Weibel-Palade Bodies in Endothelial Cells as a Marker for Angiogenesis in Brain Tumors

Pat Kumar, Shant Kumar, Henry B. Marsden, Patrick G. Lynch, and Elaine Earnshaw


ABSTRACT

A transmission electron microscope study was made of eight childhood brain tumors divided up into three zones, center, edge, infiltrating zone, and also of adjacent "normal-looking" brain. In seven of eight tumors, the numbers of Weibel-Palade bodies in endothelial cells were significantly increased in peripheral zones compared with central zones. A similar significant increase was observed after treatment of chick chorioallantoic membranes with tumor angiogenesis factor. It is suggested that large numbers of Weibel-Palade bodies may be a marker for proliferating endothelial cells in vivo.

INTRODUCTION

Several questions asked by pathologists about human brain tumors have not yet been resolved. One of the most important questions for the patient is how the behavior of an astrocytoma Grade II is to be predicted. Will it behave like a relatively benign astrocytoma Grade I or a malignant Grade III tumor? Another is the mechanism whereby relatively benign astrocytomas (Grade I) often undergo cyst formation and liquefaction. It was considered that these questions might be answered by a detailed electron microscope study of blood vessel structure in brain tumors.

Recent research has emphasized the importance of vasculature in tumor growth. Thus, the hypothesis of Folkman (4), supported by his own work (4–7), that the increase in size of a very small tumor depends on the growth into it of a blood supply from the surrounding host tissue implies that tumor invasiveness may be related to the degree of permeation of a tumor by new blood vessels. As the roughly spherical tumor grows outward, central blood vessels mature and may degenerate because of compression, so that newly growing small blood vessels are confined mainly to the periphery. This suggests that blood vessels may differ in structure or function according to their site in the tumor. The mechanism of new blood vessel growth is considered to be migration of endothelial cells at the tip of a sprout, followed by division of cells further back (1, 4–6). This does not markedly differ in tumors from the normal wound healing described by Schoeffl (21), except that tumors secrete a substance not detected in normal or healing tissues, TAF, and the end result in terms of capillary morphology and function is different. Capillaries and small capillary venules in brain tumors exhibit many ultrastructural features different from those in normal brain (10–12, 14, 15, 19, and 25). The presence of fenestrations and pores allows direct contact between the tumor and brain parenchyma, and increased permeability at interendothelial cell junctions also leads to brain tissue edema as a result of the breakdown of the blood-brain barrier. Pinocytosis by endothelial cells is increased. Endothelial cell cytoplasmic projections into the tumor capillary lumen are common and fibrosis around capillaries is more conspicuous. Associated with the increased metabolic activity of endothelial cells themselves is an increase in the number of their cytoplasmic organelles and the appearance of cytoplasmic tubular arrays and large vacuoles. WP bodies, which occur infrequently in normal brain tissue (9), are found in very great numbers in brain tumor endothelial cells (10).

No electron microscopic study of brain tumors has attempted to quantify the difference between tumor and normal blood vessels or to relate them to zones in a tumor, nor has any attempt been made to correlate these changes with tumor malignancy or invasiveness. However, Brem et al. (2) used a scoring system under the light microscope in an attempt to determine capillary density and growth rate. This was difficult, but in general the more malignant tumors of their series had higher scores than did benign ones, indicating higher capillary densities and greater endothelial cell hyperplasia in the former.

Thus, we decided to undertake a quantitative electron microscopic study of different zones in childhood brain tumors and to try to correlate the observed changes with the zone in which they were situated and possibly with the degree of tumor malignancy. It was hoped that the results would be useful in assessing prognosis in difficult cases, where it was often impossible to predict the behavior of a tumor.

TAF extracted from many sources, from both solid tumors and tumor cells in culture, has been used to demonstrate the induction of new capillary growth when implanted in test systems such as the rat dorsal air sac and chick CAM (7, 17, 18). Chick CAM’s treated with TAF were compared with untreated controls to see if there were any changes induced by treatment and if these changes resembled the situation in human tumors in vivo.

MATERIALS AND METHODS

Materials

Eight childhood brain tumors were examined, and these comprised 6 medulloblastomas, 1 juvenile astrocytoma, and 1 oligodendroglioma. Brain specimens used as controls were obtained from diagnostic biopsies of cerebrum of 3 children with mental retardation, one 69-year-old man with dementia, and from a normal young adult rat (cerebellum). Five CAM’s...
from 14-day-old chick embryos treated with TAF using an extraction procedure described by Phillips and Kumar (17) were compared with an untreated control CAM.

### Processing of Tissues

Childhood brain tumor specimens were obtained directly from the operating theater. Pieces of tissue 1 to 2 mm in diameter were removed by surgeons from the tumor center, tumor edge, and adjacent tissue designated "normal-looking" brain and were placed in fixative for transmission electron microscopy (2.5% glutaraldehyde in M/15 Sorensen’s phosphate buffer, pH 7.4). The tissue was transferred to buffer alone following the final wash, dried 3 times during the next 24 hr, and stored at 4°C Postfixation was in 1% osmic acid in M/5 Sorensen’s phosphate buffer, pH 7.4, followed by dehydration in graded ethanols and propylene oxide, and embedding in an Epon-Araldite mixture at 60°C. Semi thin resin sections (1 μm) were cut and examined by light microscopy to confirm the nature of the tissue in each block, whether tumor center, tumor edge, or normal-looking brain. Sometimes blocks contained both tumor tissue and normal-looking brain, and these were designated infiltration zones. Ultrathin sections were made from all specimens containing a reasonable number of blood vessels and were stained with lead citrate and uranyl acetate.

Grids were scanned systematically in the electron microscope, and every blood vessel cross-section that was wholly visible was photographed so that there was no bias in sampling. Since grid bars were evenly spaced, it was considered that blood vessels were excluded from analysis in a random manner.

Specimens of brain tissue taken for another study from children and adults with undiagnosed forms of dementia were processed in the same manner as brain tumors and used as controls.

CAM’s were removed from 14-day-old chick embryos, untreated or treated with TAF, and isolated from rat Walker carcinoma according to the procedure used by Phillips and Kumar (18). These membranes were processed for electron microscopy as described above.

### Quantitative Measurements on Blood Vessels

The study was carried out blind; i.e., all measurements were recorded without knowledge of the tumor type or of the zone within the tumor.

**Blood Vessel Diameter.** Since the diameter of the lumen was variable in any capillary, measurement was taken from inner edge of basement membrane to inner edge of basement membrane surrounding a single layer of endothelial cells or a spherical cluster of endothelial cells. The smallest diameter was measured, since transverse sections might have been oblique. Capillaries and venules with a diameter greater than 20 μm were excluded, as were arterioles.

**Number of Endothelial Cells.** The number of endothelial cells lining a blood vessel cross-section was counted after first identifying interendothelial tight junctions. Since large numbers of WP bodies are one of the most remarkable features of brain tumors, WP bodies were counted in each cell and calculation was made of the average numbers per endothelial cell for each block of tissue. Large dense bodies and multivesicular bodies were not included in the counts.

**Amount of Fibrosis.** Estimates using a semiquantitative scale were made of the amount of fibrosis (collagen) surrounding a blood vessel, the number of projections into the lumen, and the degree of pinocytosis exhibited by every endothelial cell.

**Gaps.** Gaps between endothelial cells and fenestrae in their cytoplasm were also noted when they occurred.

### Analysis of Results

Differences in the average number of WP bodies per endothelial cell between tumor zones or between TAF-treated and control CAM’s were tested for statistical significance, using Student's t test and the χ² test.

The distribution of WP bodies among individual endothelial cells in each tumor zone was analyzed using a χ² goodness-of-fit test against a Poisson distribution.

### RESULTS

**Capillary Diameter.** The average capillary diameter for every tumor zone studied was less than 10 μm, with the exception of the cystic central zones of 2 low-grade tumors, the astrocytoma and the oligodendroglioma. These 2 zones had values of 15.3 and 16.3 μm, compared with 7.6 and 9.7 μm in the corresponding outer zones, i.e., infiltrating zone and normal-looking brain.

**WP Bodies.** The number of endothelial cells lining a transverse section of capillary or capillary venule ranged from 1 to 13.

Figs. 1 and 2 illustrate WP bodies in longitudinal and transverse section. Multivesicular bodies (one is visible in Fig. 1) were relatively easy to distinguish from WP bodies by their greater size and more heterogeneous content. Dense bodies were also excluded because of their large size. The tubular arrays and large vacuoles containing tubules described by Hirano (11) were rarely seen.

The number of WP bodies in an endothelial cell section ranged from 0 to 25 in capillaries and capillary venules. The average numbers of WP bodies per endothelial cell for each zone of 8 brain tumors and one cerebellar biopsy from a child with a suspected tumor are illustrated in Table 1. These values are an average of 100 to 200 readings on endothelial cells per zone. The highest value for each tumor (italicized in Table 1) was found in the edge, infiltrating zone, or normal-looking brain in 7 of 8 tumors. The exception was a desmoplastic medulloblastoma. The lowest numbers occurred in central zones of 7 tumors, and each of these showed a highly significant difference from the greater numbers recorded in peripheral zones of the same tumors.

Table 2 shows the average number of WP bodies per endothelial cell in specimens of brain tissue from 4 patients with dementia. The results are pooled and ranged from 1.0 to 1.6 for individual patients, i.e., significantly lower than in any of the tumors examined. No budding capillaries were observed in these patients.

Attempts were made to determine the significance of WP bodies in the brain tumor situation. It was obvious from examination of photographs that WP body distribution was in no way related to the cross-sectional area of the cell. It also became apparent that the distribution was a nonrandom one; some endothelial cells contained none of these organelles, whereas some contained very many (Figs. 3 and 4). A gradient effect...
Av. no. of WP bodies/endothelial cell (100–200 cells quantified) in Tumor zone

<table>
<thead>
<tr>
<th>Tumor diagnosis</th>
<th>Av. no. of WP bodies/endothelial cell</th>
<th>Statistical analysis (Student’s t test)</th>
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<tbody>
<tr>
<td></td>
<td>C^a E T + N N</td>
<td></td>
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<tr>
<td>Astrocytoma Grade I</td>
<td>2.51 2.31 7.70^b</td>
<td>Center vs. edge: not significant</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>2.89 2.92 3.87</td>
<td>Center vs. edge: not significant</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>2.07 5.96 5.50</td>
<td>Center vs. edge: not significant</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>2.25 2.18 2.44 4.67</td>
<td>Center vs. T + N: p &lt; 0.01</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>3.09 1.51 3.01</td>
<td>Center vs. edge: p &lt; 0.01</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>1.24 2.42 2.16</td>
<td>Center vs. edge: p &lt; 0.01</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>1.40 1.00 2.30</td>
<td>Center vs. edge: not significant</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>1.42 1.70 2.78</td>
<td>Center vs. edge: p &lt; 0.01</td>
</tr>
<tr>
<td>Brain biopsy of cerebel-</td>
<td>2.78</td>
<td>Center vs. edge: not significant</td>
</tr>
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<td>-lum (no tumor found)</td>
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^a C, central zone; E, edge of tumor; T + N, infiltrating zone; N, normal-looking brain.

^b Numbers in italics, highest value for each tumor.

seems to be operating in the budding capillary of Fig. 4. A plot of their distribution was made and compared with a Poisson distribution for every tumor zone studied. Chart 1 shows a typical distribution resembling a Poisson one with a very long tail. The χ^2 goodness-of-fit test revealed a significant difference from a Poisson distribution in every case. The difference which lay in the tail implied that in tumors there were small but significant numbers of endothelial cells with a very high WP body count. Longitudinal sections of small blood vessels also show this pattern. In Fig. 5, several consecutive endothelial cells containing no WP bodies lie next to a cell that is filled with them. In another capillary, one cell containing many WP bodies appeared to be migrating into the lumen (Fig. 6).

**Other Morphological Changes.** Pinocytic vesicles, cytoplasmic projections and fibrosis around the capillary were assessed in brain tumors, in a semiquantitative fashion using a grading system from 0 to 3, representing complete absence to greatest amount or number. When average values had been determined for every tumor zone, it was obvious that none of the 3 features above were preferentially located in a particular tumor zone. Since increased pinocytosis and frequently occurring cytoplasmic projections are both indicative of greater endothelial cell metabolic activity, it might be expected that either of these features would show a positive correlation with WP bodies, if the latter is also the result of increased cellular metabolic activity. Log-ranking statistical tests revealed positive correlations between WP bodies and pinocytic vesicles in only 5 of 22 tumor zones studied and between WP bodies and projections in a different 5 zones of the 22 studied.

It was assumed that extensive fibrosis around a capillary was indicative of capillary maturity or even a "shutdown." Indeed, fibrosis did seem to be confined mainly to central tumor zones. However, the expected negative correlation between WP bodies and fibrosis occurred in only 3 of 22 zones. Thus, attempts to understand the function of WP bodies in terms of other endothelial cell activities were inconclusive. This may have been caused by limitations of the semiquantitative grading system.

**Gaps and Fenestrae.** Gaps in capillary walls are rare, occurring in degenerating central zones and almost always plugged by platelets (Fig. 7). Fenestrae (limited to the hypothalamus, infundibulum, choroid plexus, and pituitary in normal brain), were seen mainly in infiltrating zones of medulloblastoma (Fig. 8). They were common in all zones of the desmoplastic medulloblastoma.

**Effects of TAF on CAM Capillaries.** Table 3 shows that treating CAM's with TAF increased the average number of WP bodies per endothelial cell compared with untreated CAM's. Plotting the distribution of WP bodies in treated CAM's produced a Poisson-like curve, but with a very long tail. The untreated CAM did not have such a long tail (not shown). A χ^2 test using the long tail of the treated CAM's revealed a highly
Thus, the effect of TAF on CAM capillary growth in producing an increase in the number of WP bodies per endothelial cell was observed to parallel the situation in brain tumors, but to a lesser extent.

Presence of Mast Cells during Angiogenesis. Mast cells were observed on chick CAM's after treatment with TAF where they had never previously been seen. These cells were often closely associated with capillaries in peripheral zones of brain tumors (Figs. 9 and 10).

DISCUSSION

Several important facts have emerged from our study. The presence of a large number of WP bodies in endothelial cells seems to be a marker for actively growing capillaries. Thus, examination of brain tissue in senile dementia and results from central tumor zones, where there is no evidence of capillary proliferation, suggest that 1.5 to 2.9 WP bodies per endothelial cell represents a resting value in cerebral cortex and cerebellum. A biopsy from a child where an astrocytoma was suspected, but not found, gave a value of 2.8. Any significant increase above this value probably represents active capillary growth.

One of the 2 relatively benign tumors studied, an oligodendroglioma, showed some evidence of capillary proliferation in the infiltrating zone, and the other (a cystic astrocytoma, Grade I) had a value of 7.7 in adjacent normal brain. If WP bodies are associated with growing capillaries, it is possible that this latter value represents a growth spurt by a previously dormant tumor.

It is impossible as yet to answer one question around which this study is centered, namely, the relationship of prognosis for the patient with ultrastructural changes observed in capillaries. A study of a larger series of tumors will be necessary before conclusions can be drawn.

The 2 relatively benign tumors were the only ones to show significant increases in average capillary diameter in their central zones. Values of 15.3 and 16.3 μm in central zones, compared with 7.6 and 9.7 μm in the corresponding normal-looking brain and infiltrating zones, and diameters below 10.0 μm in all of the other tumors, imply that cystic central areas of benign tumors may progress to that state via the formation of large sinus-like blood vessels.

The distribution of WP bodies among individual endothelial cells was also consistent with our hypothesis that they are a marker for actively growing endothelial cells. Since the growth of capillaries is confined to a few cells at the tips of sprouts or buds (1, 5, 6), one would expect to find only a small proportion of endothelial cells actually undergoing migration-mitosis. This was indeed the case in the tumors studied, where only a small proportion of endothelial cells contained large numbers of WP bodies.

TAF, which has been demonstrated to cause capillary proliferation in vivo (7, 17, 18) and endothelial cell proliferation in vitro (22), also causes an increase in WP bodies in endothelial cells of chick CAM's. This is good evidence to support our hypothesis. The difference between treated CAM's and untreated controls was not as marked as in brain tumors, possibly because the 14-day-old chick CAM is an embryonic tissue with capillaries which are undergoing continuous proliferation. TAF in this situation then merely increases proliferation in an already active capillary network.

Further evidence to corroborate the hypothesis comes from a study of retinal capillary regeneration after surgical injury. In this study, the number of WP bodies vastly increased during regeneration after wounding. Since the discovery and original description of WP bodies by Weibel and Palade (24), the normal physiological function and their role in blood vessels has remained obscure. Burri and Weibel (3) suggested a role in coagulation of the blood. Recently, these organelles have been demonstrated to contain histamine (8).

Authors other than ourselves have observed mast cells in tumors, and these were most conspicuous in infiltrating zones (23, 25). Similarly, Kessler et al. (13) reported mast cells on chick CAM's treated with TAF, and emphasized that they had never previously seen mast cells in this site. Mast cells can secrete histamine and heparin (16, 20). It is possible that they too have a role in angiogenesis, perhaps by influencing the formation of WP bodies.

ACKNOWLEDGMENTS

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Fig. 1. Medulloblastoma. WP bodies (single arrows) in a capillary endothelial cell. A multivesicular body (double arrows) can be clearly distinguished because of its heterogeneous contents. × 62,500.

Fig. 2. Medulloblastoma. WP bodies in a capillary wall. They are sometimes aligned with microfilaments of the endothelial cells. A cross-section (arrow) contains visible microtubules. × 40,000.

Figs. 1 to 10. All of the electron micrographs are of tissues stained with uranyl acetate and lead citrate.
Fig. 3. Oligodendroglioma, infiltrating zone. Part of a capillary venule showing an uneven distribution of WP bodies (arrows). Three endothelial cells on 2 opposite sides of the lumen contain many more of these organelles than do the other endothelial cells. × 8,000.

Fig. 4. Medulloblastoma, infiltrating zone. A cluster of endothelial cells forming a bud with an incompletely formed lumen. Endothelial cells at 2 opposite sides of the bud contain nearly all of the WP bodies visible. Cells with large dense droplets (single arrows), considered to be mast cells because of their metachromatic staining with toluidine blue, lie close to the cells containing WP bodies. A gradient effect is apparent in these organelles in one area (double arrows). × 4,000.
Fig. 5. Part of a large capillary wall in a 14-day-old chick CAM treated for 96 hr with TAF isolated from rat Walker carcinoma. One endothelial cell (arrow) has a large number of WP bodies, while its neighbors in the capillary venule wall have virtually none. × 5,000.

Fig. 6. An endothelial cell containing many WP bodies (arrow) which seem to be migrating into the lumen (L). The neighbors of this cell contain hardly any of these organelles (cf. Fig. 6). × 5,000.
Fig. 7. Medulloblastoma. A capillary with a gap between 2 of its endothelial cells (arrows). × 10,000.

Fig. 8. Medulloblastoma. A capillary with many fenestrae (arrows) in a degenerating part of the tumor. × 7,500.
Fig. 9. Medulloblastoma, infiltrating zone. This appears to be the tip of a small blood vessel with 2 adhering mast cells enclosed in basement membrane (arrows). × 12,500.

Fig. 10. Medulloblastoma, edge of tumor. Longitudinal section through a small blood vessel showing 2 elongated endothelial cells with many WP bodies (arrows). Mast cells lie on either side. × 4,000.
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