normal tissues was studied using immunohistochemistry on frozen sections and three different anti-HMW-MAA MAbs.

In the tumor stroma, anti-HMW-MAA MAbs 225.28S, 763.74 or TP41.2 stained capillaries with strong intensity (Table 2), corresponding with the vascular staining of anti-endothelial MAb PAL-E in consecutive sections (Figure 1). All anti-HMW-MAA MAbs reacted with equal intensity and similar distribution. The strongest positivity was found in colon and brain tumors in areas with apparent capillary proliferation as characterized by glomeruluslike structures (Figure 2). Occasionally, endothelial cells unstained for HMW-MAA were seen at the luminal side of the DAB reaction product (Figure 2). This observation suggested that the antigen was expressed by nonendothelial microvascular cells. Of the tumors tested, staining of tumor cells was found in melanoma and hemangiopericytoma. In addition, myoepithelial cells in breast carcinoma were occasionally stained. Vascular structures in two spontaneously developed feline breast carcinomas and the rabbit VX2 carcinoma also were stained by anti-HMW-MAA MAb 225.28S, but not by the other anti-HMW-MAA MAbs. Similar results were obtained when 50-μ vibratome sections were stained for HMW-MAA (Figure 3).

In chronically inflamed synovia and in granulation tissue of wound healing, anti-HMW-MAA MAbs 225.28S, 763.74, or TP 41.2 stained capillaries with high intensity (Table 2 and Figure 4) In contrast, adjacent scar tissue and normal skin were not stained (not shown).

The vascular staining pattern observed with anti-HMW-MAA monoclonal antibodies in normal adult tissues contrasted strongly with the results obtained in tumor le-

Table 3. *Staining with Anti-HMW-MAA Monoclonal Antibodies* of Frozen Sections of Normal Adult Tissues from Humans, Cat, Cow, and Rabbit†*

Vascular structures	Staining	Nonvascular structures: Tissue	Nonvascular structures: Cell type	Staining
Arteries, arterioles		Cartilage	Chondrocytes	++
Endothelium	−‡	Hair follicle	Basal keratinocytes	++
Smooth muscle cells	++	Gut	Smooth muscle cells	+
Capillaries	*	Glomerulus	Mesangial cells	+

* Staining results obtained only by anti-HMW-MAA MAb 225.28S.
† Staining results obtained in the species mentioned were similar.
‡ −, no staining; +, moderate staining; ++, marked staining; *, sporadic weak staining.

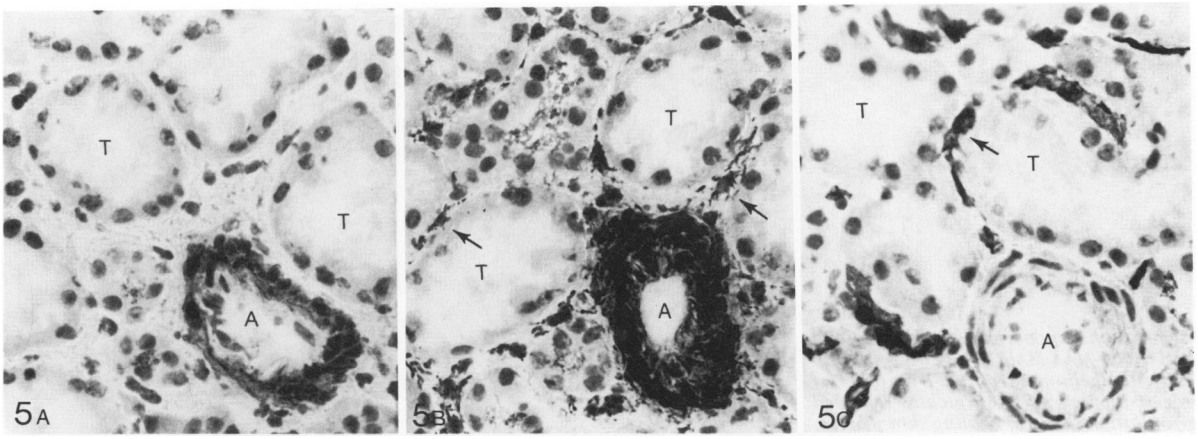

Figure 5. *Immunoperoxidase staining with anti-HMW-MAA MAb 225.28S* **(A)**, *with MAb anti-alpha-sml* **(B)** *and with anti-endothelium MAb PAL-E* **(C)** *of serial frozen sections of a surgically removed normal kidney. MAb 225.28S stains smooth muscle cells in the wall of an arteriole* **(A)**. *Peritubular capillaries are not visible. MAb anti-alpha-sml stains smooth muscle cells of arteriole* **(A)** *and pericytes of peritubular capillaries (arrows). MAb PAL-E stains endothelium of capillaries (arrows). T = tubuli (×450).*

sions. Immunoperoxidase staining of frozen tissue sections with MAb 225.28S showed that HMW-MAA is barely detectable in capillaries in normal tissues of adult man, cat, cow, and rabbit (Table 3, Figure 5). Smooth muscle cells in arterioles and arteries (Figures 5 and 6) and mesangial cells in renal glomerulus, however, stained consistently. Outside the microvasculature, basal keratinocytes in hair follicles, chondrocytes in cartilage, and smooth muscle cells were stained (Table 3). Anti–HMW-MAA MAbs 225.28S, 763.74 or TP 41.2 strongly stained blood vessels in placenta (Figure 7) and in tissues of a 20-week-old fetus (not shown).

Expression of HMW-MAA by Cultured Cells

To confirm the preferential staining for HMW-MAA by pericytes, pericytes were isolated and characterized, and stained by immunocytochemistry.

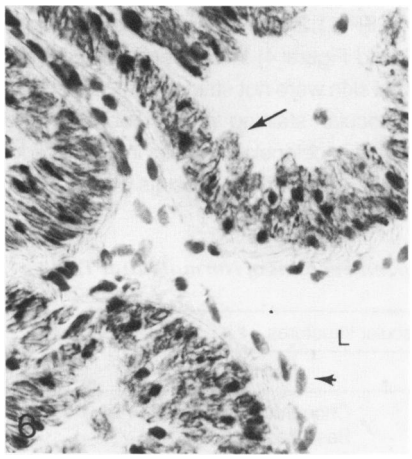

Figure 6. *Immunoperoxidase staining with anti–HMW-MAA MAb 763.74 of a frozen section of a surgically removed umbilical cord. MAb 763.74 stains smooth muscle cells (arrow) in media of umbilical vein, but does not stain the endothelium (arrowhead). L = lumen (×450).*

Characterization of Pericytes

The phenotype of pericytes cultured from human brain were stained by MAb anti-alpha-sm1 (not shown), indicating that they were not fibroblasts. Between 60% and 70%

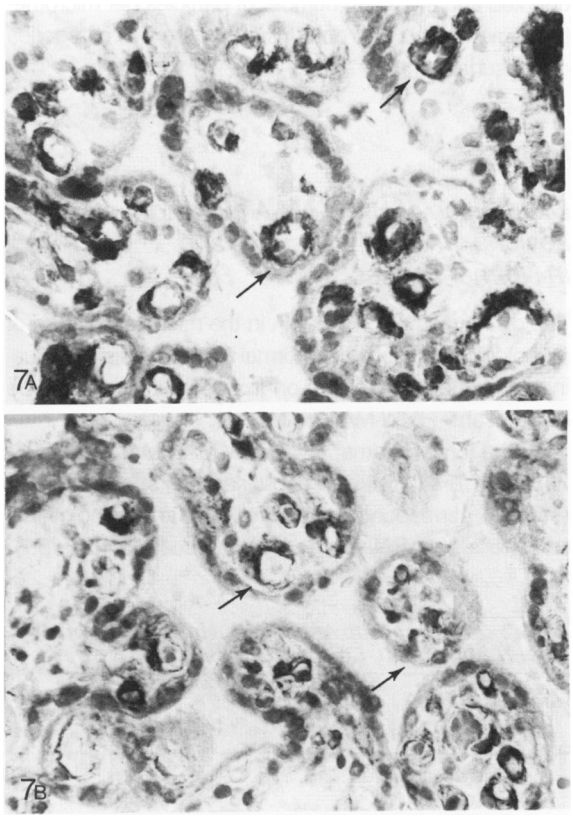

Figure 7. *Immunoperoxidase staining with anti–HMW-MAA MAb 225.28S* **(A)** *and with MAb anti-alpha-sml* **(B)** *of frozen sections of a surgically removed full-term placenta. MAb 225.28S and MAb anti-alpha-sm1 stain fetal capillaries (arrows) (×450).*

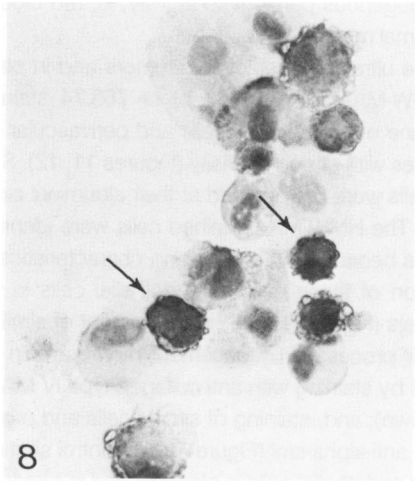

Figure 8. *Immunoperoxidase staining with anti-pericyte MAb 3G5 of a cytospin preparation of human brain pericytes. MAb 3G5 stains pericyte membrane (arrows) (×450).*

of cells were also stained by the MAb 3G5 (Figure 8), recognizing a gangliosidelike antigen expressed by cultured pericytes but not detectable in cultured smooth muscle cells. No staining was detected with anti-desmin MAb RD301 (not shown). Furthermore, the cells did not express the morphologic characteristics of smooth muscle cells (not shown), both by light, (ie, cell shape, high number of nucleoli and knobs in cellular extensions), and by electron microscopic analysis, (ie, abundant microfilament bundles and plasmalemmal densities). No staining of cells was detected with anti-von Willebrand factor MAb CLB-35 and with the anti-endothelial cell MAb EN4 and PAL-E, indicating that they are not of endothelial origin

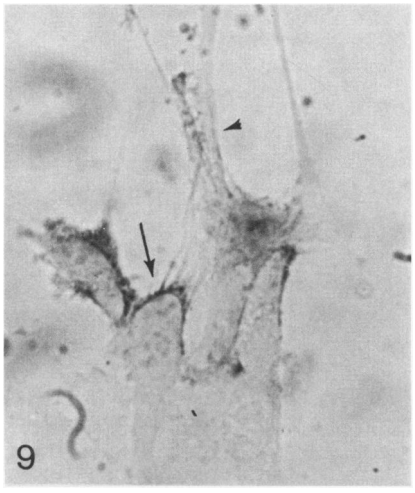

Figure 9. *Immunoperoxidase staining with anti-HMW-MAA MAb 225.28S of human brain pericytes cultured on plastic coverslip. No counterstaining. MAb 225.28S stains pericyte membrane in areas with clusters of cellular processes (arrow). Other areas of the membrane are not stained (arrowhead) (×450).*

Table 4. *Staining of Cultured Human Cells with MAb EN4, MAb 3G5, and Anti-HMW-MAA MAbs 225.28S, 763.75, or TP41.2* *

	MAb		
	Anti-HMW-MAA	3G5	EN4
Foreskin microvascular Endothelium	−	−	++
Umbilical vein Endothelium	−	−	++
Brain pericytes	++	++	−
Fibroblasts	−	−	−
Smooth muscle cells	++	−	−
Melanoma cells	++	−	−

* The three different anti-HMW-MAA MAbs gave similar results.
−, no staining; ++, marked staining.

(not shown). No staining of cells was detected with antibodies to glial fibrillary acidic protein (GFAP) and to neurofilaments (not shown), arguing against their neural origin. Based on all these identification criteria, the cells cultured from human brain were classified as pericytes.

Staining With Anti-HMW-MAA Monoclonal Antibodies

The anti-HMW-MAA MAb AMF-6, 225.28S, 763.74 or TP41.2 stained cultured brain pericytes (Figure 9), smooth muscle cells, and melanoma cells with a strong intensity (Table 4). The staining of the cell membrane of pericytes was not homogeneous, but was restricted to sites of multiple cellular processes (Figure 9). No staining was detected of endothelial cells from umbilical vein and from microvascular endothelium and of fibroblasts in culture (not shown).

Molecular Profile of the Antigen Recognized by Anti-HMW-MAA Monoclonal Antibodies in Pericytes

To analyze the nature of the antigen recognized by anti-HMW-MAA monoclonal antibodies on human pericytes, the components immunoprecipitated from biosynthetically labeled human brain pericytes were analyzed by SDS-PAGE. Their electrophoretic profile was compared with that of HMW-MAA immunoprecipitated from intrinsically labeled cultured melanoma cells. Figure 10 shows that the antigen immunoprecipitated by ascites fluid containing anti-HMW-MAA MAb AMF-6 from 14-leucine-labeled pericytes has the characteristic two-chain structure of HMW-MAA with a 420-kd and a 250-kd component.[1-3,15,22] When a 35S-sulfate-labeled pericyte extract is the antigen source, only the large 420-kd component containing the labeled glycosaminoglycan side chains is visualized (Figure 10), because the core protein, devoid

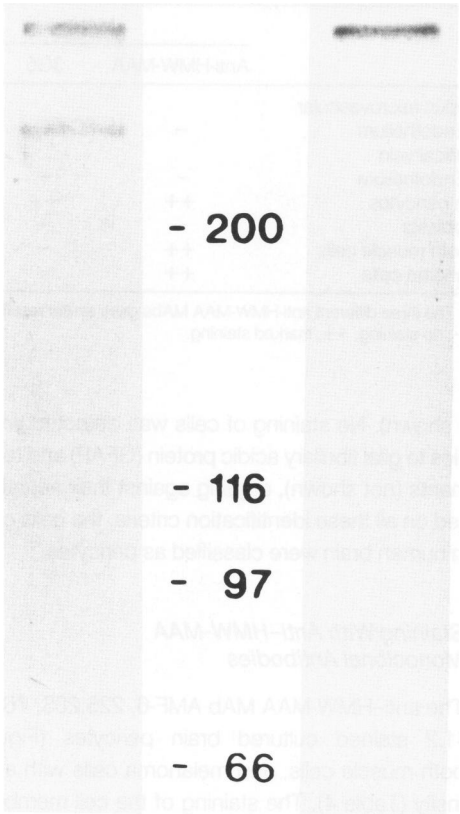

Figure 10. *SDS-PAGE analysis of components immunoprecipitated with ascites fluid containing anti-HMW-MAA MoAb AMF-6 from 14-leucine and 35S-sulphate-labeled human brain pericytes. The results using MoAb AMF-6 containing culture supernatant were identical (not shown).*

of sulfate groups, remains unlabeled. The results were identical using culture supernatant containing anti–HMW-MAA MAb AMF-6.

Immunoelectronmicroscopy

To determine the nature of the microvascular cells stained by anti–HMW-MAA MAbs, immunoelectronmicroscopy was used, using thick vibratome tissue sections and cell suspensions.

Pre-embedding Immunoperoxidase Staining of Tissues

In control vibratome sections, where incubation with specific first antibody was omitted, both light- and electronmicroscopy showed electron-dense reaction product (DAB) in occasional intracellular lipid droplets and in sites with endogenous peroxidase activity, ie, red blood cells and stromal macrophages.

At the ultrastructural level in tumors and in placenta, anti–HMW-MAA MAbs 225.28S or 763.74 stained the membrane of perivascular cells and perivascular cellular processes with strong intensity (Figures 11, 12). Some of these cells were only stained at their albuminal side (Figure 11). The HMW-MAA–stained cells were identified as pericytes because of the following characteristics: close apposition of these cells to endothelial cells in the microvessels (Figures 11, 12); envelopment of similar cells and their processes by basement membrane in control sections by staining with anti-collagen type IV MAb 1042 (not shown); and, staining of similar cells and processes by MAb anti-alpha-sml (Figure 12) in control sections. All vascular endothelial cells in placenta and in tumor stroma were surrounded by a sheath of pericytes and their processes (Figures 11, 12). The morphologic appearance of these pericytes, ie, a plump cell body, lobed nucleus, and increased number of cell organelles, suggested an activated state (Figure 12). In the examined tumor lesions, placenta, and normal organs, no staining of endothelial cells was detected with anti–HMW-MAA MAbs 225.25S or 763.74.

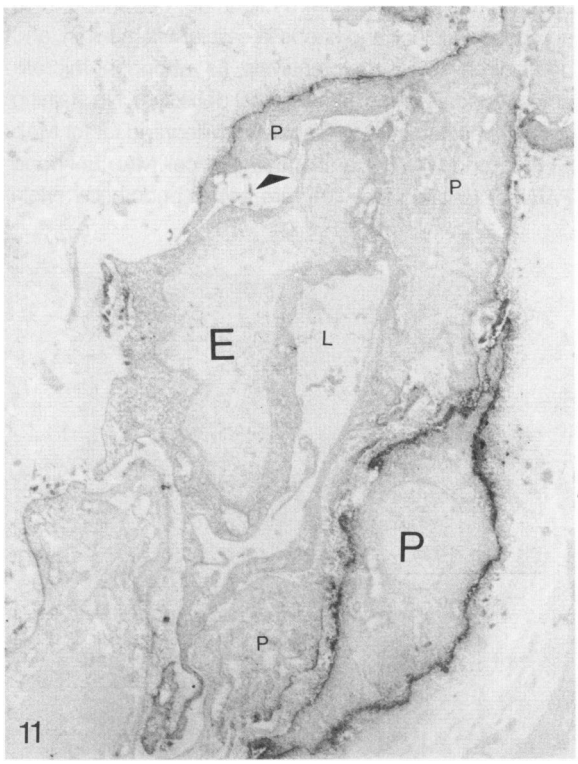

Figure 11. *Electronmicroscopy of immunoperoxidase staining with anti–HMW-MAA MAb 763.74 of a surgically removed colon carcinoma lesion. MAb 763.74 stains perivascular cells (P). Some perivascular cells are not stained on luminal side (arrowhead). E = endothelium; L = lumen; (×4000).*

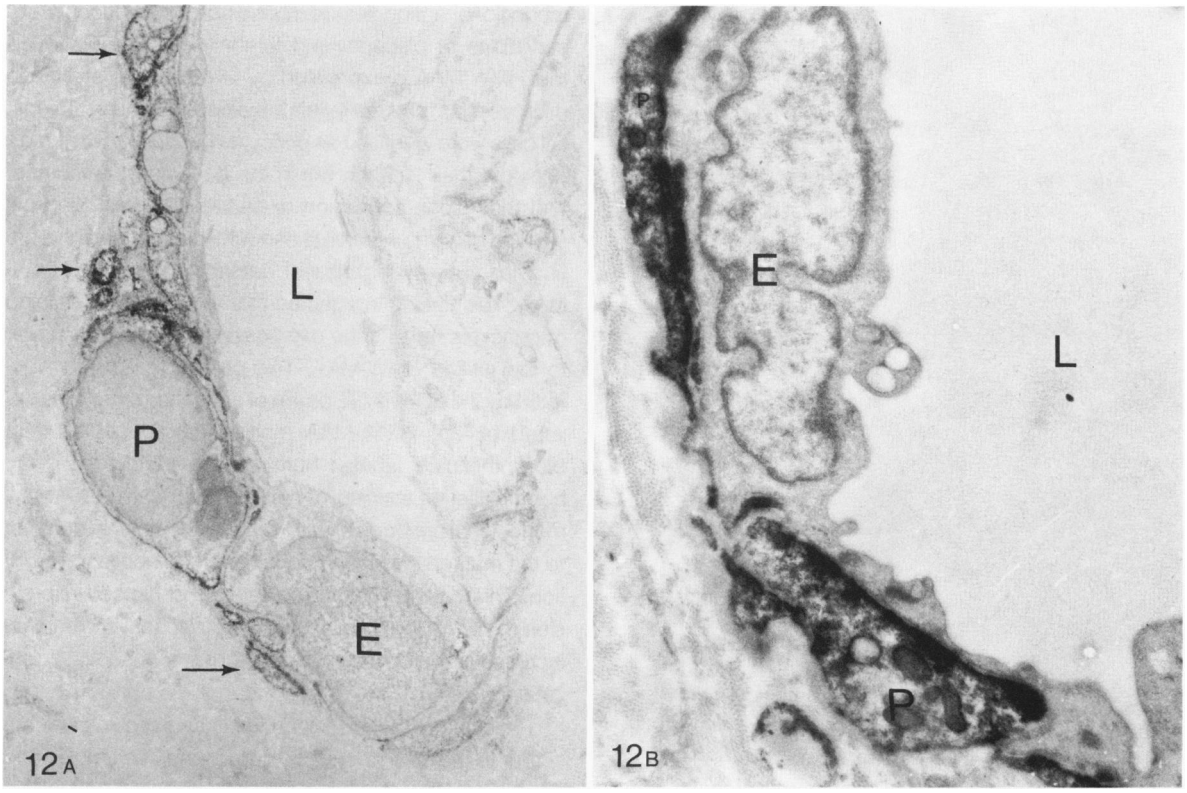

Figure 12. *Electronmicroscopy of immunoperoxidase staining with anti-HMW-MAA MAb 763.74* (A) *and with MAb anti-alpha-sml* (B) *of serial vibratome sections of a surgically removed placenta. MAb 763.74 stains perivascular cells (P) and perivascular cellular processes (arrows). MAb anti-alpha-sm1 stains actin in perivascular cells (P). E = endothelium; L = lumen* (A: ×4000 *and* B: ×7800)

Pre-embedding Immunogold Staining of Cultured Cells

Many (80 to 200/cell) gold particles were found at the cell membrane of cultured human brain pericytes stained with anti-HMW-MAA MAb 763.74. Staining was preferentially localized at the membrane of microspikes (Figure 13) usually situated in clusters on one side of the cell. These processes were 1 to 3 μ in length and had an approximate diameter of 100 nm. We quantitated the number of gold particles per micrometer of cell membrane of the processes and underlying cell membrane. At the membrane of microspikes, 1 to 3 particles/micrometer were observed, compared with 0.1 to 0.5 particles/micrometer at the remaining cell surface. No gold particles were found on the membrane of endothelial cells incubated with MAb 763.74 and of pericytes incubated with the control antibody MAb EN4 (not shown).

Quantitation of Immunohistochemical Staining Results

To gain an impression on the pericyte coverage of the microvasculature in different conditions, the proportion of HMW-MAA and of alpha smooth muscle actin-staining microvascular cross-sections was estimated.

In normal adult thyroid, kidney, and striated muscle, the proportion of capillary cross sections stained by anti-HMW-MAA monoclonal antibodies was much lower than that stained by MAb anti-alpha-sm1, indicating that most pericytes in normal tissues are not stained by anti-HMW-MAA MAbs 225.28S, 763.74, or TP 41.2 (Figures 5, 14).

In contrast, in tumors, in granulation tissue, and in full-term placenta, most capillary cross sections were stained by anti-HMW-MAA MAbs 225.25S, 763.74, or TP 41.2 (Figures 1, 14). Based on the previous immunoelectronmicroscopic finding of the preferential staining with anti-HMW-MAA MAbs in pericytes in tumor tissues, this pattern is indicative for a high number of pericytes with marked expression of HMW-MAA. It is remarkable that in these tissues a lower number of capillaries is stained by MAb anti-alpha-sm1 than by anti-HMW-MAA monoclonal antibodies. This difference suggests that in these conditions not all pericytes express alpha-sm1 (Figure 14).

Discussion

This study shows that in conditions associated with neovascularization, capillaries are stained by anti-HMW-MAA

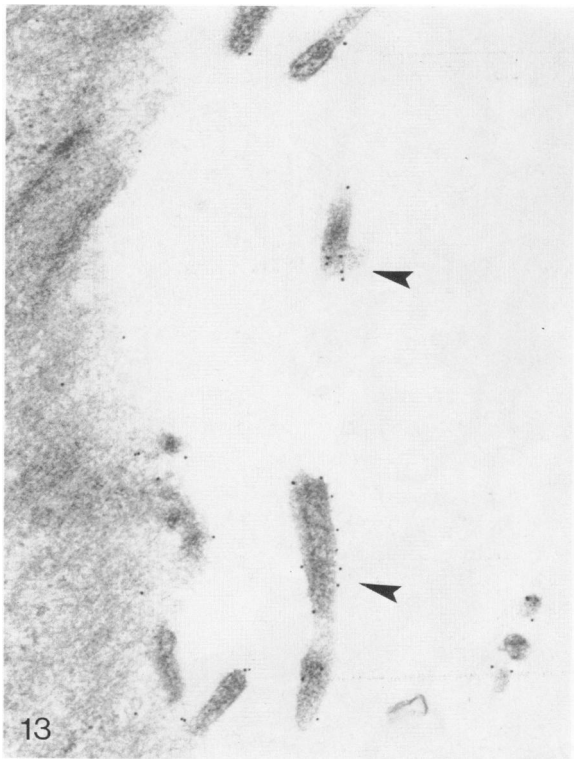

Figure 13. *Electronmicroscopy of immunogold staining with anti-HMW-MAA MAb 763.74 of human brain pericytes cultured on melinex. Immunogold staining is preferentially localized on microspikes (arrows) (×40,000).*

monoclonal antibodies. Immunoelectronmicroscopic observations of placenta and tumor tissue demonstrated that HMW-MAA is expressed by adventitial cells in these microvessels, and not by endothelial cells. These adventitial cells were identified as pericytes because of their morphology, their location within the basement membrane, and their close apposition to endothelial cells. Identical staining patterns were obtained with three monoclonal antibodies recognizing distinct determinants of the HMW-MAA. This finding suggested that the observed staining of pericytes reflects the expression of an antigen similar to that of the HMW-MAA. This possibility was corroborated by the SDS-PAGE profile of antigens immunoprecipitated by anti-HMW-MAA monoclonal antibodies from biosynthetically labeled human brain pericytes.[1-3,22] In normal tissues staining of capillaries by anti-HMW-MAA monoclonal antibodies was hardly detectable, in contrast to the marked staining in conditions with neovascularization. This differential staining reflects enhanced expression of HMW-MAA by pericytes and also may indicate an increase in their number in conditions with neovascularization.

Pericytes are found in capillaries and venules and are similar to the smooth muscle cells found in arterioles and arteries.[22,23] Interest in these cells has increased with *in vitro* propagation[24,25] and identification of markers.[10,11,26-29]

Several functions have been attributed to pericytes, such as a role in regulation of angiogenesis,[30-32] micro-

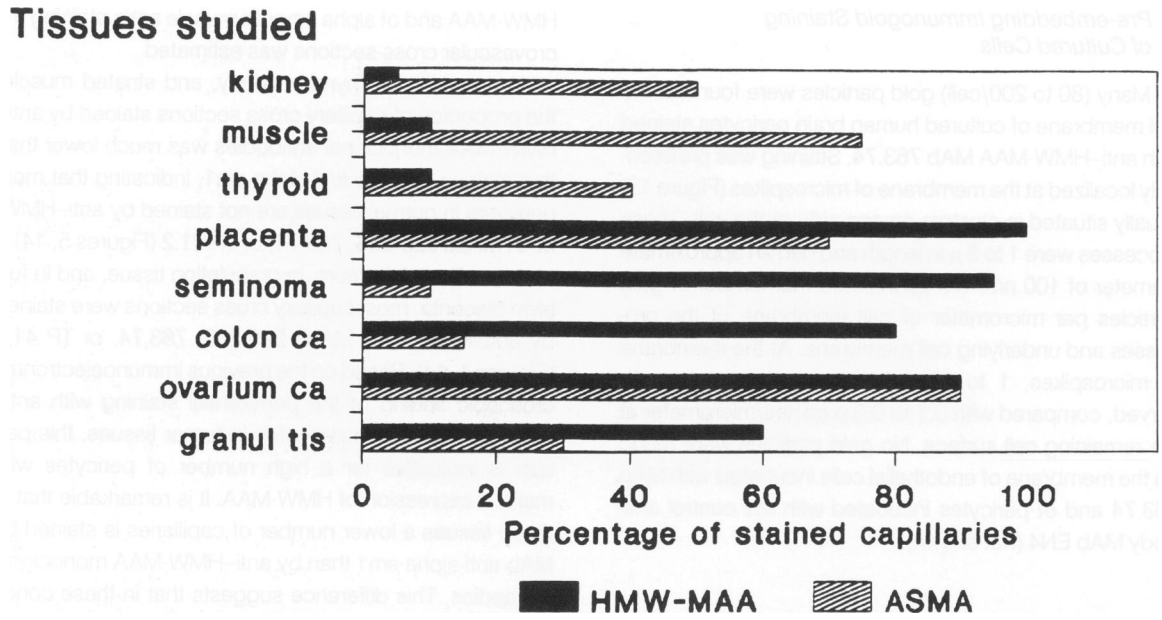

Figure 14. *Proportion of pericyte coverage of capillaries in normal adult tissues and conditions with neovascularization as based on immunohistochemical visualization using anti-HMW-MAA or anti-alpha-smooth muscle actin (ASMA) antibodies. The pericyte coverage is expressed as percentage of the total number of capillaries present (based on MAb PAL-E staining). The mean percentage of three samples of each normal tissue is given. Standard deviations of these values are 5% and 10% for kidney stained with anti-HMW-MAA 225.28S and MAb anti-alpha-sml, respectively; 10% and 30%; for striated muscle stained with anti-HMW-MAA MAb 225.28S and with anti ASMA MAb anti-alpha-sm1, respectively; and 5% and 20% for thyroid stained with anti-HMW-MAA MAb 225.28S and with anti-ASMA MAb anti-alpha-sm1, respectively.*

vascular contractility, and phagocytosis.[23,33] Serologic and immunocytochemical assays detected the HMW-MAA in cultured human brain pericytes. The latter were differentiated from smooth muscle cells because of their antigenic phenotype and their morphology.[24,25,32] However, it has been suggested that pericytes and smooth muscle cells are two closely related cell types and that pericytes may differentiate into smooth muscle cells.[23] If so, these two types of microvascular adventitial cells may be two functionally and phenotypically different forms of cells with the same common origin. This possibility is supported by our finding that both cell types express HMW-MAA. The preferential expression of the HMW-MAA by pericytes in lesions with neovascularization is remarkable. Based also on their morphology, we assume that under these circumstances pericytes are in an 'activated' state and that the HMW-MAA can therefore be used as a marker for this kind of pericyte activation. It is further notable that the HMW-MAA appears on normal pericytes when these cells are grown in culture. This is in analogy with the reported expression on cultured melanocytes,[34] which *in situ* lack the HMW-MAA.[1] These findings suggest that culture conditions might resemble those found in regenerating or in otherwise stimulated tissues. In our cultured brain pericytes, we found that HMW-MAA is expressed on microspikes, 1- to 2-μ processes present on the cell surface, a phenomenon also observed in cultured melanoma cells.[3] In other cell types, these microspikes are known to be involved in migration and contact-inhibition.[35] The localization of HMW-MAA on these microspikes on pericytes provides further evidence for an association with an activated state of these cells.

Our estimations regarding the number of pericytes in tissues with neovascularization are surprising in light of the proposed role of pericytes in the regulation of endothelial cell proliferation during angiogenesis.[32] By co-culture studies *in vitro*, it was shown that pericyte–endothelial contact via pericytic processes inhibits endothelial proliferation,[32] and it was suggested by these authors that *in vivo* a similar mechanism might occur. In the present study, however, we found immunohistochemical evidence for an almost continuous layer of pericytes and pericyte processes surrounding the endothelium in tumors, placenta, and granulation tissue, contrasting with the observed scattered distribution in normal adult tissues. In ultrastructural studies, other authors have also noted increased numbers of pericytes in tumor stroma[36,37] or healing wounds.[31,38] Considering the proposed inhibitory role of pericytes, these findings are unexpected, as endothelial proliferation in tumor vasculature has been reported to be much higher than in normal resting tissues.[39,40] Microphotographs have been published earlier showing endothelial cells undergoing mitosis in close relation with pericytes in wound healing.[31,41,42] Under certain circumstances, the proposed inhibitory effect of pericytes might therefore be absent or overruled by the influence of locally released factors. Most of the studies on the vasculature in tumors have focused on the endothelial cell.[43–46] The data presented here suggest that pericytes also contribute to the particular nature of vessels in conditions with regenerating vasculature.[47–51] An immunohistochemical marker for an 'activated' state of this cell type can make a contribution to our knowledge on the biologic role of the enigmatic microvascular pericyte. In addition, such pericytes may serve as a target for radiolabeled monoclonal antibodies for immunodiagnosis or immunotherapy of tumors *in vivo*.[52] Because anti–HMW-MAA MAb 225.28S recognizes pericytes of a number of animal species, this hypothesis may be tested in preclinical animal studies.

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