

CLINICAL NEUROSCIENCES IN THE DECADE OF THE BRAIN  
HYPOTHESES IN NEURO-ONCOLOGY

The Genesis of Peritumoral Vasogenic Brain Edema  
and Tumor Cysts: A Hypothetical Role for Tumor-Derived  
Vascular Permeability Factor

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Cerebral edema and fluid-filled cysts are common accompaniments of brain tumors. They contribute to the mass effect imposed by the primary tumor and are often responsible for a patient's signs and symptoms. Cerebral edema significantly increases the morbidity associated with tumor biopsy, excision, radiation therapy, and chemotherapy. Both edema and cyst formation are thought to result from a deficiency in the blood-brain barrier, with consequent extravasation of water, electrolytes, and plasma proteins from altered tumor microvessels. The resultant expansion of the cerebral interstitial space contributes to the elevated intracranial pressure observed with brain tumors. Departure from the typical blood-brain barrier microvascular architecture may only partially explain the occurrence of edema and tumor cyst formation. Biochemical mediators have also been implicated in vascular extravasation. Vascular permeability factor or vascular endothelial growth factor (VPF/VEGF) is a protein that has recently been isolated from a variety of tumors including human brain tumors. VPF<sup>b</sup> is an extraordinarily potent inducer of both microvascular extravasation (edemagenesis) and the formation of new blood vessels (angiogenesis). Its role in tumor growth and progression would therefore appear pivotal. Herein, the author presents an updated account of the investigation of VPF. Historical and clinical perspectives of the study and treatment of tumor associated edema are provided. The efficacy of high-dose dexamethasone in the treatment of neoplastic brain edema is discussed. A hypothetical role for VPF in edemagenesis is presented and discussed. It is hoped that an expanded understanding of the mechanisms responsible for the genesis of edema will ultimately facilitate therapeutic intervention.

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<sup>b</sup>Abbreviations used: VPF, vascular permeability factor; CSF, cerebrospinal fluid; ICP, intracranial pressure; CT, computerized tomography; MRI, magnetic resonance imaging; BBB, blood brain barrier; VPI, vascular permeability induction; SIADH, syndrome of inappropriate secretion of anti-diuretic hormone, VBE, vasogenic brain edema; CMR, cell membrane receptor; PDGF, platelet-derived growth factor, EGF, epidermal growth factor; TAFs, tumor angiogenesis factors; HG-VPF, human glioma-derived vascular permeability factor; IgG, immunoglobulin G; EGTA, ethylene-glycol-bis(β aminoethylether)-N,N'-tetra-acetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamine gel electrophoresis; VPF, human vascular permeability factor; VSM, vascular smooth muscle cells; HUVEC, human umbilical vein endothelial cells; PKC, protein kinase C; VEGF, vascular endothelial growth factor; IL-1, interleukin-1; IL-2, interleukin-2; PET, positron emission tomography; FGF, fibroblast growth factor; EI, edema index; GFRAP, glial fibrillary acidic growth protein; RAS, reticular activating system; CN, cranial nerve.

## INTRODUCTION: HISTORICAL AND CLINICAL PERSPECTIVE

The word edema is derived from the Greek word for swelling. Early Greek authors were the first to associate brain swelling with compound skull fractures. Hippocrates' work, *On Injuries of the Head*, advocated trepanation within the first three days of injury prior to any further deterioration related to inflammation, hemorrhage, or tissue swelling. In his work, *On the Sacred Disease*, Hippocrates recognized the associations between prolonged seizure activity, cerebrovascular engorgement, and consequent brain swelling [1]. In 1761, Morgagni's work *De Sedibus et Causis Morborum (The Seats and Causes of Disease)*, reported the occurrence of vasogenic edema in association with a left capsular infarction in a patient with aphasia, right hemiplegia, and right hypoesthesia [2]. Monro further elucidated the occurrence of vasogenic brain edema in his treatise *Observations on the Structure and Function of the Nervous System* which was published in 1783 [3]. Observations in this work formed the basis of the Monro-Kellie hypothesis which, in essence, recognized that intracranial hemorrhage, tumor, or edema could only be accommodated by exclusion of other components of the normal cranial contents (brain, blood, and cerebrospinal fluid). Samuel Solly, a surgeon at St. Thomas' Hospital, reported in 1847, the occurrence of cerebral edema in association with infantile hydrocephalus [4]. In 1874, Sir John Bucknill and Daniel Tuke, physicians at the Devon County Lunatic Asylum, put forth a definition of cerebral edema in their *Third Edition of the Manual of Psychological Medicine* [5]. It was said: "Oedema of the brain, a state in which the tissue of the organ is permeated by water or serosity".

In 1905, Reichardt furthered our pathological understanding of cerebral edema by dividing it into "hirnödém", where the swollen brain is soft and water drips from the cut surface, and "hirschwellung", where the brain is firm and the cut surface is dry [6]. In 1908, Harvey Cushing recommended a subtemporal craniectomy for decompression of brain swelling associated with bursting fractures and added that evacuation of CSF from the subarachnoid space opened at operation would facilitate brain relaxation and surgical exposure [7,8]. One year later, he advocated the use of cisternostomy for CSF drainage when lumbar puncture was contraindicated [9]. Weed and McKibben, working at the Army Neurosurgical Laboratories of the Johns Hopkins Medical School in 1919, investigated the effects of intravenous solutions of varying osmolalities on the brain. Their paper in the *American Journal of Physiology* demonstrated that brain bulk in the cat was increased by intravenous infusion of hypotonic distilled water and decreased by intravenous infusion of hypertonic 30% saline or saturated sodium bicarbonate [10]. Another major therapeutic advance occurred in 1961 when Galicich and French published their observations on the "Use of dexamethasone in the treatment of cerebral edema resulting from brain tumors and brain surgery" [11].

In his Presidential Address to the American Association of Neuropathologists, Igor Klatzo in 1966 advanced an explanation of the pathogenic mechanisms of the two types of edema [12]. He defined "vasogenic" edema (hirnödém) as an accumulation of water and plasma constituents outside cells, resulting from injury to the walls of cerebral blood vessels. "Cytotoxic" edema (hirschwellung) was characterized by intracellular swelling due to a disturbance of cell membrane permeability, and was associated with an intact blood-brain barrier. In 1967, Fishman defined "interstitial" edema as that resulting from the transependymal spread of ventricular CSF into the surrounding periventricular white matter, as seen in the setting of acute obstructive hydrocephalus [13]. Miller further expanded the classification in 1979 by identifying "hydrostatic" edema as that occurring when an imbalance in Starling's equilibrium across the blood vessel wall exists [14]. The latter imbalance results in extravasation of a protein-free plasma ultrafiltrate into the extracellular space. Similarly, "hyposmotic" edema is seen in association with free water

overload. The resulting excess water accumulation occurs in the extracellular compartment initially but is soon followed by cellular imbibition and swelling in both gray and white matter (Table 1).

Cerebral edema contributes significantly to neurological morbidity in patients with brain tumors. Patients with intracranial neoplasms typically present with a constellation of signs and symptoms which are in part determined by the tumors location in the brain, as well as the extent to which it elevates ICP. The degree of elevation in ICP is in turn determined by the contribution of the primary tumor mass and any associated edema surrounding the lesion. Progressively worsening headache, nausea, vomiting, blurred vision, double vision, ataxia, seizures, diminished cognitive skills, and depressed level of consciousness are amongst the more typical indicators of intracranial hypertension (Table 2). Failure to recognize, diagnose, and treat the underlying cause may result in a patient's rapid demise.

Edema associated with brain tumors represents an ultrafiltrate of plasma containing water, electrolytes, and plasma proteins. Edema fluid emanates from the brain tumor microvasculature (vasogenic) and infiltrates the white matter surrounding the tumor in a diffuse manner. Passage into the finger-like projections of subcortical white matter (white matter edema) results in a characteristic appearance on CT and MRI studies, as the adjacent cortex is relatively spared (Figure 1). Similarly, a focal accumulation of cerebral interstitial tissue fluid can result in the formation of a tumor-associated cyst (Figure 2). Edema and cyst formation result in distortion of intracranial structures, and contribute to elevation of the intracranial pressure. As a result, vascular extravasation is frequently as culpable for a brain tumor patient's clinical signs and symptoms as the primary intracerebral neoplasm.

Vasogenic brain edema is commonly seen in association with a variety malignant and benign brain tumors. It results from a flaw in the integrity of the blood-brain barrier. The blood-brain barrier is both an anatomical and physiological system which normally regulates the entry and egress of substances between the cerebral interstitial and intravascular

**Table 1. Classification of brain edema.**

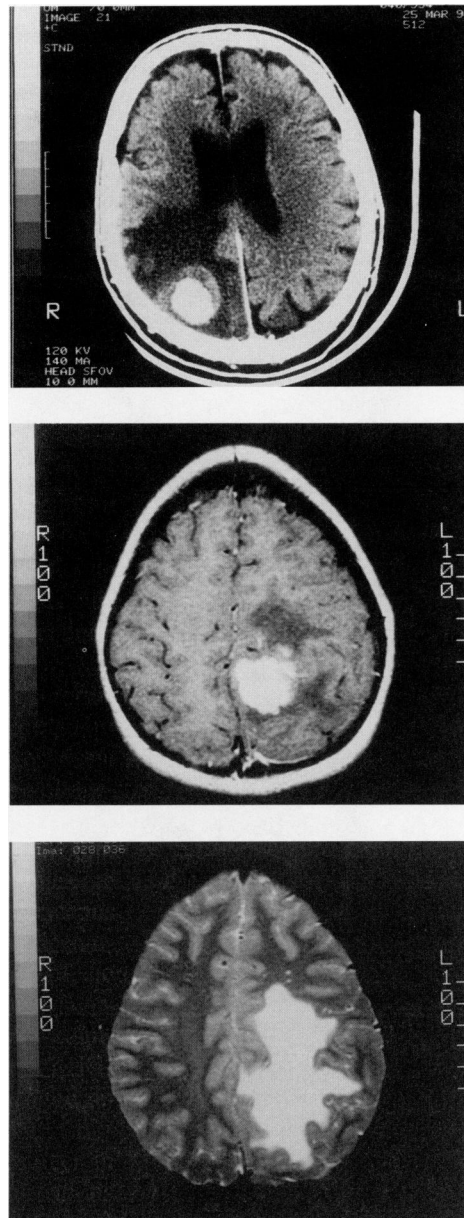
Edema Type	Current Terminology	Clinical Association
Type I	Vasogenic Brain Edema	tumor, abscess
Type II	Cytotoxic Brain Edema	hypoxia, ischemia, toxins
Type III	Interstitial Brain Tumor	hydrocephalus
Type IV	Hypoosmotic Brain Edema	SIADH, water intoxication, malignant hypertension

SIADH, syndrome of inappropriate antidiuretic hormone secretion.

**Table 2. Clinical indicators of elevated intracranial pressure.**

Clinical Symptoms	Clinical Signs	Clinical Pathophysiology
Progressive headache	NA	dural and vascular traction
Vomiting	NA	medullary compression
Blurred vision	papilledema	venous congestion
Double vision	paresis of CN VI	compression of CN VI
Depressed consciousness	altered mentation	diffuse effect on RAS
Gait disturbance	wide based, apraxia	compression of corticobulbar fibers

CN, cranial nerve; RAS reticular activating system.



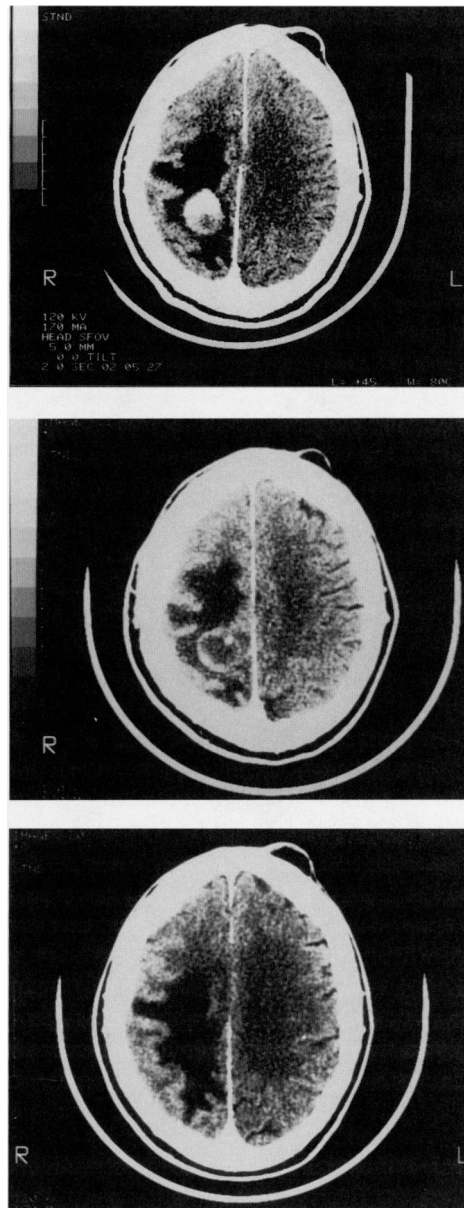
**Figure 1.** CT and MRI of two patients who presented with a history of progressively worsening headache, vomiting, double vision, and impaired mentation. Enhanced images obtained using intravenously administered iodinated (CT) or ferromagnetic (MRI) contrast agents reveal bright regions of contrast extravasation in areas of frank blood-brain barrier disruption. The dark CT (low beam attenuation) and corresponding bright MRI (high T2 signal intensity) region of vasogenic brain edema emanates primarily from within the tumor vascular bed (a and b). Spread of the edema fluid in a centrifugal fashion results in a characteristic finger-like penetration into the subcortical white matter (a and c). It is evident in both cases that cerebral edema is contributing as much to the overall mass effect and brain distortion as the primary neoplasm. Notably, within 24 hrs of starting high-dose dexamethasone (10 mg intravenous every 4 hrs), both of these patients became asymptomatic and were able to undergo an uneventful gross total excision of their tumor.



**Figure 2.** MRI images depicting a low grade glioma of the cerebellum. Note the irregular, brightly enhancing tumor nodule and associated cyst (a). The fluid content of the cyst is best seen on a T2-weighted MRI image where it is depicted as a bright or high signal intensity (b). As in the case of diffuse interstitial edema, the cyst is contributing substantially to mass effect. Both edema and cyst formation are thought to be related but distinct epiphenomena of microvascular extravasation.

compartments. As such, it provides a critical interface between the brain and essential blood-borne nutrients, including glucose, amino acids, vitamins, minerals, trace elements, and a variety of hormonal regulatory signals. The functional components of the blood-brain barrier occur primarily at the level of the microvascular endothelial cell. The basement membrane, pericyte, and astrocytic foot processes completely invest the cerebral microvasculature and contribute to barrier integrity by inducing and regulating the expression of specialized endothelial cell junctional characteristics. Ultrastructural examination of normal brain microvessels in comparison with brain tumor microvessels supports the hypothesis that altered tumor microvessels account for the abnormal extravasation of fluid and protein into the interstitial space. However, a focused pathophysiological explanation for these anatomical observations has not heretofore evolved despite extensive study.

Treatment of brain tumor patients with high-doses of a glucocorticosteroid (dexamethasone) will result in an improvement or resolution of their ICP-related signs and symptoms. Clinical improvement occurs predictably within 12 to 48 hrs of initiation of this therapy. This response is of practical significance as it allows the physician, patient and patient's family, additional time prior to surgery to discuss issues related to the disease process, surgical therapy, and perioperative care. Furthermore, patients undergoing



**Figure 3.** CT images of a patient with a metastatic brain tumor who presented with severe headache, a left-sided visual field cut, and left-sided weakness. (a) CT image demonstrating peritumoral vasogenic brain edema and tumor enhancement prior to initiation of dexamethasone therapy. (b) Partial resolution of both edema and tumor enhancement are evident on a CT performed 24 hrs later. The patient's headache was markedly diminished at that time. (c) CT image taken 72 hrs after the initiation of high-dose dexamethasone therapy. Tumor enhancement is no longer evident and edema fluid continues to resolve. The patient had no residual headache and was neurologically normal at the time of this scan. The tumor was successfully removed without event on the following day. It has been three decades since the initial recognition of dexamethasone's efficacy in the setting of peritumoral brain edema. Recent studies have at once suggested a role for VPF in tumor edemagenesis, and a mechanism for dexamethasone efficacy by its inhibitory actions on VPF expression and activity.

brain tumor surgery are more likely to have a better surgical outcome if their neurologic deficits are minimized preoperatively. The clinical efficacy of dexamethasone therapy correlates with a partial resolution of vasogenic brain edema and a reduction in tumor enhancement evident on CT studies (Figure 3). Experimental studies indicate that this relates to a retardation in vascular extravasation and edema fluid formation. Notably, the clinical responsiveness of brain edema to dexamethasone has been shown to correlate with cytosolic glucocorticoid-receptor concentrations in several types of intracranial tumor. The clinical use of high-dose dexamethasone as an adjunct to brain tumor therapy now spans three decades. Nevertheless, the pharmacological basis for this remarkable clinical, physiological, and radiographic response remains open to conjecture, and the rationale for glucocorticoid use in this setting remains largely empirical. It follows that further study of the mechanism, and specificity of dexamethasone's actions in the setting of neoplastic brain edema, would be worthwhile as it might reveal novel insight into the very mechanism by which brain edema evolves, as well as suggest alternative therapeutic avenues.

### THE BLOOD-BRAIN AND BLOOD-CSF BARRIERS DEFINED

Paul Ehrlich, a 19th century bacteriologist, first noted that intravascular injection of vital dyes stained all organs except the brain. His interpretation, that this related to a differential organ affinity for the dye was later proven incorrect when in 1913 a student of his, Edwin E. Goldmann, showed that injection of trypan blue into the cerebrospinal fluid readily stained the brain but failed to enter the bloodstream. The BBB is now recognized to be a complex, highly selective anatomical and physiological barrier, which regulates the entry and egress of cerebral nutrients and biological substances essential for the maintenance of cerebral metabolism and neuronal activity [15–17]. Although Goldmann was first to postulate that brain capillaries were the anatomic basis for the blood-brain barrier, direct confirmation did not occur until the 1960's when electron microscopy was used to demonstrate the occurrence of continuous pentalaminar tight junctions between adjoining endothelial cells. In an elegantly designed series of experiments, Thomas S. Reese and Morris J. Karnovsky at the Harvard Medical School, and Reese and Milton W. Brightman at the National Institutes of Health, demonstrated the inability of an electron-dense macromolecular marker (horseradish peroxidase) to pass from either the intravascular or interstitial compartments respectively, through the interendothelial tight junctions [18, 19].

Endothelial cells of the cerebral vasculature display several features attesting to their unique participation in blood-brain barrier function (Table 3). The single most important feature is the high-resistance pentalaminar tight junction that fuses endothelial cells together in a continuous layer, and effectively forms a physical barrier between circulating molecules and cells, and the cerebral interstitial space. Additional specialized features include continuous capillary basement membranes, a paucity of endothelial micropinocytotic activity, an abundance of endothelial cell mitochondria, the absence of endothelial

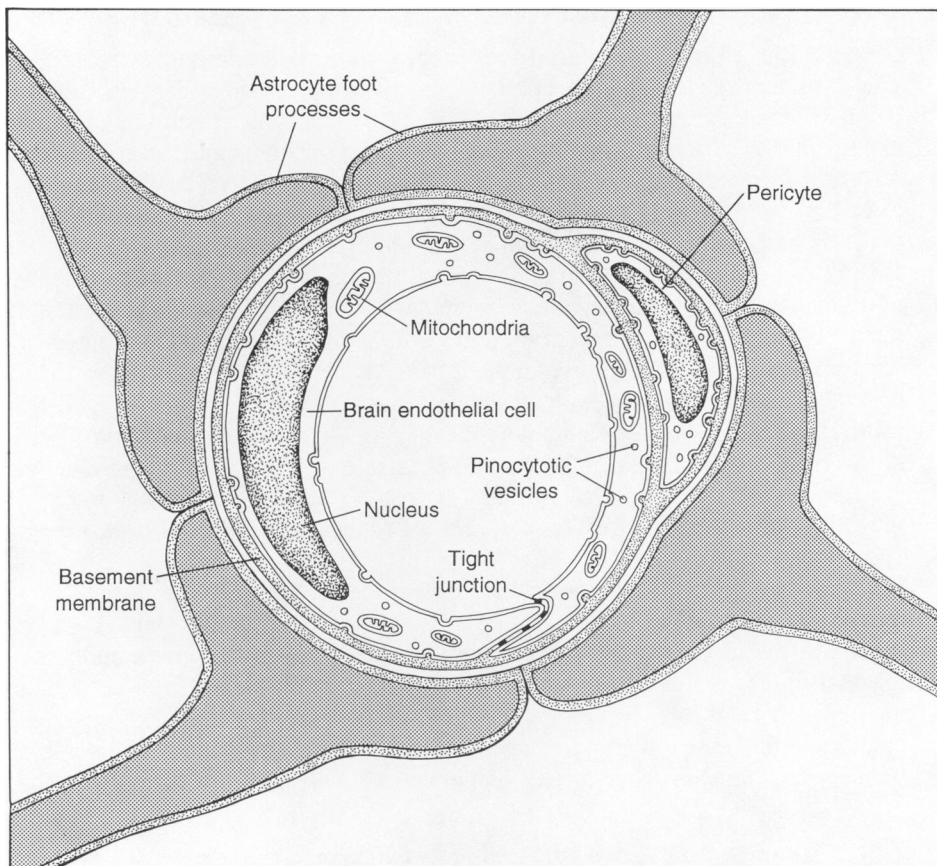
**Table 3. Ultrastructural features of blood-brain barrier microvessels.**

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Endothelial cell pentalaminar tight junctions
Continuous capillary basement membranes
Paucity of endothelial cell micropinocytotic activity
Abundance of endothelial cell mitochondria
Absence of endothelial cell membrane fenestrations
Investiture of capillary basement membrane by astroglial foot processes

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membrane fenestrations, and complete investiture by astroglial foot processes (Figure 4) [17–24]. Therefore, virtually all macromolecules are excluded by the blood-brain barrier, and passive diffusion of substances into the brain largely depends upon their physical characteristics such as molecular size, electrostatic charge, and lipophilicity. Lipid solubility, as quantitated by an oil-water partition coefficient, is the most important chemical property determining a substance's ability to penetrate the blood-brain barrier. Amino acids, glucose, biogenic amines and other essential brain nutrients may gain entry by a complex system of membrane transporters. A variety of molecular entry mechanisms exist including active transport (energy-requiring), facilitated transport (not energy-requiring), and enzymatic modification of a molecule prior to entry. The blood-cerebrospinal fluid barrier is an analogous but distinct entity whose function is governed by the selective secretory activity of the choroid plexus epithelium. Together, the blood-brain and blood-CSF barriers regulate the composition of the cerebral interstitial fluid and cerebrospinal fluid within well-defined limits. Disturbances in any of the compo-



**Figure 4.** Artistic interpretation depicting the normal cytoarchitectural relationships between a blood-brain barrier microvessel and the surrounding glial cells (astrocytes). The critical component of this permeability barrier occurs at the interendothelial cell junction (pentalaminar tight junction). Intimate intercellular contacts and chemical signalling are believed responsible for the highly specialized features expressed by these microvessels under normal circumstances (Type A morphology).



nents of the blood-brain or blood-cerebrospinal fluid barriers, if significant, will result in cerebral edema.

### **PATHOPHYSIOLOGY AND CLASSIFICATION OF BRAIN EDEMA**

Brain edema is generally defined as a condition whereby disturbed cerebral homeostatic mechanisms result in an abnormal increase in brain tissue volume that is largely attributable to an increase in water content. Many pathological processes are known to result in brain edema formation. Although the pathophysiological mechanisms are not completely understood, it is apparent that several distinct varieties of cerebral edema exist [12, 15, 25]. Vasogenic brain edema (Type I) is associated with marked alterations of cerebral microvascular elements. This disturbance in the blood-brain barrier results in extravasation of plasma-like fluid into the white matter interstitial space (white matter edema). Alternatively, edema fluid may become sequestered into a single loculated cavity or cyst. Diffuse vasogenic edema is seen clinically in the setting of brain tumor, abscess, intracerebral hemorrhage, malignant systemic hypertension, and after prolonged seizure activity. Cyst formation is seen exclusively in the setting of certain neoplasms. Cytotoxic brain edema (Type II) occurs when direct damage to cortical cells causes impaired cellular membrane homeostasis and increased permeability. Intracellular accumulation of sodium and water results in cellular swelling (gray matter edema) at the expense of the interstitial space. Cytotoxic brain edema is seen clinically in the setting of cerebral anoxia, ischemia or infarction, diabetic coma, hepatic encephalopathy, Reye's syndrome, and pseudotumor cerebri. Interstitial brain edema (Type III) refers to an infiltration of CSF from the ventricular cavities into the periventricular white matter. Cerebral interstitial fluid normally communicates freely with the ventricular cavities and bulk diffusion into them occurs in a centripetal fashion. Interstitial brain edema occurs in the setting of acute hydrocephalus wherein CSF under increased pressure is forced to flow centrifugally across the ependymal surface lining the ventricular cavities, and into white matter interstitial space. Blood-brain barrier function is not therefore altered. Osmotic/hydrostatic brain edema (Type IV) occurs when a sudden disturbance in Starling's equilibrium results in the development of an osmotic gradient between plasma and cerebral tissue. Initially, the edema consists largely of free water without electrolytes or plasma proteins. The brain eventually loses electrolytes without gaining significant quantities of water. Clinical impairment is more likely related to cellular potassium loss and hyponatremia, as elevated intracranial pressure is not a consistent association. Osmotic or hydrostatic brain edema occurs with primary water intoxication, rapid hemodialysis, and the inappropriate secretion of antidiuretic hormone (SIADH).

This classification of cerebral edema is quite sound and clinically useful provided one recognizes its limitations. It is well recognized that disparate types of brain edema may coexist. For example, vasogenic and cytotoxic edema can occur together in certain settings depending upon the nature and severity of the initial insult. Furthermore, if one considers the variety of distinct pathological events that result in expression of the same pattern of edema, a lack of pathophysiological specificity becomes self-evident. Nevertheless, this template rightfully remains the standard for all further discussion regarding edema ultrastructure, pathology, and physiology.

### **BRAIN TUMORS AND NEOPLASTIC BRAIN EDEMA**

#### *The clinical and pathological spectrum of human brain tumors*

Tumors of the central nervous system pose a major challenge to the field of oncology. Intracranial neoplasms account for 2% of all cancer-related deaths. They continue to

be associated with a high degree of morbidity and mortality, despite advances in neurosurgery, radiotherapy, and chemotherapy [26–34]. Primary tumors of the central nervous system account for approximately 10% of all malignancy. Furthermore, cancer is second only to trauma as a cause of death in childhood, and brain tumors represent the second most common childhood malignancy. About 20,000 new primary brain tumors and an additional 20,000 secondary or metastatic brain tumors are diagnosed each year in the United States.

Approximately 40% of all central nervous system tumors are primary tumors derived from neuroectodermal supporting cells (glial cells) such as astrocytes, oligodendrocytes, and ependymal cells. Astrocytomas comprise 60 to 70% of glial cell tumors (gliomas). Several attempts have been made to create a practical grading system for these lesions by correlating histological features with their biological behavior. One of the more commonly employed systems assigns a numerical grade from I through IV. Grade I (benign astrocytoma) and Grade II (cellular astrocytomas) lesions are both generally considered histologically and biologically benign tumors characterized by variable degrees of hypercellularity, and relatively prolonged survival after treatment with surgical excision or radiation therapy. Grade III (anaplastic astrocytoma) and Grade IV (glioblastoma multiforme) astrocytomas display malignant cellular features such as extreme hypercellularity, pleomorphism, increased nucleocytoplasmic ratio, mitotic figures, nuclear hyperchromatism, multinucleation, and bizarre giant cell forms. Malignant stromal changes such as necrosis, pseudopalisading, edema fluid accumulation, intravascular thrombosis, hypervascularity, and microvascular endothelial proliferation, occur almost exclusively in the most highly malignant tumor varieties (glioblastoma multiforme).

Survival in patients with malignant gliomas is poor and ranges from 3 to 6 months untreated. Therapy with dexamethasone alone may only extend survival by several months at most. Combined surgical excision, radiation, and chemotherapy significantly improve survival but mortality still approaches 95 to 100% at 5 years post-diagnosis. A peculiarity of primary brain tumors is the rarity with which they metastasize beyond the central nervous system. Exceptions to this behavior are well documented in the literature and most commonly occur with medulloblastoma and glioblastoma multiforme. Nevertheless, in most instances, malignant brain tumors are locally invasive, and therefore, limit their growth to the fixed confines of the intracranial cavity. Clinically significant recurrences after surgical excision and radiation therapy are most likely to occur at the site of the original tumor (>95% of gliomas recur within 2 cm of the original resection margin). Certain tumors demonstrate a predilection for seeding malignant cells into the subarachnoid space throughout the leptomeninges and spinal cord along CSF pathways (primitive neuroectodermal tumor, medulloblastoma, ependymoma, pineoblastoma, ependymoblastoma). This behavior has also been reported for anaplastic astrocytoma and glioblastoma multiforme when these lesions have anatomical access to the subarachnoid space. Extension, spread, and dissemination of a brain tumor in any of the latter fashions would naturally pose additional diagnostic and therapeutic challenges.

Brain tumors most likely to be associated with clinical and CT-evident vasogenic cerebral edema include most primary malignant tumors (anaplastic astrocytoma and glioblastoma multiforme), many secondary malignant tumors (metastases from lung, breast, renal, thyroid, and colon cancer, and malignant melanoma), and certain benign tumors (meningioma). In addition, fluid-filled macrocysts are frequently seen in association with benign astrocytomas and hemangioblastomas, and likely represent a distinct epiphenomenon of vascular extravasation. Management of brain tumors is complicated by the presence of peritumoral edema, which frequently limits a patient's tolerance for essential therapies (surgery, radiotherapy, and chemotherapy), and which may,

themselves, initially contribute to cerebral swelling. Furthermore, the risk of a poor outcome after neurosurgical excision of a brain tumor is increased substantially in the setting of severe, symptomatic brain edema. For these reasons, an expanded understanding of the pathophysiology of peritumoral brain edema is essential to the effective management of patients with intracranial tumors.

#### *Ultrastructure of the tumor microvasculature and vasogenic brain edema*

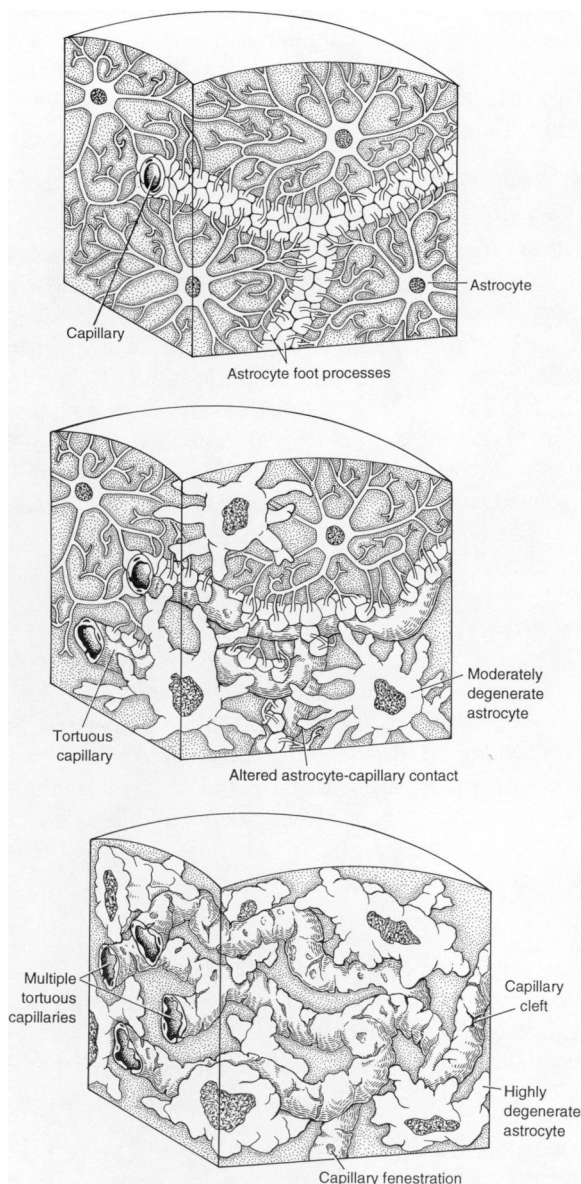
Prior reports have described the increased permeability characteristic of the microvasculature within primary and metastatic malignant brain tumors, as well as certain benign tumors [35–37]. The attention of initial studies primarily focused on the morphology of cerebral edema, and the altered vascular ultrastructure of intrinsic tumor and peritumoral blood vessels. Vasogenic brain edema (VBE) was characterized by infiltration of the white matter interstitial spaces by an ultrafiltrate of plasma (water, salts, protein) [12, 15, 16, 25]. It has been postulated that the specialized features of normal brain endothelial cells result from cellular contact with normal brain tissues (basement membrane, pericytes, interstitial stroma, astrocytes) [20, 21, 35–37]. The latter consists largely of astrocytes, whose cytoplasmic processes completely invest these microvessels (Table 4 and Figure 5a). It would therefore follow that the vastly altered milieu inherent in brain tumors, where microvessels develop amongst a varying mixture of normal and abnormal astrocytes (low-grade gliomas) (Table 4 and Figure 5b), or in the presence of either markedly degenerate astrocytes (high-grade gliomas) or completely in the absence of astrocytes (metastatic tumors) (Table 4 and Figure 5c), might account for a dedifferentiation of brain endothelial cells into a less specialized phenotype (i.e., not expressing tight junctional architecture). One can further speculate that microvessels growing into minimally altered environments might retain some anatomical and functional blood-brain barrier features as seen in low-grade astrocytomas associated with minimal or no brain edema (Figure 6). In contradistinction, the microvasculature of highly anaplastic gliomas and brain metastases retains little or no semblance to normal blood-brain barrier microvessels (associated with extensive brain edema) (Figure 7).

#### *Statement of working hypothesis*

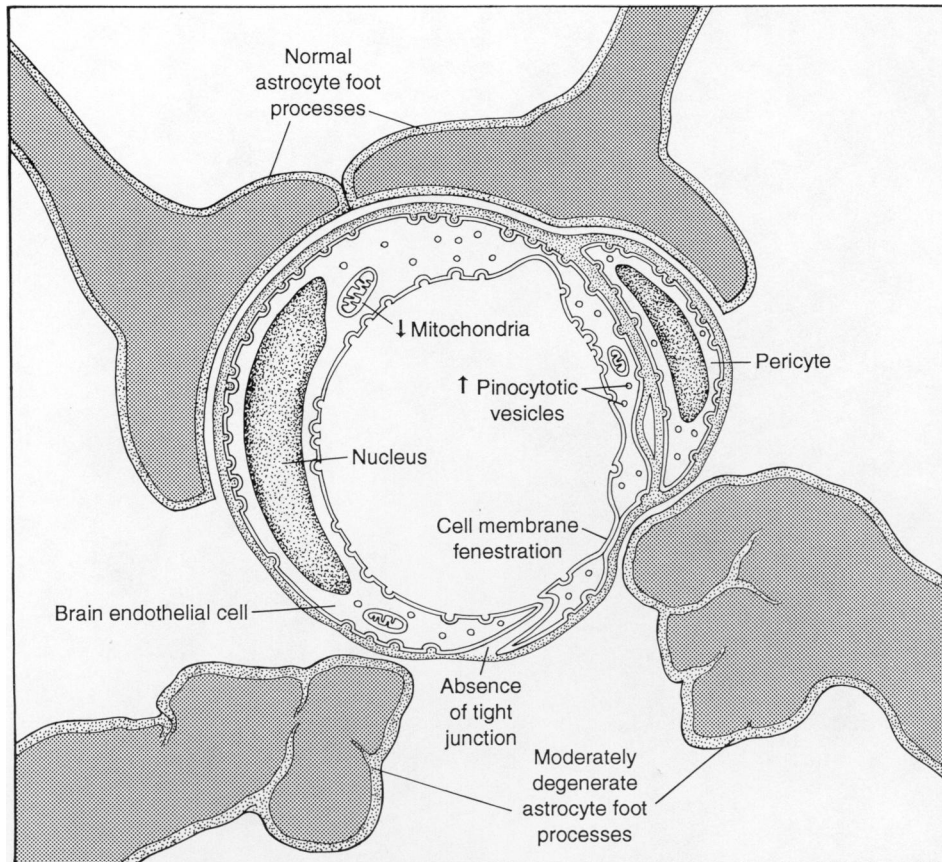
Several features of tumor-associated vascular endothelium, such as widened intercellular junctions, discontinuous tight junctions, membrane fenestrations, noncontiguous basement membranes, active micropinocytosis, and paucity of mitochondria, contrast sharply with the normal architecture of the blood-brain barrier (Table 5 and Figures 4, 6

**Table 4. Proposed functional and morphological classification of brain microvessels.**

Microvessel Type	Departure from Barrier Vessels	Tumor Differentiation/ Type	Vascular Extravasation	VPF Expression
Type A Morphology	none (Figures 4 and 5a)	normal glia (no tumor) normal brain	none	none
Type B Morphology	moderate (Figures 5b and 6)	low grade (partially differentiated solid or cystic astrocytoma)	minimal or cyst formation	present
Type C Morphology	extensive (Figures 5c and 7)	high grade (undifferentiated) anaplastic astrocytoma glioblastoma multiforme metastatic tumor	extensive edema formation	present



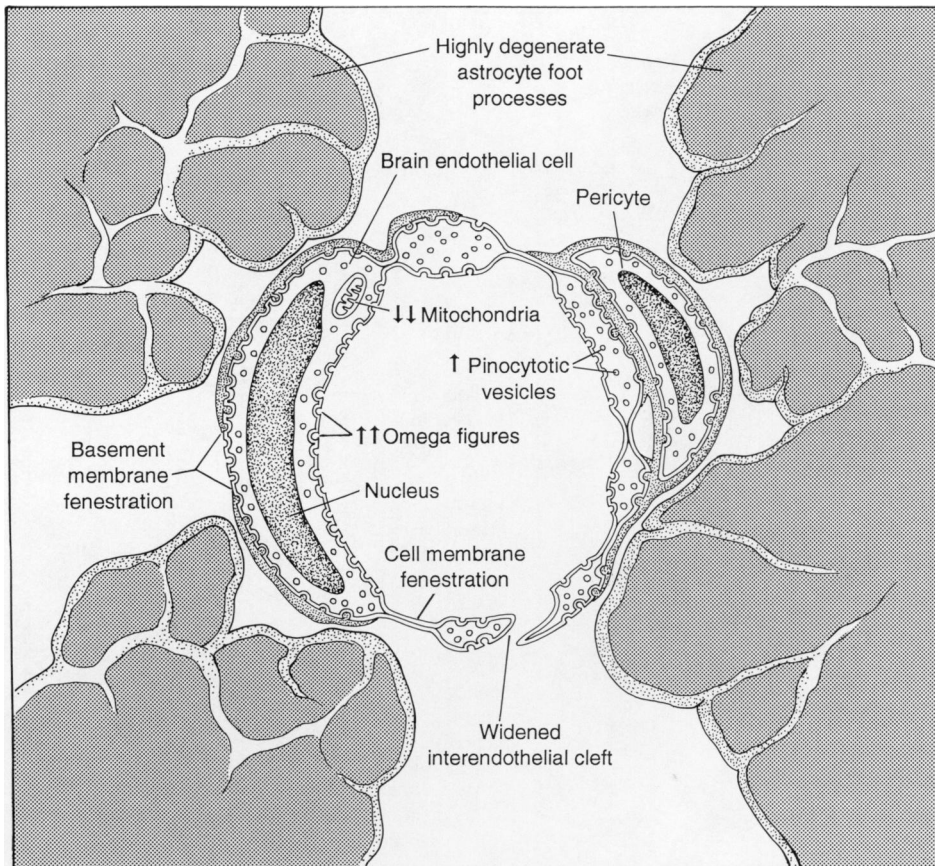
**Figure 5. Artistic interpretation of the three dimensional relationships between microvessels, normal astrocytes, less differentiated neoplastic astrocytes, and undifferentiated tumor cells. (a) Normal cellular interactions confer a typical pattern to the brain microvascular bed (Type A morphology). (b) Partial replacement of the astrocytes by less differentiated neoplastic glial cells is thought to result in disturbed cellular contacts and perturbations of chemical signaling. Type B morphology is observed with certain low grade gliomas. Vascular extravasation is less consistent in this setting but may be expressed as variable degrees of contrast enhancement, edema formation, or tumor cyst formation. (c) Ultimately, extreme degrees of departure from the normal microvascular architecture occur in the setting of highly malignant astrocytic and metastatic brain tumors. This figure depicts a vascular bed that is markedly altered by both the absence of normal cellular contacts as well as the influence of chemical signalling by tumor angiogenesis factors (Type C morphology). In this instance the stage is set for permeability induction by chemical mediators such as VPF.**



**Figure 6.** Artistic interpretation depicting a slightly atypical microvascular architecture in response to the altered environment imposed by the replacement of normal glial cells with low-grade (partially degenerate) neoplastic astrocytes. One can speculate that microvessels growing into minimally altered environments might retain some anatomical and functional blood-brain barrier features (Type B morphology). Tumors retaining this architecture are associated with minimal brain edema and tumor enhancement. Tumor-associated cyst formation, an epiphenomenon of vascular extravasation, may occur if VPF is expressed by these lesions.

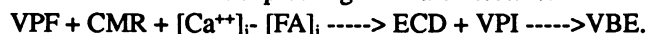
and 7) [16–19, 35–43]. However, many of these heterotypical cellular features of brain tumor microvessels actually typify the endothelium lining the otherwise normal peripheral vasculature (not involving the blood-brain barrier). While the peripheral vasculature is not inherently permeable to macromolecules, it does appear to be exquisitely sensitive to vascular permeability induction (VPI) by physiologically occurring substances such as histamine, bradykinin, serotonin, and prostaglandin [44–47]. Notably, the normal cerebral blood vessels appear totally unable to respond to these potent mediators of microvascular extravasation.

It now appears unlikely that the absence of continuous pentalaminar tight junctions would solely account for the abnormal permeability of brain tumor microvessels. Nevertheless, alteration of this critical component of the blood-brain barrier may at once render brain tumor microvessels similar to the peripheral microvasculature in anatomical form, and physiological reactivity to a variety of permeability-inducing substances. Recent studies have suggested tumor-induced permeability induction to be linked to a



**Figure 7.** Artistic interpretation depicting the highly degenerate microvascular architecture observed in the complete absence of normal glial-endothelial cell contacts. Normal glial cells are completely replaced by undifferentiated tumor cells derived from astrocytes, or systemic cancers that have metastasized to the brain. The microvasculature of highly anaplastic gliomas and brain metastases retains little or no semblance to normal blood-brain barrier microvessels (Type C morphology). These tumors are commonly associated with extensive brain edema and tumor enhancement. Immunohistochemical evidence of VPF expression by these lesions has recently been demonstrated.

cellular cascade entailing: tumor-derived VPF-CMR binding, a rapid and transient elevation in endothelial cytosolic calcium  $[Ca^{++}]_i$ , reduction in cellular F-actin content  $[FA]_i$ , and endothelial cytoarchitectural distortion (ECD) culminating in retraction of the interendothelial junctions and consequent extravasation of water and macromolecules through the widened interendothelial clefts. It is believed that all endothelial cells (including BBB endothelium), are capable of responding to the initial steps of the cascade. It is therefore postulated that the lack of VPF-CMR induced extravasation in BBB endothelium reflects a physical inability to open intercellular clefts and that this physical constraint reflects that imposed by the pentalaminal junctional structure. Therefore, it follows that in tumor microvessels not expressing BBB architecture:



This gross oversimplification of the mechanism of permeability induction retains its

**Table 5. Ultrastructural features of brain tumor microvessels.**


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Widened interendothelial junctions
Discontinuous endothelial cell tight junctions
Endothelial cell membrane fenestration
Noncontiguous basement membranes
Abundant endothelial cell micropinocytosis
Paucity of endothelial cell mitochondria

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**Table 6. Acute management of peritumoral brain edema.**


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Surgical cytoreduction (tumor excision)
Fluid restriction
Diuresis (furosemide and mannitol)
High-dose intravenous dexamethasone
Ventricular CSF drainage

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utility as a template for further investigative efforts. The background and ongoing research seminal to this hypothesis is thoroughly discussed throughout the remainder of this work.

#### *Clinical approach and management of peritumoral brain edema*

Patients with brain tumors frequently present with clinical manifestations of elevated intracranial pressure. They typically complain of headache, nausea, vomiting, drowsiness and double vision. Seizures and focal neurologic deficits are also commonly present, and their precise pattern, distribution, and severity is dictated largely by the location and size of the tumor and the extent of the surrounding cerebral edema. Progressive elevation of the intracranial pressure will result in further clinical deterioration to lethargy, stupor, and coma. Ultimately, intracranial hypertension leads to cerebral herniation with consequent fatal brain stem compression. In this regard, peritumoral vasogenic cerebral edema deserves reemphasis as a frequent and important accompaniment of intracranial tumors. The combined effects of rapid tumor growth and cerebral swelling within the confines of the rigid (fixed volume) cranial vault, dictate the rapidly progressive clinical decline observed in patients with malignant brain tumors. Methods of acutely managing these patients include tumor excision (surgical cytoreduction), fluid restriction, diuresis with intravenous furosemide and mannitol, high-dose intravenous dexamethasone, and ventricular CSF drainage (Table 6). Tumor excision addresses the tumor mass and its vascular bed directly, and as a consequence, eliminates the source of vasogenic edema fluid. Fluid restriction and diuretics decrease intracranial pressure by contracting the intravascular space and facilitating the egress of edema fluid from the cerebral interstitial space and into the vascular compartment. Furosemide is thought to act additionally by curtailing CSF production through its inhibitory action carbonic anhydrase, an enzyme key to CSF production. Ventricular CSF drainage lowers intracranial pressure both by reducing the volume of the cranial contents, and by facilitating the egress of edema fluid centrifugally into the ventricular system. Therapeutic CSF drainage is most efficacious in the specific setting of hydrocephalus related to a tumor obstructing CSF outflow. However, in the setting of a focal mass lesion with impending brain herniation, CSF decompression may be contraindicated as it may hasten the shift intracranial contents, thus facilitating herniation.

Glucocorticoids such as dexamethasone are remarkably effective in reducing the

neurological deficits and intracranial hypertension associated with malignant brain tumors [11, 48–50]. Their efficacy is not the result of tumor cytorreduction as *in vivo* and *in vitro* studies have failed to demonstrate any cytolytic action of steroids on brain tumors in general, and primary glial tumors in particular. However, the cytolytic action of glucocorticoids on peripheral lymphoid tumors is well described and does have a CNS correlate with primary and secondary CNS lymphomas. Nevertheless, the latter instances are exceptionally rare and a substantial body of CT data clearly indicate a direct action upon the peritumoral edema [51–54]. Moreover, myriad studies have clearly shown neoplastic vasogenic brain edema to be the only type of brain edema that responds clinically to glucocorticoid therapy [55–59]. Once a patient has been stabilized with dexamethasone, the brain tumor may be more safely neurosurgically excised. The need for additional therapy postoperatively is largely governed by the extent of tumor resection and histopathological diagnosis. Definitive treatment plans are proposed only after consultation between the neurosurgeon, neurologist, medical oncologist, and radiation therapist. Regretably, it is not unusual for patients with residual tumor and edema (i.e., those in greatest need of further therapy) to have adjunctive radiation or chemotherapy interrupted as a result of exacerbation of the edemagenic component. Once again, dexamethasone, employed in doses yet higher than those used initially, may control the brain edema sufficiently to allow adjunctive therapy to resume.

### MEDIATORS OF MICROVASCULAR PERMEABILITY: AN OVERVIEW

#### *Introduction: Classical and novel mediators of microvascular permeability*

The microvasculature of many solid tumors exhibits increased permeability in comparison with normal tissues. Certain pathological reactions associated with neoplastic growth are likely to result from permeability induction by tumor cells. Clinical manifestations of this process include decreased total serum albumin, production of malignant effusions and ascites, soft tissue edema, and paraneoplastic arthropathy [60–65]. Neoplastic vasogenic brain edema derives from a physiological alteration in the blood-brain barrier that is manifested by ultrastructural changes in tumor microvessels, and the extravasation of water, salts, and serum proteins into the peritumoral white matter [12, 16, 35–37]. Several biochemical mediators have been implicated in the pathogenesis of brain edema. Substances such as histamine, serotonin, bradykinin, glutamic acid, polyamines, leukokinins, lymphokines, prostaglandins, thromboxane, prostacyclin, kallidin, lymphocyte permeability factors, kallikrein, interleukin-2 (IL-2), and plasminogen activator, are potent inducers of microvascular permeability associated with a variety of allergic, traumatic, ischemic, infectious, and inflammatory conditions [44, 63–77]. Furthermore, attention has recently been focused on the potential role of oxygen free radicals in the formation of traumatic and peritumoral brain edema (Table 7) [78,79].

#### *Initial studies of tumor-derived vascular permeability factors*

A series of proteinaceous VPFs have stirred considerable interest in the past several years [60–65, 72, 74, 76, 80–94]. The existence of VPF was first recognized when guinea pig line 10 hepatocarcinoma cells promoted the accumulation of ascites fluid after intraperitoneal implantation [60]. This factor (gVPF) was found to be a 34 to 42 kDa basic protein with a strong affinity for immobilized heparin and a permeability-inducing potential three orders of magnitude greater than histamine. The presence of gVPF activity has been routinely determined by the Miles assay, a quantitative bioassay for induction of microvascular permeability [45]. Investigators found that gVPF-induced microvascular extravasation was rapid in onset (1 min) and of short duration (20 min), suggesting a



**Table 7. Potent mediators of microvascular permeability.**


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Histamine  
 Serotonin  
 Bradykinin  
 Leukotrienes  
 Prostaglandins  
 Lymphokines  
 Kallikreins  
 Interleukin-2  
 Oxygen free radicals  
 VPF/VEGF

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direct action upon the microvascular endothelial cell. Furthermore, gVPF activity induced a period of desensitization wherein microvessels previously exposed to this substance became temporarily refractory (20 or 30 min) to permeability induction by further gVPF perturbation. Light and electron microscopy showed that gVPF did not induce endothelial cell damage, mast cell degranulation, or an inflammatory cell infiltrate when injected into the guinea pig subcutaneous microvasculature. Moreover, intravascular injection of colloidal carbon resulted in the labeling of diaphragmatic, mesenteric, peritoneal, and gastrointestinal serosal post-capillary venules, thus supporting an action of gVPF upon these critical microvessels [70, 71, 95]. Rabbit-derived immunoglobulin (polyclonal IgG) raised to partially purified guinea pig hepatocarcinoma-derived gVPF neutralized essentially all permeability-inducing activity present in tumor ascites fluid and conditioned medium from line 10 cell cultures. This antibody also blocked the ascites formation that follows intraperitoneal injection of line 10 tumor cells. In addition, VPF activity from guinea pig line 104 C1 fibrosarcoma and Walker rat carcinoma lines was similarly inactivated by anti-gVPF antibody. It was suggested that secretion of VPF may be a common feature of tumor cells accounting for the abnormal accumulation of fluid associated with neoplasms [60, 61].

Soon thereafter, a tumor-derived capillary endothelial cell growth factor was identified and purified from rat chondrosarcoma extracellular matrix [96]. This 18 kDa cationic polypeptide had a marked affinity for heparin, and therefore differed from PDGF and EGF. Nanogram quantities of this substance stimulated both proliferation and mobilization of capillary endothelial cells in culture, activities thought to be key components of angiogenesis induction. Other studies showed that while heparin potentiated tumor-induced angiogenesis on the chorioallantoic membrane, angiogenesis was inhibited when both heparin and cortisone were administered simultaneously. Thus, glucocorticoids were once again identified as having an inhibitory action on a specific process essential to the biology of tumor growth and metastasis [97, 98]. At this point, investigators interested in factors acting directly upon endothelial cells diverged into two groups: those studying tumor angiogenesis factors (TAFs) and other endothelial, fibroblast, and smooth muscle cell mitogens, and those investigators studying VPFs [60–65, 80, 83–86]. Interest in these heparin-binding molecules has only recently reconverged with the publication of the amino acid sequence of a VPF molecule (vascular endothelial growth factor or VEGF) exhibiting both permeability-inducing and angiogenic mitogen capabilities (Table 8) [80–82, 87–93, 97, 98].

Partial purification and characterization of VPF from a human HT-29 colon adenocarcinoma cell line conditioned medium showed it to be a 45 kDa acidic protein which lacked a specific binding affinity for heparin [65]. Like guinea pig hepatocarcinoma gVPF, HT-29 permeability-inducing activity was not inhibited by antihistamines ( $H_1$  or

**Table 8. VPF/VEGF action/activity *in vitro*.**

Physiological Action/Activity	Assay Performed/Method	References Cited
Microvascular permeability	Miles assay <sup>125</sup> I-albumin extravasation	60, 80, 82, 83, 85, 86
Endothelial calcium transients	fluorescence spectrophotometry fura-2/AM calcium ion probe	84
Endothelial cell migration	chick chorioallantoic membrane nude mouse subrenal capsule visual inspection/scoring	81, 91, 93, 152, 153
Endothelial cell replication	endothelial cell culture cell counts after VPF incubation Coulter counter	81, 91, 93
Endothelial cell binding affinity	<sup>3</sup> H-thymidine incorporation <sup>125</sup> I-VPF/cellular cross-linking quantitative autoradiography	125-128

H<sub>2</sub> receptor blockade), antikinins, pepstatin A, or indomethacin. Several other human tumor lines have since been tested for VPF activity. One study demonstrated significant VPF expression by human osteogenic sarcoma, bladder carcinoma, cervical carcinoma, and fibrosarcoma. VPF activity from these tumors comigrated closely with guinea pig line 10 gVPF (34 to 42 kDa) and was neutralized by antibodies raised to line 10 gVPF. Furthermore, the VPF expressed by two human tumorigenic cell lines was also characterized by heparin affinity and an apparent size of 38 kDa [62]. Demonstration of the highly conserved nature of VPF molecules across species lines suggested a broader purpose for VPF-mediated permeability induction by tumors (i.e., extravasation of nutrients to support tumor growth in the extracellular matrix) [62, 97, 98]. Studies comparing VPF production by matched pairs of nontumorigenic and tumorigenic cell lines showed tumorigenic cell lines to secrete at least 14-fold more VPF activity, thus lending further support for a specific role of VPF and enhanced microvascular permeability in tumor biology and growth [62].

#### *Identification of human brain tumor vascular permeability factor*

Serum-free conditioned media derived from cultures of primary human malignant brain tumors (anaplastic astrocytoma and glioblastoma multiforme) have been shown to contain a factor that induces microvascular extravasation of fluid and protein in the Miles permeability bioassay [80]. VPF activity was not expressed by cultured human fibroblasts, meningioma cells, or several lines of low-grade astrocytomas (gliomas not usually associated with CT-evident brain edema), however it was strongly expressed by a number of rapidly-growing malignant gliomas (associated with CT-evident edema). Furthermore, fluid derived from the cysts associated gliomas was shown to contain potent VPF activity. HG-VPF appears to differ from commonly recognized mediators of vascular permeability. Early studies did, however, demonstrate several similarities to guinea pig line 10 hepatocarcinoma-derived gVPF. HG-VPF is a 41 to 56 kDa basic cationic polypeptide which avidly binds heparin [83, 84]. Like gVPF, the time course of permeability induction by HG-VPF is rapid and transient suggesting a direct action upon endothelial cells. As in previous gVPF studies, prior exposure to HG-VPF results in a period of refractoriness, during which further induction of microvascular extravasation by HG-VPF does not occur. Expression of HG-VPF activity is inhibited by incubation of glioma cell cultures with cycloheximide or dexamethasone. Finally, a rabbit-derived polyclonal IgG raised to gVPF completely inactivated HG-VPF activity in partially purified conditioned media

(U251 human malignant glioma), thus further supporting homology between these substances (polyclonal anti-VPF IgG was kindly provided by Donald R. Senger, Ph.D., Beth Israel Hospital, Harvard Medical School, Boston) [80].

In order to eliminate variations in HG-VPF expression associated with use of several different tumor lines, serum-free conditioned medium derived from low-passage cultures of a single cloned human malignant glioma line (U251) were employed for all subsequent HG-VPF studies. A partially purified U251 glioma protein product containing HG-VPF, has been shown to induce rapid and reversible elevations in cytosolic calcium in several types of cultured endothelial cells [83–85]. The HG-VPF induced intracellular calcium ion changes exhibited dose-responsiveness and were inhibited by nonspecific calcium channel blockers (lithium, cobalt, manganese, and lanthanum ions) suggesting that influx of extracellular calcium ions was responsible for the observed cytosolic calcium transients. Re-exposure of endothelial cells to the HG-VPF stimulus failed to produce a second calcium ion transient increase suggesting that a period of refractoriness similar to that observed *in vivo*, had been induced by the initial HG-VPF exposure. Most significantly, HG-VPF induced calcium changes were completely inhibited in endothelial cells previously exposed to dexamethasone, thus suggesting a second explanation for its clinical efficacy.

## INITIAL BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF HUMAN BRAIN TUMOR VPF

### *HG-VPF activity, expression, and behavior in a permeability bioassay*

Serum-free conditioned medium from low-passage confluent monolayer cell cultures of human malignant astrocytoma lines evoked macromolecular extravasation (quantitated by measurement of <sup>125</sup>I-BSA accumulation) in the Miles cutaneous microvascular permeability assay [80]. Intradermal injection of this factor results in a permeability change with a rapid onset at 1 to 5 min, a peak at 5 to 15 min, and reversibility by 20 to 30 min. In addition, there is a characteristic period of desensitization, refractoriness or tachyphylaxis whereby injection of HG-VPF into previously exposed sites results in no further induction of permeability. Conditioned medium from benign astrocytoma, meningioma, or fibroblasts demonstrated no significant VPF activity. Fluid aspirated from a cystic glioblastoma contained very high HG-VPF activity, whereas no activity was evident in samples of cerebrospinal fluid from a normal volunteer, a patient with a sacral chordoma, or a patient with a malignant cerebral glioma. HG-VPF activity increased as the duration of culture incubation was lengthened.

### *Physical and chemical characterization of human glioma-derived VPF*

HG-VPF is an acid-stable heat labile 41–56 kDa molecular weight polypeptide, which is hydrophobic and positively charged under physiological conditions [83]. HG-VPF activity was abolished by treatment with trypsin or pepsin, but was unaffected by ribonuclease A, chondroitinase A,B,C, hyaluronidase or lipase. Similarly, HG-VPF activity is not inhibited by soybean trypsin inhibitor or hexadimethrine (both known antagonists of tissue plasminogen activator, Hageman factor, and serum kallikrein), aprotinin (an antagonist of both plasmin and tissue kallikrein), phenylmethanesulfonyl fluoride (a serine esterase (elastase) inhibitor), or by pepstatin A (an acid protease inhibitor which inactivates vascular permeability-inducing leukokinins). Treatment of HG-VPF with dithiothreitol abolished permeability-inducing activity, indicating the presence of at least one essential disulfide bond in this molecule. VPF displays a marked affinity for immobi-

lized heparin (heparin-Sepharose). In addition, we found 90–95% binding of sample activity to hydrophobic resin (phenyl-Sepharose), and hydroxylapatite. While 40–45% of HG-VPF activity was bound by negatively charged resin (carboxymethyl-Sepharose), only 5% of sample activity was bound to positively charged resin (diethylaminoethyl-Sepharcel). The latter resin sequestered 80–85% of sample protein. Employing a heparin-Sepharose column and a NaCl gradient generator, it was determined that peak elution of HG-VPF activity occurs at a salt concentration of 0.45 N NaCl. Specific activity was increased 26-fold by this step. When this partially purified sample was applied to an HPLC sizing column, HG-VPF activity eluted in the 41–56 kDa fraction. Furthermore, an additional 25-fold increase in specific activity was realized with this step, thus increasing the total approximate purification to 1,000-fold.

#### *Dexamethasone inhibition of HG-VPF expression and activity*

Studies to determine how dexamethasone affects HG-VPF expression by cultured human glioma cells failed to show a direct toxic effect as measured by cell viability (>98% by trypan blue exclusion), and final cell counts [83]. Although HG-VPF expression was significantly inhibited by the presence of glucocorticoid, this effect was not associated with a general inhibition of cellular protein synthesis as determined by <sup>3</sup>H-leucine incorporation. We had previously determined that treatment of test animals with dexamethasone immediately before performing the permeability assay did not confer protection against HG-VPF induced extravasation. Coinjection of dexamethasone with HG-VPF also failed to impart a membrane stabilizing protective effect as extravasation was unhindered. In fact, significant inhibition of HG-VPF activity *in vivo* only occurred in test animals given dexamethasone at least 1–2 hrs before performing the permeability assay. To determine whether the inhibitory action of dexamethasone was mediated by *de novo* protein synthesis, a series of *in vivo* studies were performed whereby actinomycin D was administered 2 hrs prior to dexamethasone treatment. *In vivo* inhibition of HG-VPF activity by dexamethasone was found to be partially reversed in animals given actinomycin D prior to steroid. This suggested that *de novo* synthesis of a specific polypeptide intermediate is required for the protective effect of dexamethasone to occur. These observations strongly argue against a nonspecific steroid-induced membrane stabilization as the key physiological event.

#### *HG-VPF induction of cytosolic calcium ion flux in endothelial cells*

Recent advances in our understanding of endothelial cell growth requirements have allowed their routine culture for investigational purposes [19–21, 24, 99–110]. Endothelial cells derived from brain capillaries have received considerable attention because of their unique barrier characteristics [20, 21]. Cytosolic free Ca<sup>2+</sup> plays a pivotal role in endothelial cytoskeletal alterations and subsequent microvascular extravasation [84–86, 111–120]. Studies of cytosolic calcium changes have been greatly facilitated by the development of a series of novel fluorescent calcium ion probes (quin-2/AM, indo-2/AM, fura-2/AM, fura-3/AM) possessing molecular structures similar to EGTA [113, 117]. They differ from EGTA in that they contain aromatic rings capable of electrostatic interactions with the functional groups that participate in the chelation of free cytosolic calcium ions [Ca<sup>2+</sup>]<sub>i</sub>. Chelation of divalent cations changes the fluorescence and ultraviolet light-absorption properties of these molecules. Alterations in fluorescence are in turn detected and quantitated by a spectrofluorometer. Fura-2/AM has proven particularly useful with its high sensitivity and specificity for calcium ions. Because of its lipophilicity,

fura-2/AM is rapidly internalized by endothelial cells. Once internalized however, deesterification of the acetoxy-methyl group (fura-2/AM  $\rightarrow$  fura-2) converts it to a lipophobic free acid, with only minimal capability for diffusion out of the cell. Cytosolic calcium ion changes in the nanomolar range are readily detectable with this probe.

Partially purified HG-VPF has been shown to induce significant calcium ion transients in several varieties of endothelial cells sustained in monolayer cultures [84]. It did not, however, elicit a calcium response in two non-endothelial cell lines (U251 glioma and fibroblasts). The endothelial cytosolic calcium changes were typically rapid or only slightly delayed in onset (15–45 sec), and varied in magnitude depending upon the cell type being studied. The largest responses were observed in human endothelial cells. It has already been postulated that such variations may relate to some degree of molecular species-specificity despite the highly conserved nature of VPF activity [61]. Peak HG-VPF induced intracellular calcium ion elevations were observed within 60 sec, exhibited a dose-response phenomenon, and were followed by a sustained elevation above baseline for 5 to 10 min thereafter. In contrast, exposure of the endothelial cells to the flow-through product of HG-VPF containing glioma conditioned medium after binding of all HG-VPF activity to a heparin-Sepharose affinity column, did not produce a change in intracellular calcium. The brief time course of the HG-VPF induced cytosolic calcium changes *in vitro* are compatible with the *in vivo* kinetics, and is supported by other data showing prolonged stimulus-response coupling to occur after only transient intracellular calcium elevations [115].

Exposure of endothelial cell suspensions to elevated extracellular potassium chloride (120 mM KCl) failed to induce cytosolic calcium transients. Furthermore, verapamil (10  $\mu$ M) failed to inhibit HG-VPF induced calcium changes. These data suggests that HG-VPF increases cytosolic calcium by a route other than verapamil-sensitive voltage-gated calcium channels. Addition of 2 mM lithium, cobalt, manganese, or lanthanum ions inhibited HG-VPF induced calcium transients, as did the absence of extracellular calcium after chelation with EGTA. This indicates that HG-VPF induced calcium ion changes are largely dependent upon the influx of extracellular calcium  $[Ca^{2+}]_e$  through membraneous calcium channels. Finally, incubation of endothelial cells for 2 hrs with 10  $\mu$ M dexamethasone before exposure to HG-VPF containing medium resulted in inhibition of the HG-VPF induced cytosolic calcium changes. This inhibition was not observed in similarly treated cells exposed to ATP, nor did it occur when cells were incubated with dexamethasone for less than 1 hr, suggesting the steroid-induced inhibition was specific for HG-VPF induced calcium flux, and that dexamethasone may mediate its effect through an intermediary requiring *de novo* synthesis.

Like VPF, another polycationic substance (protamine), known to induce changes in vascular permeability, was also shown to induce physiologically significant perturbations in endothelial intracellular calcium when present in concentrations as low as 10  $\mu$ g/ml [121–123]. Induction of calcium release by protamine was rapid in onset, peaked within 30 seconds, and sustained a level above baseline for several minutes thereafter. The presence of extracellular divalent cations (2 mM cobalt or manganese) reduced peak  $[Ca^{2+}]_i$ , but also more completely affected the post-maximum levels resulting in a rapid down-slope and normalization of intracellular calcium. These findings indicated that combined mobilization of intracellular calcium stores (rapid upslope and peak levels), as well as influx from the extracellular compartment (peak and sustained levels), are involved in the response to HG-VPF and protamine. It also suggests that neutralization of the anionic endothelial cell surface charge imposed by heparin-like moieties, by certain polycationic substances (VPF, protamine, angiogenesis factors) may play a role in mediating the

actions of these substances. This is supported by ultrastructural evidence that exposure of renal glomeruli to protamine results in disorganization of the epithelial cell foot process architecture (podocytes) investing the microvascular glomeruli, and subsequent leakage of protein into the urine. Endothelial surface charge neutralization by polycations may simply eliminate the electrostatic barrier that exists to the vast majority of proteins (negatively charged), allowing them to escape through pores in fenestrated vessels.

### MOLECULAR GENETICS: CHEMICAL AND BIOLOGICAL DEFINITION OF VPF

#### *Purification and sequencing of human vascular permeability factor*

A VPF has recently been purified to homogeneity from serum-free conditioned medium of human histiocytic lymphoma cell line U937 [62, 81, 82, 91]. The cDNA sequence of hVPF was shown to code for a 189-amino acid 38–40 kDa polypeptide with two identical 20–24 kD subunits that became evident on electrophoresis in reducing gels (SDS-PAGE). Available data suggests that native hVPF is a dimer composed of disulfide-linked subunits, each with the same NH<sub>2</sub>-terminal amino acid sequence. The NH<sub>2</sub>-terminal region of the predicted amino acid sequence of U937 hVPF was found to be 78% identical to the analogous region of line 10 (guinea pig) VPF. The hVPF molecule also bore some similarities in structure to the  $\beta$  chain of platelet-derived growth factor (PDGF- $\beta$ ), as well as several PDGF/*v-sis* oncogene-related proteins. Similarly, a heparin-binding VEGF has recently been purified from conditioned media derived from bovine pituitary folliculostellate cells and phorbol ester-activated human (HL60) promyelocytic leukemia cells [93]. The cDNA sequence of human VEGF is similar to that of hVPF except for an additional 24 amino acids in the hVPF sequence. VPFs with similar immuno-cross reactivity have been detected in conditioned media derived from a variety of human and rodent tumor cell lines. In addition to its permeability-inducing activity, hVPF also appears to specifically stimulate endothelial cell growth and angiogenesis [87–93]. The hVPF molecule did not stimulate [<sup>3</sup>H]thymidine incorporation or promote growth of other non-endothelial cell types such as vascular smooth muscle cells (VSM) or fibroblasts. This feature distinguished it from several other endothelial cell growth factors, such as the heparin-binding acidic and basic fibroblast growth factors (FGFs), which promote growth and replication in non-endothelial, as well as endothelial cell lines. Conversely, several endothelial cell growth factors including epidermal growth factor (EGF), fibroblast growth factors (aFGF and bFGF), transforming growth factor (TGF), interleukin-1 (IL1), tumor necrosis factor (TNF), and platelet-derived growth factor (PDGF) failed to exhibit VPF-like permeability-inducing activity when tested in the Miles assay. Other studies have confirmed the permeability-inducing activity of IL2, however this protein bears no size similarity or sequence homology to the hVPF molecule. <sup>125</sup>I-hVPF was shown to bind specifically and with high affinity to endothelial cells *in vitro*, and could be chemically cross-linked to a high-molecular weight cell surface receptor, thus suggesting a specific site of interaction between hVPF and the vascular endothelial cell. Complex formation was blocked by excess unlabeled hVPF and anti-hVPF serum, but not by the addition of excess quantities of the aforementioned growth factors. Permeability induction in the Miles assay was observed with as little as 1 nanomolar (8 nanograms) hVPF.

#### *The human gene for vascular permeability factor*

In 1991, Tischer et al. showed VSM cells synthesize VEGF mRNA and secrete biologically active VEGF [124]. Their analyses of VEGF transcripts using polymerase chain

reaction and cDNA cloning revealed three different forms of the VEGF coding region as had been reported in HL60 cells. The three forms of human VEGF protein chain from these coding regions are 189, 165, and 121 amino acids in length. Comparison of cDNA nucleotide sequences with sequences derived from human VEGF genomic clones indicates that the VEGF gene is split among eight exons and that the various VEGF coding region forms arise from this gene by analogous alternative gene splicing: the 165 amino acid form of the protein is missing the residues encoded by exon 6, whereas the 121 amino acid form is missing the residues encoded by exons 6 and 7. Analysis of the VEGF gene promoter region revealed a single major transcription start, which lies near a cluster of potential Sp1 factor binding sites. The promoter region also contains several potential binding sites for the transcription factors AP-1 and AP-2; consistent with the presence of these sites, Northern blot analysis demonstrated that the level of VEGF transcripts is elevated in cultured vascular smooth muscle cells after treatment with the phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate.

The finding that cultured VSM cells secrete VEGF raises the possibility that VSM cells act as a source of VEGF that could function as a paracrine factor both to maintain the integrity of the vascular endothelium and to repair endothelial damage. Since VEGF binds heparin, locally synthesized VEGF might be stored in the extracellular matrix bound to heparin-like molecules, as appears to occur with bFGF. Whether VSM cells make VEGF *in vivo* remains to be confirmed by *in situ* hybridization or by immunohistochemistry.

#### *Binding sites for human VPF/VEGF on vascular endothelial cells*

Specific binding of human VPF/VEGF to a variety of endothelial cells has now been demonstrated by several investigators. Olander et al., in 1991, used highly purified gVPF in cross-linking experiments to demonstrate <sup>125</sup>I-gVPF-receptor complexes of two types [125]. Most of the complexes migrated slowly in SDS-PAGE, indicating that they were of very high molecular weight and probably highly cross-linked. A portion of the molecules migrated as 270 kDa complexes, indicating that the molecular weight of the endothelial cell VPF receptor is about 230 kDa. In 1991, Myoken et al. purified VEGF to homogeneity from protein-free culture conditioned medium of A-431 human epidermoid carcinoma cells [126]. This VEGF was characterized as a homodimer composed of 22 kDa subunits with an N-terminal sequence that was similar to VEGFs produced by human HL60 leukemic and U937 histiocytic lymphoma cells. A431 VEGF was used to identify specific and saturable binding sites for VEGF on HUVEC. By affinity cross-linking, VEGF-binding site complexes of 230, 170, and 125 kDa were detected on human umbilical vein endothelial cells (HUVEC). VEGF specifically induced the tyrosine phosphorylation of a 190 kDa polypeptide, which was similar in mass to the largest binding site detected by affinity cross-linking.

In 1992, Jakeman et al., using recombinant human VEGF (rhVEGF), showed the colocalization of <sup>125</sup>I-rhVEGF binding with Factor VIII-like immunoreactivity, thus demonstrating binding sites associated with vascular endothelial cells of both fenestrated and nonfenestrated microvessels as well as the endothelium of large vessels, while failing to demonstrate evidence of displaceable binding on nonendothelial cells [127]. Specific binding was associated with quiescent as well as proliferating vessels. Those findings were thought to support the hypothesis that VEGF plays a specific role in both the maintenance and in the induction of growth of vascular endothelial cells. In the central nervous system, <sup>125</sup>I-rhVEGF binding sites were associated with Factor VIII immunoreactivity in arteries, veins, and microvessels. The density of binding was greatest in the gray

matter of the brain and spinal cord. In the cerebral cortex, the binding pattern followed the distribution of penetrating vessels, extending from the outer meninges toward the deeper laminae. Displaceable binding was also associated with the meninges and the large vessels on the surface of the brain and spinal cord, and was seen along vascular elements in the choroid plexus. No binding was seen along the ependyma of the ventricles. Examination of pituitary sections revealed binding in the *pars distalis* and *pars nervosa*, while essentially no binding was seen in the poorly vascularized *pars intermedia*.

In 1992, Gitay-Goren et al. reported that VEGF binding to its cell surface receptors was clearly dependent on the presence of cell surface-associated heparin-like molecules [128]. Heparin, at concentrations ranging from 0.1 to 10  $\mu\text{g/ml}$ , strongly potentiated the binding of  $^{125}\text{I}$ -VEGF to its receptors on endothelial cells derived from the bovine aorta and human umbilical vein. Scatchard analysis of  $^{125}\text{I}$ -VEGF binding indicated that 1  $\mu\text{g/ml}$  heparin induces a 8-fold increase in the apparent density of high affinity binding sites for VEGF, but does not significantly affect the dissociation constant of VEGF. Cross-linking experiments showed that heparin strongly potentiates the formation of the 170-, 195-, and 225-kDa  $^{125}\text{I}$ -VEGF-receptor complexes on endothelial cells. At high  $^{125}\text{I}$ -VEGF concentrations (4 ng/ml), heparin preferentially enhanced the formation of 170- and 195-kDa complexes. Preincubation of the cells with heparin, followed by extensive washes, produced a similar enhancement of subsequent  $^{125}\text{I}$ -VEGF binding. The binding of  $^{125}\text{I}$ -VEGF was completely inhibited following digestion of endothelial cells with heparinase and could be restored by the addition of exogenous heparin to the digested cells. The enhancing effect of heparin facilitated the detection of VEGF receptors on cell types that were not known previously to express such receptors (bovine granulosa cells, bovine corneal endothelial cells). The results suggested that cell surface-associated heparin-like molecules are required for the interaction of VEGF with its cell surface receptors.

#### *Regulatory mechanisms involved in VPF expression*

VPF/VEGF is highly expressed in areas of active vascular proliferation and in a variety of human tumor cell lines and also in many normal tissues. It is a heparin-binding mitogen and permeability-inducing polypeptide structurally related to PDGF and existing in four species which are generated by analogous alternative gene splicing. The extracellular ligands and components of the signal transduction pathway which lead to VEGF expression remain unclear. Isoenzymes of PKC are thought to play an essential role in the pathway leading to VPF/VEGF expression. Four AP-1 binding sites have been identified in the promoter region of the VEGF gene. Phorbol ester treatment of vascular smooth muscle cells and U937 cells induces VEGF mRNA expression. Transforming growth factor- $\beta$ 1 also causes a slight increase in VEGF mRNA in U937 cells. In 1992, Finkenzeller et al. showed that both platelet-derived growth factor and phorbol ester cause an increase in vascular endothelial growth factor (VEGF) mRNA expression in control NIH 3T3 fibroblasts and NIH 3T3 fibroblasts overexpressing human PKC $\alpha$  as compared to control cells [129]. In cells stimulated with PDGF or phorbol ester, induction of expression was lost after down-regulation of PKC. This indicates that PKC is involved in signal transduction leading to VEGF expression. Claffey et al. recently reported a series of experiments employing phorbol esters, calcium ionophore, and cAMP analogues which demonstrated that VEGF mRNA expression is stimulated in preadipocytes (3T3-F442A) by both PKC and protein kinase A-mediated pathways. Their results suggest that VEGF mRNA levels are closely linked to the process of cellular differentiation. They also demonstrated that VEGF expression is specifically regulated in a transformed cell line (pheochromocytoma



PC12) by a mechanism possibly involving aberrant activation of cellular second messenger pathways. The transformed undifferentiated cells express moderate levels of VEGF mRNA whereas expression is essentially extinguished when the cells differentiated into non-malignant neuron-like cells [130].

Additional insight into VEGF regulatory mechanisms derives from a report that studied *fms*-like tyrosine kinase (*Flt*) in *Xenopus laevis* oocytes. *Flt* is a transmembrane receptor in the tyrosine kinase family. It was determined that *Flt* expression in oocytes conferred specific high-affinity binding of VPF/VEGF and caused the cells to release calcium in response to VPF/VEGF, thus indicating that *Flt* encodes a receptor for VPF/VEGF. The *Flt* transcript has also been detected in HUVEC that respond to VPF/VEGF. Whether or not *Flt* is the only receptor for VPF/VEGF has yet to be determined. Similarly, it is not known whether the *flt* receptor is responsible for either permeability or angiogenesis induction by VPF/VEGF [131]. A brief tabulation of documented VPF/VEGF actions/activities has been provided (Table 8).

### THE ROLE OF VPF IN TUMOR EDEMA GENESIS AND ANGIOGENESIS

Angiogenesis is a process by which new capillary structures are formed. The new microvessels eventually coalesce, organize, and enlarge into larger vascular structures by the process termed neovascularization. Examples of physiologic angiogenesis include the cyclical development of the *corpus luteum* and normal wound healing. Neovascularization plays a critical, albeit self-limited, role in a variety of pathological processes such as arthritis, retrolental fibroplasia, atherogenesis, pyogenic granuloma, and keloid formation. Tumor-induced angiogenesis is subservient to the metabolic needs of the neoplasm which in turn is self-sustaining and not self-limited. Tumor cell replication continues provided a vascular network allowing for adequate nutrient delivery develops in parallel. It is now apparent that tumor cells induce and control new vessel growth by secreting a variety of factors capable of inducing endothelial cell migration and replication. Most notable are a group of heparin-binding polypeptides which include acidic and basic FGF, and the more recently defined VPF/VEGF. Copper ions and heparin likely play key roles as modulators or potentiators of angiogenesis. VPF/VEGF is most novel for a variety of reasons including: (1) its dual role in permeability induction and angiogenesis (FGF has no permeability-inducing properties.); (2) its specific action on endothelial cells (FGF and other non-VPF/VEGF angiogenesis factors act upon vascular smooth muscle cells and fibroblasts as well as endothelial cells.); and (3) its active secretion by tumor cells (The VPF/VEGF molecule possesses a peptide secretor sequence and unlike FGF does not rely on cellular demise for release of intracellular factor stores.)

The microvasculature of brain tumors displays several distinctive ultrastructural features that allow ready distinguishment from normal cerebral microvessels. The presence of widened endothelial cell junctions, discontinuous tight junctions, cellular membrane fenestrations, noncontiguous basement membranes, active micropinocytosis, and paucity of mitochondria, sharply contrast with normal blood-brain barrier endothelial cytoarchitecture [12, 15, 16, 20, 21, 25, 35–37]. These features, which also typify endothelium lining the normal peripheral vasculature, have previously been associated with the water and protein extravasation, and consequent cerebral edema that occurs with malignant brain tumors [15, 17, 25, 132]. Like blood-brain barrier microvessels, however, the normal peripheral vasculature is not inherently permeable to macromolecules. Nevertheless, tumors occurring outside the central nervous system also exhibit microvascular extravasation that is clinically manifested as malignant effusions, ascites, and tissue edema [60–65].

The normal peripheral vasculature is exquisitely sensitive to permeability-induction by histamine, bradykinin, serotonin, prostaglandins, and a variety of other physiological substances [44–46]. In contradistinction, normal cerebral microvessels are totally incapable of responding to these substances when tested *in situ*. Similarly, HG-VPF, which clearly is active in the guinea pig and rat cutaneous microvasculature, failed to evoke extravasation of fluorescein-albumin, <sup>125</sup>I-bovine serum albumin, or edema fluid as evidenced by tissue specific gravity determinations, when injected into normal rat brain. However, injection of HG-VPF into C6 gliomas that had been implanted into rat brains showed brain tumor microvessels to be capable of responding with increased permeability *in situ* (unpublished observations, G. R. Criscuolo and E. H. Oldfield).

Vascular endothelial cells from peripheral and central nervous system sources are known to possess a complex cytoskeletal architecture [101, 111]. The endothelial cytoskeleton is composed of actin, myosin, and tropomyosin proteins that serve to regulate cellular configuration. Therefore, cellular mobility and vascular permeability are processes that are likely to result from changes in intracellular calcium ions that occur in response to angiogenesis factors and VPFs. Stimuli that induce cytosolic calcium transients and change endothelial cell shape, ultimately result in opening of interendothelial junctions at the level of the postcapillary venule, which in turn results in vascular extravasation [44, 84, 116]. Histamine-induced cytosolic calcium changes have been shown to correlate with changes in endothelial F-actin content, and passage of albumin across endothelial monolayers derived from human umbilical veins [115]. In fact, many of the commonly recognized mediators of microvascular permeability have been shown to induce transient elevations in endothelial cytosolic calcium, irrespective of the variety of endothelial cell tested [44, 84, 112, 115]. Significantly, normal brain microvascular endothelial cells are equally capable of responding with calcium ion transients, and, by conjecture, cytoskeletal alterations that would culminate in vascular extravasation. These findings, and the morphological similarities between brain tumor vessels and the normal peripheral vasculature, suggest an alternative hypothesis for the induction of vascular permeability in brain tumors.

Perhaps the reason BBB microvessels are incapable of responding to permeability mediators *in situ*, relates solely to their interendothelial junctions being physically joined together by continuous pentalaminal tight junctions. The extreme integrity of this junction is supported by experimental studies of osmotic disruption of the BBB by mannitol, wherein investigators showed the interendothelial clefts to be closed, and the tight junctions to be continuous and intact after exposure to that potent physical agent: opening of the barrier by mannitol was associated with an increased transgression of micropinocytotic vesicles between the luminal and abluminal endothelial surfaces [69]. Further support derives from the knowledge that peritumoral brain edema emanates directly from the tumor bed, and is not the result of HG-VPF induced extravasation in the surrounding blood-brain barrier microvessels [54]. Therefore, one may reasonably put forth the hypothesis; that an alteration or absence of such a critical component of BBB as the interendothelial tight junction may at once render brain tumor microvessels similar to the peripheral microvasculature, both anatomically, and with respect to their ability to respond to permeability mediators. As a result, brain tumor microvessels would be capable of responding to the HG-VPF secreted by surrounding tumor cells, and production of vasogenic cerebral edema would result.

Although the study of VPFs has already yielded valuable insight into a mechanism of tumor-mediated vascular permeability, many questions remain unanswered. Several questions pertinent to neoplastic brain edema immediately arise: Why does peritumoral brain edema not follow an even more malignant course? If brain tumor microvessels remain

constantly exposed to HG-VPF, and respond with a constant rate of plasma extravasation, why would a patient not succumb to a rampant increase in intracranial pressure within perhaps minutes, hours, or a few days at most? This may be partly explained in part by known mechanisms of brain edema resolution. Excess extracellular fluid normally travels through the brain interstitium in a centripetal direction (toward the CSF-containing ventricular system) by a passive process referred to as bulk flow. In addition, astrocytic cells are known to be actively involved in the imbibition of excess tissue fluid. This issue may be further explained by observations indicating that HG-VPF is capable of inducing a period of unresponsiveness, refractoriness, or tachyphylaxis, whereby consecutively applied stimuli will not result in cytosolic calcium changes or further vascular extravasation [60, 80, 83, 84]. Supporting this notion is the common occurrence of a period of relative unresponsiveness (refractory period) in many physiological cascades, as well as in a variety of physiologically excitable cells (neurons, photoreceptor cells, muscle cells).

#### EFFICACY OF DEXAMETHASONE FOR NEOPLASTIC BRAIN EDEMA

The clinical efficacy of dexamethasone in the setting of peritumoral brain edema has been well described and documented [11, 48–56, 80, 83–86]. Clinical trials were initiated by Kofman et al., who in 1957 reported remarkable temporary improvement in neurological symptoms in 14 out of 22 patients with brain metastases from breast cancer treated with prednisolone [133]. Galicich and French subsequently established a standard regimen of dexamethasone for primary and metastatic brain tumors and for postoperative brain edema [11]. Using this treatment regime, patients will predictably improve within 8–24 hrs of the first intravenous dose and will subsequently resolve most of their signs and symptoms within the next 2–5 days thereafter. Although significant resolution of peritumoral edema is not often readily apparent on imaging studies, several investigators have provided direct evidence of reduction in both tumor enhancement and edema on CT scans taken several days post initiation of steroid therapy [134, 135]. Positron emission tomography (PET) employing  $^{68}\text{Gd}$  as a tracer, has been used to demonstrate that increased capillary permeability associated with brain tumors occurs only within the tumor vascular bed and not in the adjacent brain tissue [136]. Furthermore, in studies employing  $^{82}\text{Rb}$  as a PET tracer, Jardan et al., demonstrated that in patients receiving high-dose dexamethasone treatment (100 mg *i.v.* followed by 24 mg every 6 hrs), the permeability constant ( $K_1$ ) decreased significantly in tumor tissue (mean of 30%) [137]. A quantitative autoradiographic study reported by Austin et al., demonstrated that in a rat C6 glioma model, capillary permeability of the tumor and adjacent brain decreased significantly as early as 1 hr after administration of dexamethasone (10 mg/kg *i.p.*) [138]. Complementing these studies are reports indicating that ( $^3\text{H}$ )-dexamethasone given intravenously concentrates in the brain tumor bed. Moreover, steroid receptor studies have demonstrated the occurrence of receptors in a variety of brain tumors, with the greatest concentrations being observed in metastatic tumors. This is notable in view of the fact that metastatic tumors are at once most likely to express the greatest amount of edema and most likely to respond to dexamethasone therapy. These data support the hypotheses that edema fluid emanates from the tumor's angiogenic vascular bed, and that dexamethasone acts primarily by retarding the rate of edema fluid formation by a mechanism involving steroid receptor binding. As a result, patients respond early to the consequent reduction in both fluid extravasation and tissue pressure (ICP). Subsequently, and in a more delayed fashion, CT- or MRI-evident edema will slowly resolve as normal physiological mechanisms for clearing excess interstitial fluid prevail.

We have been able to demonstrate several specific levels at which dexamethasone may specifically exert its beneficial effects in this focused pathophysiological setting

[80]. Concentrations of dexamethasone that occur in our patients' brain tumors will specifically suppress production of HG-VPF by cultured human malignant glioma cells [83]. This finding alone suggests a powerful mechanism for steroid efficacy. In addition, a distinct and separate mechanism of steroid action may occur at the level of the key biological component for this process; the microvascular endothelial cell. This is supported by the finding of calcium transient suppression by dexamethasone in cultured endothelial cells [84]. Lastly, a glimpse into the mechanism by which dexamethasone effects target cells (glioma cells and endothelial cells) is provided by the following findings: (1) coinjection of HG-VPF with dexamethasone did not alter the extent of vascular extravasation [80], (2) pretreatment of test animals with dexamethasone 1–2 hrs before HG-VPF injection substantially inhibited vascular extravasation whereas pretreatment less than 1 hr before resulted in no inhibition of HG-VPF activity [80, 83, 85], (3) pretreatment of test animals with actinomycin D before dexamethasone exposure resulted in significant, albeit slightly reduced, HG-VPF induced extravasation [83]. The implication is that dexamethasone exerts its actions indirectly, by inducing *de novo* synthesis of a polypeptide intermediary after receptor-mediated membrane traversal, rather than by nonspecific membrane stabilization. This concept has been put forth as an explanation for the protective effect of dexamethasone in rats affected by global cerebral ischemia [139, 140]. Furthermore, a second messenger polypeptide, (variably referred to as "macro cortin", "lipocortin", "endocortin", or "renocortin"), has been identified, characterized, and found to mediate the effect of glucocorticoids by inhibition of phospholipase-A2 [140]. Induction of macrocortin synthesis requires steroid receptor occupation and *de novo* protein synthesis. It therefore appears possible that HG-VPF may act by inhibiting a rate-limiting enzyme in the prostaglandin cascade (phospholipase-A2) [83–85]. This is corroborated by the work of others who have shown prostaglandins to be potent inducers of cytosolic calcium release in endothelial cells [141, 142].

Additional investigations using a completely purified vascular permeability factor product are clearly warranted and should better define the kinetics, refractoriness to sequential stimulation, and dose-responsiveness of VPF-induced microvascular extravasation. Furthermore, comparison of endothelial cells derived from different anatomical sources (brain, retina, lung, adrenal, umbilical cord), from differing sized vessels (arterial, arteriolar, capillary, venous, venular), and from several species (human, bovine, rodent), should determine the extent, and degree of specificity of VPF activity with regard to these variables.

Information to date strongly favors a direct action of VPF on the vascular endothelial cell. The rapid and reversible kinetics of VPF activity *in vivo* and *in vitro*, its remarkable affinity for immobilized heparin, and its ability to induce significant changes in endothelial cytosolic calcium lend solid support to, if not affirmation of, this premise [80, 83–85]. A well-defined negatively charged glycocalyx, composed primarily of sulfated glycosaminoglycans, is present on capillary endothelial cell surfaces [101]. This cell coat is largely composed of polyanionic heparan sulfate and sialic acid residues. Its primary function is thought to be the selective regulation of endothelial cell binding and transcapillary passage of macromolecules. The cationic nature of the VPF molecule at physiological pH would therefore suggest a means for direct, albeit nonspecific, electrostatic attraction of the VPF molecule to the endothelial cell surface. This in turn, would likely facilitate the subsequent binding and interaction between the active site on the VPF molecule and a specific membrane receptor.

The investigations outlined herein support an important role for VPF in the expres-

sion of peritumoral brain edema. In addition, an explanation for the efficacy of dexamethasone in the treatment of symptomatic brain edema associated with cerebral neoplasms is strongly suggested. The clinical and experimental findings are also supported by the occurrence of steroid receptors in cerebral tumors [48–54], and the lack of steroid efficacy in the setting of ischemic, toxic, hemorrhagic, and traumatic cerebral edema [55–59]. Inhibition of VPF activity by glucocorticosteroids such as dexamethasone appears to be related to highly specific actions at the cellular level (i.e., inhibition of tumor cell expression of VPF, and inhibition of VPF-induced endothelial cell calcium responses). Perhaps a better understanding of the subcellular mechanisms involved in steroid-induced VPF inhibition, will suggest either worthwhile refinements in our use of these agents, or the means to develop novel methods that are safer and more effective in the treatment of patients with neoplastic vasogenic brain edema.

### **ONGOING STUDIES OF VPF IN BRAIN TUMOR EDEMA GENESIS AND ANGIOGENESIS**

Discussions in this section have grown out of experiments carried out in the Brain Edema Research Laboratory of the Yale University School of Medicine, Section of Neurological Surgery (submitted for publication in a work authored by John G. Strugar, William N. Harrington, David Rothbart, and Gregory R. Criscuolo). Informal discussion of our preliminary findings in this work is meant only to provide current ideological insight for the reader, and is done with the mutual consent of all authors.

We are now in the process of immunostaining low grade and high grade human gliomas with purified rabbit polyclonal IgG-anti-VPF/VEGF. Our goal is to further elucidate the role of VPF in neoplastic vasogenic brain edema and tumor cyst formation. To study VPF in relation to angiogenic activity, immunostaining for von Willebrand factor, an endothelial cell surface specific antigen, is performed and vessels are examined for their number and morphological changes. Clinical correlation will then be made with respect to VPF staining, the presence and extent of peritumoral brain edema, the occurrence of fluid-filled tumor cysts, and tumor enhancement, using T2 weighted unenhanced and gadolinium enhanced T1 weighted MRI images. Preliminary results indicate that the presence or degree of VPF staining does not necessarily correlate with the tumor grade, although higher grade lesions generally stained more consistently for VPF. Peritumoral brain edema is quantitated and reported as an edema index (EI) derived from gadolinium-enhanced T1 and T2-weighted MRI images. The magnitude of the edema index does not appear to necessarily correlate with the intensity of VPF staining. A significant difference between the average EI of the positive and negative VPF groups has been noted. There is a statistically significant difference between the negative VPF group and the two positive VPF groups taken together. All tumors associated with large cysts have thus far stained positively for VPF, despite the absence of apparent peritumoral white matter edema in three of those lesions. The degree of gadolinium enhancement appeared to correlate to some extent with tumor grade in that all high grade lesions demonstrated significant enhancement, while most of the non-enhancing specimens were identified as gliosis or low grade tumors did not. It is interesting to note however, that the four low grade lesions which enhanced with gadolinium, also stained positively for VPF. These findings support previously reported observations on the consistent occurrence of neovascularity and blood-brain barrier disruption with malignant tumors. It has also been noted that certain benign CNS tumors (low grade astrocytomas, neurilemmomas, hemangioblastomas, and meningiomas) may also exhibit contrast enhancement, peritumoral edema, and tumor cyst formation. Our preliminary results suggest, therefore, that the strongest correlation appears neither to be between tumor grade and contrast enhancement, nor tumor grade

and VPF expression *per se*, but rather a correlation between the occurrence of neovascularization, edemagenesis, cyst formation, or tumor enhancement, and the expression of the VPF molecule, irrespective of tumor type or grade. High grade tumors staining positively for VPF tended to have hypertrophic, hypercellular vessels, with vascular wall thickening and proliferation. Positively staining low grade tumors exhibited a vascular morphology intermediate between the gliosis or VPF-negative low grade lesions, and the high grade tumors positive for VPF in that areas of quiescent appearing vessels were interspersed with variable numbers of hypertrophic, hypercellular vessels. Results of this investigation suggest that tumor grade in itself does not necessarily correlate with presence or absence of VPF. The occurrence of peritumoral edema or tumor cysts appear to correlate very well however, with presence of VPF expression.

The inclusion of tumor cysts as a form of edema or more accurately, an epiphenomenon of microvascular extravasation is a relatively new concept deserving of some discussion. Tumor cysts, unlike non-neoplastic intracranial cysts such as arachnoid or leptomeningeal cysts which contain mainly CSF, have a large number of endogenous cerebral as well as vasogenic proteins within their fluid. Among the endogenous cerebral proteins present are GFAP, S-100 protein, and  $\beta$ -endorphin [143–146]. However, in tumor cysts, it appears that these endogenous proteins represent a minority, whereas greater than 92% of tumor cyst fluid protein is plasma derived [146]. The implication of this study is that an increase in the permeability of the BBB takes place, allowing passage of plasma proteins into the cystic fluid. Furthermore, an electron micrograph examination of a cystic grade II-III glioma revealed endothelial fenestrations in the capillaries lining the tumor cyst identical to those present in patients with vasogenic cerebral edema associated with solid tumors [146]. These data strongly suggests that both cerebral edema and tumor cysts share a common pathway at the endothelial level, and that they may indeed represent dramatically different expressions of the same pathophysiology. All cystic lesions examined thus far were associated with VPF staining, regardless of tumor grade. These preliminary data appear to lend support to the view that tumor cysts and vasogenic edema share a common pathophysiology involving VPF, angiogenesis and microvascular extravasation.

Our primary aim is to examine the relationship between presence of VPF and vasogenic brain edema. However, we are also studying angiogenic manifestations in our brain tumor specimens by looking at the number and morphology of tumor vessels. There is extensive documentation on the angiogenic effects of VPF/VEGF. Connolly et al. [81] has shown that VPF induces endothelial cellular proliferation *in vitro*, and angiogenesis when administered into rabbit bone grafts and corneas *in vivo*. Microvessels are inadequately visualized with routine hematoxylin and eosin staining as they are mostly collapsed in pathological specimens. Staining for factor VIII, however, takes advantage of the fact that endothelial cells uniformly express this factor and thus enhancing our assessment of microvessels. This technique was used by Weidner et al. [147] who found a significant correlation between vessel number, in staining breast cancer specimens, and potential for metastasis. An increased number of vessels counted correlated positively with a greater potential for metastasis. We are using a similar method to evaluate angiogenesis in our brain tumor specimens. Preliminary results fail to indicate any correlation between vessel number *per se*, and tumor grade, VPF staining, edema, or degree of enhancement. Several possible explanations for this result have been proposed. One is the infiltrating nature of primary gliomas which invade surrounding tissue rather than displace it. The tissue which the tumors invade is among the most abundantly vascularized in the body, thus an infiltrating tumor would encounter nutrient supplies at frequent intervals as it advances. This supply could support a fairly high rate of growth. When that

growth becomes exuberant, as in highly malignant gliomas, in the absence of adequate angiogenesis, necrosis occurs. Secondly, angiogenesis and malignancy may not always correlate. There are multiple examples of angiogenesis being present prior to the onset of malignancy [148–150]. Alternatively, angiogenesis has also been reported to occur after neoplasia has clearly developed [151]. However even if angiogenic forces are present, it is unknown how they manifest themselves in brain tumors. Weidner found that in breast cancer, metastatic potential correlated with an actual higher number of vessels.

In brain tumors, which have very low metastatic potential, tumor aggressiveness may not be manifested by an actual increase in the number of vessels, but rather as a change in vessel morphology. The data we have presented are not conclusive, yet we can confidently report the observation that tumor vessels associated with VPF positive tumors appeared morphologically altered as compared to the vessels in VPF negative tumors. Vessel wall thickening and tortuosity, both features of our VPF positive samples, correlate with the experimental findings that VPF/VEGF induce increase in vascular permeability, extravasation of fibrinogen and a formation of a fibrin rich perivascular area. Finally, as mentioned earlier in the discussion, several types of VPF/VEGFs have been isolated, each with somewhat different physiological activities. For instance, of the four types of VEGFs isolated by expression cloning from a human cDNA library, two (VEGF-189 and VEGF-206) were found to exhibit significantly more potency in inducing permeability in the Miles assay while demonstrating a markedly reduced mitogenic activity in comparison to the other forms [100]. In specimens reviewed for this report, the finding that permeability and morphological changes predominate, while mitogenesis does not, may be determined by the type of VPF expressed. The data suggest that tumors exhibiting vasogenic edema appear to express VPF.

Another avenue of research has been directed toward the suppression of tumor growth through angiogenesis inhibition. A variety of hypervascular human tumors including schwannomas, neurofibromas, and neurofibrosarcomas have been examined *in vivo* by xenograft implantation into athymic nude mice. In one study employing neurofibrosarcoma xenografts, the effect of heparin and cortisone on tumor growth and neovascularization was studied. The results were interesting in that heparin administration alone stimulated angiogenesis and resulted in tumor growth greater than in the control group. Hydrocortisone alone had a minimal effect on angiogenesis and caused a minimal reduction in tumor growth. Notably, heparin and cortisone administered together resulted in a significant inhibition of both angiogenesis and tumor growth. [152] Tumor growth and angiogenesis stimulation observed with heparin is interesting in view of *in vitro* studies demonstrating that VEGF binding to its cell surface receptors was dependent upon cell surface-associated heparin-like molecules. Furthermore, VEGF receptor expression and detection was facilitated by exogenous heparin administration [128]. The absence of angiogenesis and tumor growth inhibition when hydrocortisone alone was administered is in keeping with global clinical experience indicating a specific action by the glucocorticoid dexamethasone on edema in the absence of tumor growth inhibition. A mechanism for the synergistic effect of heparin and hydrocortisone against angiogenesis and tumor growth has yet to be determined. A recent study employing AGM-1470, a potent, fungal-derived inhibitor of angiogenesis, demonstrated the suppression of neovascularization and tumor growth of benign and malignant human Schwann cell tumors implanted into the subrenal capsule of athymic nude mice. Although the molecular mechanism by which AGM-1470 acts is poorly understood, a hypothetical action on basic fibroblast growth factor (basic FGF) was proposed [153]. The mechanisms involved in angiogenesis modulation are likely to be highly complex. The role of edemagenesis and angiogenesis inhibitors relative to VPF/VEGF expression and action remains to be deciphered.

### CLOSING REMARKS

Patients with brain tumors can enjoy an extended functional survival relating largely to aggressive surgical excision, radiotherapy, and chemotherapy. Certain brain tumors are considered histologically and biologically benign, and therefore ostensibly curable. Nevertheless, significant perioperative neurological morbidity can be associated with surgical excision of these lesions. This largely relates to the occurrence of vasogenic brain edema. One of the most significant therapeutic advances in neuro-oncology in the past three decades has been the recognition of the efficacy of corticosteroids in the symptomatic treatment of patients with brain tumors. Their salutary effects on intracranial hypertension have resulted in better tolerance of both surgical and radiation therapies. However, the extended use of high-dose corticosteroids is fraught with potential complications. Consequences of their long-term use include sodium and water retention, hypertension, diabetes mellitus, sepsis related to impaired immune function, connective tissue alterations, gastrointestinal ulceration, aseptic necrosis of the femur head, and altered mentation including mania, psychotic depression, and euphoria. Investigation into the precise mechanism by which steroids act to lessen peritumoral cerebral edema may eventually provide clinicians with safer and more effective therapeutic alternatives for patients afflicted with brain tumors. Ongoing investigations will continue to focus on the interrelationships between glucocorticoids, VPF/VEGF, and microvascular endothelial cells.

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