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Immunohistochemical demonstration of serum proteins in human cerebral gliomas*

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Summary. The leakage of different serum proteins, including immunoglobulins, into human cerebral gliomas was studied by use of the unlabeled peroxidase-antiperoxidase (PAP) method on cryostat and paraffin sections. Our series of 50 tumour biopsies included 21 isomorphic astrocytomas and oligodendrogliomas (grade II), 19 anaplastic astrocytomas and oligodendrogliomas (grade III), and 10 glioblastomas (grade IV). The immunohistochemical staining of the serum proteins was similar on paraffin and cryostat sections and graded with respect to occurrence, distribution, and intensity. Serum proteins of a small hydrodynamic radius with a low serum concentration (prealbumin) or with a high serum concentration (albumin) were diffusely present in the interstitial spaces of all glioma types. Serum proteins with a medium molecular size and variable serum concentrations, i.e. IgG, IgA, and ceruloplasmin, were detected preferentially in anaplastic gliomas and in glioblastomas (grade III and IV) displaying comparable distribution patterns but different intensities. Alpha-2-macroglobulin a serum protein with a large hydrodynamic radius was also demonstrated in grade III and IV gliomas, whereas IgM and beta-lipoprotein being the largest serum proteins tested were almost restricted to blood vessels and tumour necroses. In addition, most serum proteins occurred with high intensities in those areas of isomorphic grade II gliomas that showed a macro- or microcystic or mucinous tissue degeneration. The varying immunohistochemical staining results for the serum proteins studied indicate that the blood-brain barrier within isomorphic and anaplastic gliomas is not completely disturbed. It appears that the vascular permeability is preferentially increased for small-sized serum proteins, whereas the leakage of larger serum proteins into the glioma interstitium seems to depend on the tumour type and on increasing malignancy.

Key words: Human cerebral gliomas – Peroxidase-antiperoxidase-immunohistochemistry – Serum proteins – Blood-brain barrier

Glioma growth is characteristically accompanied by vasogenic brain edema that has been attributed to disturbances of the blood-brain barrier (BBB). However, it is well known that the edema is not restricted to the brain tumour itself but also extends, as peritumourous edema, into the adjacent brain tissue. Since is was shown by electron microscopy that the small blood vessels, particularly, within malignant brain tumours exhibit various ultrastructural abnormalities of the endothelial cell lining and the endothelial basal lamina in comparison to blood vessels of normal human brain (Long 1970; Poon et al. 1971; Weller et al. 1977), changes of the BBB within primary brain tumours were postulated to result from structural changes of the tumour blood vessels. It is, therefore, mandatory to investigate the permeability of the glioma vasculature for serum components systematically.

In experimental brain tumours tracer studies by horseradish peroxidase revealed that there are regional variations in the vascular permeability (Groothius et al. 1982). Our interest in this study was to investigate the occurrence and distribution of serum proteins in human cerebral gliomas by use of the unlabeled peroxidase-antiperoxidase (PAP) method, since it has recently been shown that immunohistochemistry is effective for the visualization of serum protein extravasation across the altered BBB in ex-

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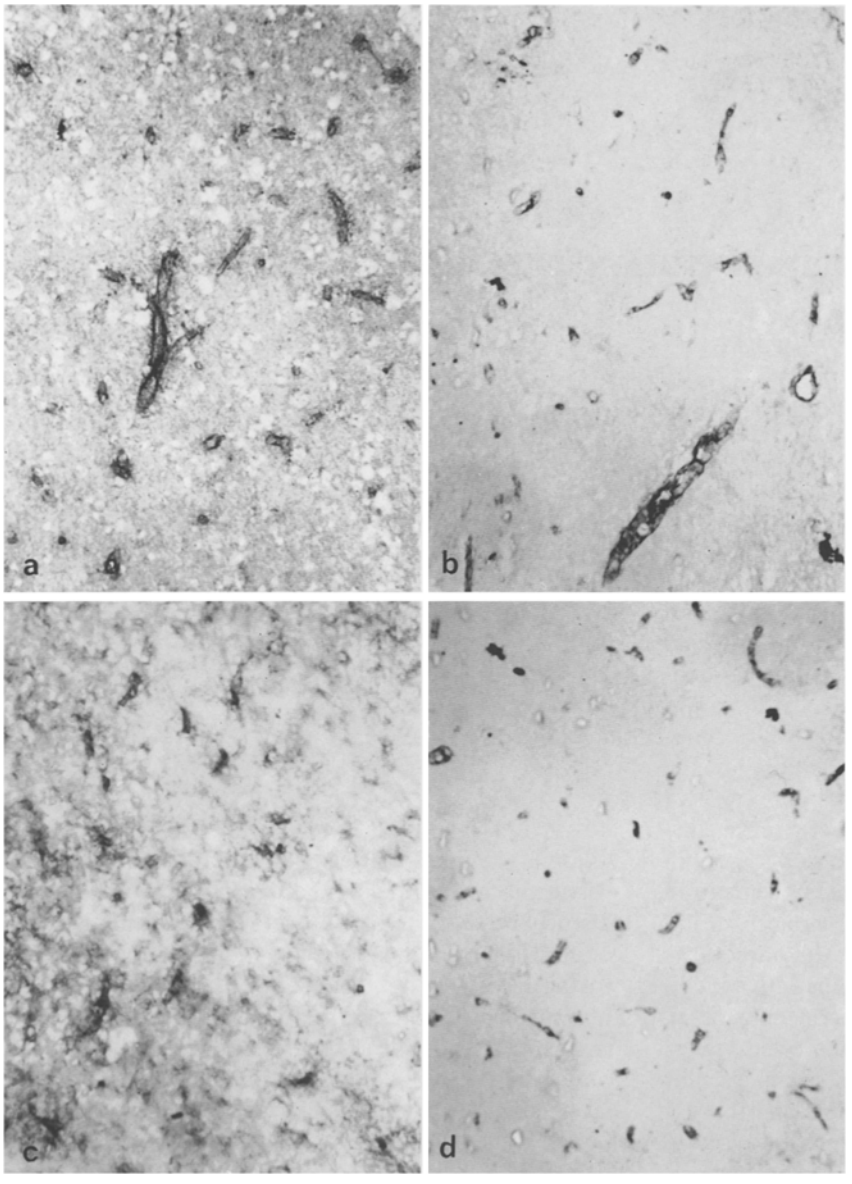

Fig. 1 a–d. Comparison of the immunohistochemical demonstration of serum proteins in human cerebral gliomas on cryostat and paraffin sections. Restriction of IgG to the blood vessels in a differentiated astrocytoma (grade II), cryostat section (**a**), paraffin section (**b**). Strongly stained blood vessels in the negative tissue of an anaplastic astrocytoma (grade III), anti-IgM; cryostat section (**c**), paraffin section (**d**). **a–d** no counterstain, × 100

perimental infarcts and hypertensive encephalopathy (Nag 1984; Barbosa-Coutinho et al. 1985).

In evaluating the immunohistochemical staining of serum proteins in formalin-fixed samples of human brain tumours, however, artificial alterations of the BBB, due to tumour resection and due to diffusion of the serum proteins from their in-situ location before fixation is complete, have to be taken into consideration. To avoid such artefacts in-vivo perfusion with fixatives as appliable in animal experiments or snap frozen tissue should, therefore, ideally be used. Since these strict conditions cannot be met in clinical neurosurgery, we used tumour samples after routine tissue processing and cryostat material of selected cases to invstigate the occurrence and distribution of a panel of serum proteins that represent indicators for the BBB function in clinical biochemistry (Felgenhauer 1974). In spite of the above out-lined limitations our findings show that the leakage of serum proteins into gliomas is predominantly increased for serum proteins of small molecular size, but varies for larger serum proteins as indicated in preliminary notes (Seitz and Wechsler 1984, 1985).

Materials and methods

Fifty cerebral gliomas of various types, grades and location were studied. The tumour samples were fixed in 4% buffered formaldehyde for 24 h and embedded in paraffin. In addition, material, which had been immediately frozen in −70° C

Table 1. Regional immunohistochemical staining intensities of serum proteins in the glioma interstitium

Serum proteins		P-Alb	Alb	Cer	IgG	IgA	A2M	IgM	BLP	FN
Molecular weight ($\times 10^3$)		61	69	140	140	160	850	900	2,500	450
Hydrodynamic radius (Felgenhauer 1974)		32.5	35.8	46.8	53.4	56.8	93.5	121.0	124.0	n.d.
Serum concentration (g/l)		0.1–0.5	35–55	0.3–0.5	13–22	0.8–2.8	1.5–4.5	0.6–1.7	4.0–14	0.3–0.49
Astrocytoma (grade II)	solid	1	4	0	0	0	0	0	0	0
	cystic	1	4	1	2–3	2–3	1–2	1	1	0
Oligodendroglioma (grade II)	solid	1	4	0	0	0	0	0	0	0
	mucinous	1	4	1	2–3	2–3	1–2	1	1–2	0
Astrocytoma (grade III)		1	4	0–1	0–3	0–3	0–2	0	0	0
Oligodendroglioma (grade III)		1	4	0–1	0–3	0–3	0–2	0	0	0
Glioblastoma		1	4	1	2–3	2–3	1–2	0–1	0	0

P-Alb: Prealbumin; Alb: albumin; Cer: ceruloplasmin; A2M: alpha-2-macroglubulin; BLP: beta-lipoprotein; FN: fibronectin; n.d.: not determined
Tumour regions, solid: vital glioma tissue; cystic (i.e. microcystic and macrocystic) and mucinous: glioma degeneration
Regional staining intensity, 4: strong; 3: moderate; 2: weak; 1: faint staining; 0: no detectable staining

isobutane, of eight selective tumours with different grades of malignancy was used. The tumours were classified according to WHO (1975) and graded as grade II astrocytomas (9 cases), grade II oligodendrogliomas (12 cases), anaplastic astrocytomas of grade III (9 cases), anaplastic oligodendrogliomas of grade III (10 cases), and glioblastomas of grade IV (10 cases).

Six-micrometer serial sections were immunohistochemically stained for nine different serum proteins by the PAP method (Sternberger et al. 1970). In a preceeding study other sections from the same material had been cytochemically stained with *ulex europaeus* I lectin (UEA I) for morphometric measurements of the glioma microvascularization (Seitz and Wechsler 1986). In the present study we used polyclonal antibodies produced in rabbits against human prealbumin, albumin, IgG, IgA, ceruloplasmin, alpha-2-macroglobulin, IgM, fibronectin, and beta-lipoprotein (Dakopatts, Copenhagn, Denmark). Paraffin sections were floated on slides that had been covered with poly-L-lysine (Sigma Chemicals, Munich, FRG), dried at 60°C for 48 h, and sequentially pretreated with 0.3% H_2O_2 and 0.0125% trypsin (Sigma) for 40 min at room temperature. Cryostat sections were fixed with 4% formaldehyde for 15 min before a 30-min treatment with 0.3% H_2O_2. In addition, the sections were preinubated for 20 min with 1 mg normal mouse IgG (Sigma), that was dissoved in 200 ml of a 5% bovine serum albumin (Sigma)/phosphate-buffered saline solution, pH 7.6 (PBSA) to block unspecific binding by Fc-receptors. Dilutions of the primary antibodies in PBSA (prealbumin 1:1000, albumin 1:10000, igG 1:10000, IgA 1:5000, ceruloplasmin 1:500, alpha-2-macroglobulin 1:2000, IgM 1:5000, fibronectin 1:2000, beta-lipoprotein 1:500) were applied for 3 h at room temperature. Subsequently, sections were incubated for 30 min at room temperature with a secondary swine anti-rabbit antibody (Dakopatts) diluted 1:50 in PBSA and thereafter with a rabbit PAP-complex (Dakopatts) diluted 1:200 in PBSA. For staining 3,3′diaminobenzidine (Sigma) was used. Most sections were counterstained with hematoxylin, while some were left unstained for Nomarski-microscopy. The staining results were compared to that in normal brain samples of a similar age group. Controls were performed by omission of the primary antibodies.

Results

The staining patterns for the serum proteins within the gliomas and in the peritumourous edema zone contrasted to normal brain. In normal brain no staining for serum proteins and immunoglobulins was observed in the grey and white matter. Here, the serum proteins were detected only in the leptomeninges and inside blood vessels with high intensities. In the peritumourous brain edema zones, however, interstitial staining of small-sized serum proteins, i.e. prealbumin and albumin, was more prominent in the white mater than in the cerebral cortex and faded with increasing distance to the brain tumour. As shown in Fig. 1, paraffin and cryostat sections exhibited corresponding results for the individual serum proteins in different glioma types. Whereas the staining patterns for serum proteins and immunoglobulins within vital glioma tissue varied in distribution and intensity with relation to the hydrodynamic radius (HR, Felgenhauer 1974) and the serum concentration (SC) of the individual proteins, tumour necroses and

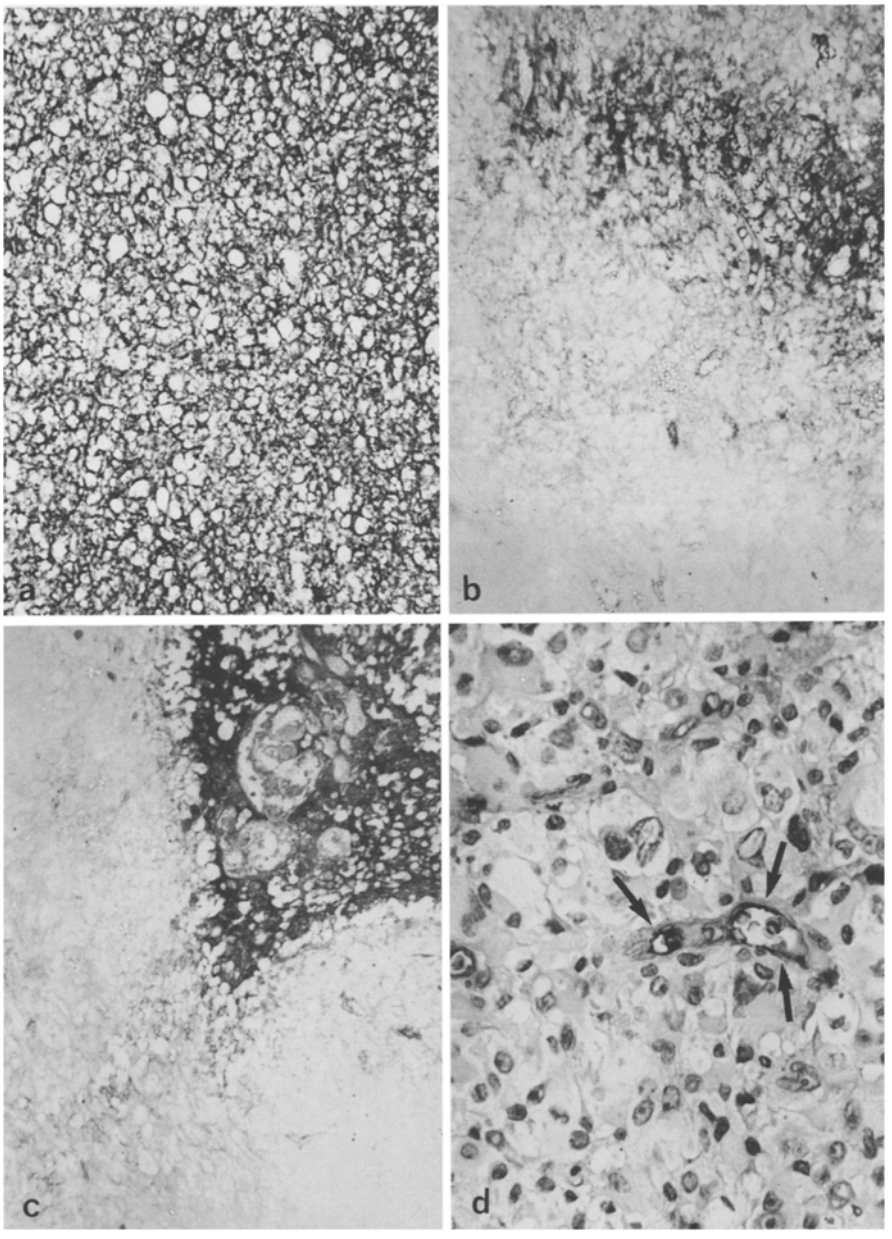

Fig. 2a–d. Immunohistochemical staining patterns of serum proteins in human cerebral gliomas. **a** Ubiquitous demonstration of albumin in the interstitium of an isomorphic oligodendroglioma (grade II), the tumour cells themselves are negative. No counterstain, ×144. **b** Variable interstitial staining intensity for ceruloplasmin in an anaplastic astrocytoma (grade III). No counterstain, ×90. **c** Strong staining for IgM in a tumour necrosis of a glioblastoma multiforme (grade IV), while the vital tumour tissue is not stained. No counterstain, ×90. **d** Glioblastoma with immunohistochemical reaction for beta-lipoprotein. Restriction of the staining products to the luminal site of a tumour vessel (*arrows*). Tumour interstitium and tumour cells are negative. ×465

tumor vessels always exhibited a strong staining for all serum proteins. For evaluation, the immunohistochemical staining in vital glioma tissue was graded semiquantitatively with respect to distribution and intensity. The results are summarized in order of increasing molecular size of the proteins in Table 1.

Prealbumin and albumin

Prealbumin (HR 32.5) and albumin (HR 35.8) occurred diffusely in the interstitium of all tumours, however with different intensities. While albumin (SC 35–55 g/l) was demonstrated with high intensity (Fig. 2a), prealbumin (SC 0.1–0.5 g/l) exhibited only a faint staining (Table 1).

IgG, IgA and ceruloplasmin

Staining for IgG (HR 53.4, SC 13–22 g/l) and IgA (HR 56.8, SC 0.8–2.8 g/l) was fairly strong but of less intensity than for albumin (Table 1).

Ceruloplasmin, a serum protein of similar molecular size (HR 46.8) but of markedly lower serum concentration (0.3–0.5 g/l), showed a distribution comparable to those of IgG and IgA, although the staining intensity was clearly weaker (Table 1). All these serum

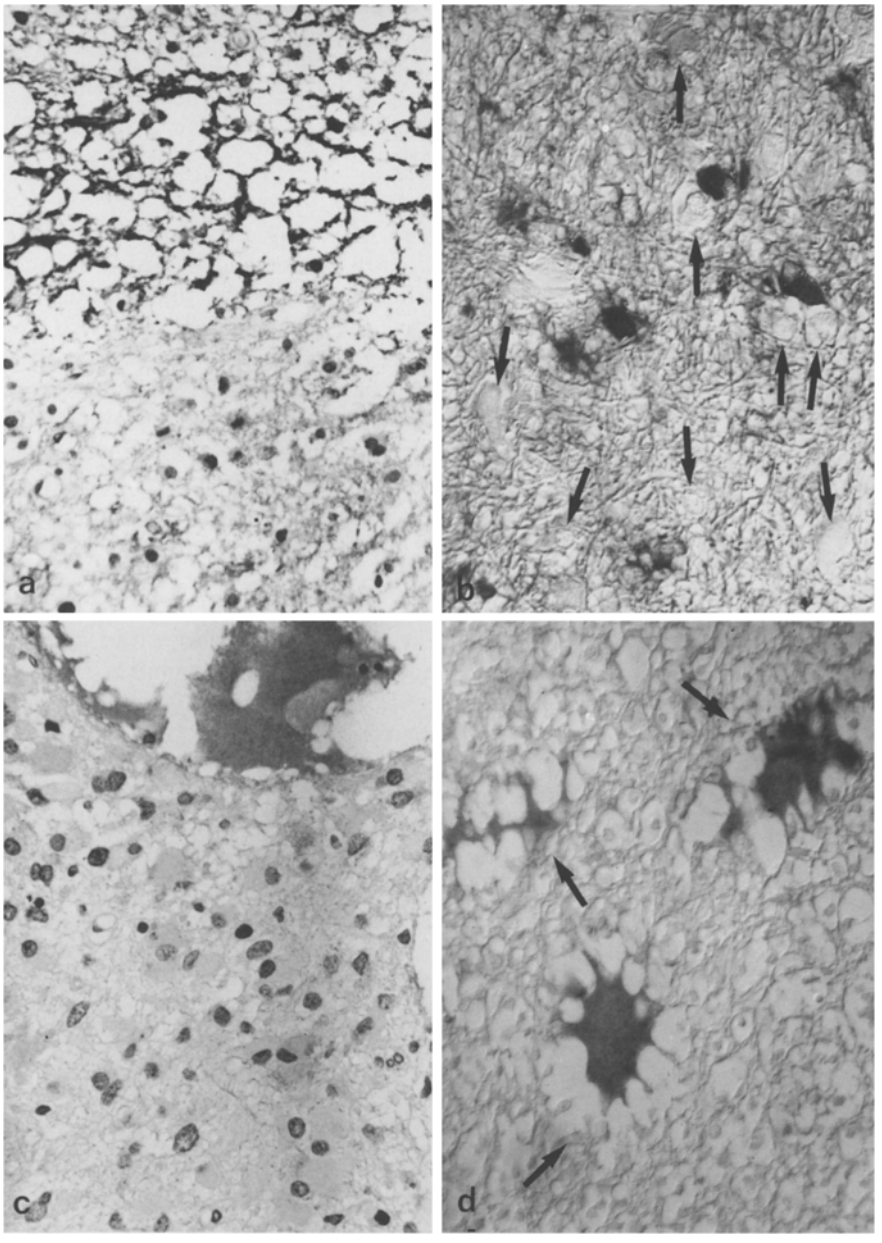

Fig. 3a–d. Localization of serum proteins in areas of tumour degeneration in grade II gliomas. **a** Strong staining for IgA in a cystic astrocytoma, while the adjacent solid tumour portions are negative (*bottom*). ×144. **b** Staining for IgG in an area of microcystic degeneration in an astrocytoma. Diffuse interstitial staining of moderate intensity. Strong staining of the cytoplasm of individual astrocytes indicating cellular up-take of protein from the interstitial space. Similar looking adjacent astrocytes are not stained (*arrows*). Nomarski, ×225. **c** Aspect of a macrocyst (*top*) in an astrocytoma. Staining of fluid remnants of the cyst for alpha-2-macroglobulin. ×360. **d** Three interstitial lakes of plasma exsudation (*arrows*) with reactivity for beta-lipoprotein in an oligodendroglioma. Nomarski, ×225

proteins were not detectable in solid glioma areas of grade II gliomas. They were, however, detected in the vicinity of perivascular lymphocytic infiltrates and, in particular, in areas with degenerative alterations. In those microcystic or macrocystic areas of astrocytomas the surrounding glioma tissue was diffusely stained, while adjoining tumour parts were negative (Fig. 3a). Occasionally, the proteinaceous fluid within such cysts had been preserved during tissue processing and also gave a strong immunohistochemical staining. Moreover, these serum proteins were also detected in areas of mucinous change in oligodendrogliomas, that are histologically characterized by interstitial metachromatic plasma exsudations. The cytoplasm of neoplastic oligodendrocytes, however, remained consistently negative.

In anaplastic gliomas (grade III and IV) the distribution and intensity of the immunohistochemical staining products for IgG, IgA and ceruloplasmin varied regionally. While solid tumour areas with densely packed glioma cells remained negative, intensive staining was observed in highly anaplastic tumour areas consisting of polymorphic glioma cells (Fig. 2b). Also the perivascular glioma parts around tortuous glioma vessels were positively stained.

Regularly, in astrocytomas of grade II and III, with interstitial staining for IgG, IgA and ceruloplasmin, numerous gemistocytic astrocytes including their cell

processes were strongly stained, while adjacent similar looking astrocytes were negative (Fig. 3b). Staining of multinuclear cells in anaplastic astrocytomas or glioblastomas was not observed.

Alpha-2-macroglobulin

Alpha-2-macroglobulin (HR 93.5, SC 1.3–4.5 g/l) was detected in vital tumour tissue of grade III and IV gliomas with low intensity. It was observed in the tumour interstitium around large tumour vessels and close to necroses. In contrast, it was almost absent in gliomas of grade II (Table 1). Only in those areas of isomorphic gliomas (grade II) with degenerative changes was a slight staining apparent. Its distribution was similar to those of IgG, IgA, and ceruloplasmin (Fig. 3c).

IgM and beta-lipoprotein

IgM (HR 121.0, SC 0.6–1.7 g/l) was localized by strong immunoreactivity within blood vessels and tumour necroses, while vital solid tumour tissue was not stained (Fig. 2c, d; Table 1). Only around tortuous capillary proliferations, i.e. glomerulum-like formations in glioblastomas, was a faint interstitial staining for IgM occasionally detected. Moreover, IgM was also demonstrated in the proteinous fluid of cystic portions of grade II astrocytomas.

Similar to IgM, beta-lipoprotein, whose molecular size (HR 124.0) is a little above that of IgM, did not occur in solid tumour tissue, despite its high SC (4.0–14 g/l). However, beta-lipoprotein was detected in areas with degenerative changes of grade II gliomas in an identical distribution to IgG, IgA, ceruloplasmin, and alpha-2-macroglobulin. Whereas the staining intensity was only moderate in the interstitium and the cytoplasm of some gemistocytic astrocytes in microcystic areas of astrocytomas, a strong reaction was obvious in the intercellular mucinous plasma exsudations of oligodendrogliomas (Fig. 3d).

Fibronectin

Fibronectin is a plasma protein that occurs in a dimeric form with a total molecular weight of about 450,000, the SC ranges from 0.31 to 0.49 g/l (Mosesson and Amrani 1980; Erikson et al. 1982). Activity for fibronectin was readily detected in tumour necroses and inside blood vessels, but in the glioma interstitium or in vital glioma cells no immunoreactivity occurred. Fibronectin was even not observed in degenerative areas of grade II gliomas that were stained for other serum proteins. In contrast, endothelial cells of arteries, veins and small blood vessels were intensely stained in all gliomas, corresponding to comparable immunohistochemical findings by Kochi et al. (1983).

Discussion

The concept of the BBB was elaborated in animal experiments showing that tracers, like evans-blue and horseradish peroxidase, do not enter the brain parenchyma after intravenous application (reviewed by Davson 1976; Lee 1982). In humans the presence of a BBB can easily be observed in icteric patients, who show a yellow colouration only of the choroid plexus or around a cerebral lesion, e.g. a tumour, while the brain tissue itself remains free. By immunohistochemistry it is now also possible to analyse the normal or pathologically altered BBB for serum proteins. This is of considerable physiological relevance, since the serum proteins are constantly present in the blood circulation and thereby serve as endogenous barrier tracers (Felgenhauer et al. 1976; Felgenhauer 1980).

Using a panel of nine antibodies against nine different serum proteins of varying molecular weight or HR (Felgenhauer 1974), we have shown that the blood-tissue barrier in human cerebral gliomas is not completely broken down. In all gliomas serum proteins of low molecular size like prealbumin and albumin were present within the tumour interstitium. Serum proteins of larger molecular size, however, occurred only in the interstitium of anaplastic gliomas or in areas of isomorphic gliomas with degenerative changes. Although immunohistochemistry cannot distinguish between proteins that are synthesized in situ, such as immunoglobulins and fibronectin, our findings indicate that the occurrence of the serum proteins within the various gliomas is related to the molecular size of the individual serum proteins. Since Felgenhauer (1974) demonstrated that the vascular permeability for serum proteins at human fluid barriers is a function of the steric configuration of the proteins, which he defined as HR, our immunohistochemical results (Table 1), particularly the absence of the large-sized IgM and of the dimeric fibronectin from tumour interstitium even of malignant gliomas, are likely to point out that Felgenhauer's concept holds true even for the pathological blood-tissue barrier in human gliomas. In constrast, the SC of the proteins seemed to be of minor importance for the vascular permeability, since the staining patterns for the individual proteins did not show a correlation to their SC. Rather the SC appeared to influence the amount of leakage across the altered barrier in that staining was intense for those proteins with a high SC and weaker for those with a lower SC. For

methodological reasons, however, our qualitative immunohistochemical staining results can provide only a semiquantitative scale.

Our findings are paralleled by biochemical studies showing that albumin represents the major proteinous constituent of tumour-induced brain edema (Cumings 1961; Manz 1974). Since particularly the highly capillarized oligodendrogliomas of grade II (Seitz and Wechsler 1986) do not exhibit an increased staining for serum proteins, these newly formed tumour capillaries apparantly already possess a low permeability. This observation is of great interest, since it was shown by quantitative autoradiographic studies on experimental brain tumours that capillary permeability and capillary surface area are the most important factors that determine the blood-to-tissue transport in malignant gliomas (Blasberg 1980; Blasberg et al. 1983). It is suggested that our immunohistochemical results stress the relevance of the capillary permeability for the altered BBB function in human gliomas.

As shown by electron microscopy, tumour blood vessels exhibit marked abnormalities compared to blood vessels in normal brain. While in normal brain the endothelial cells of the cerebral vessels possess specialized biochemical and morphological properties to maintain the integrity of the BBB (Spatz and Mrsula 1982; Goldstein and Betz 1983), the blood vessels in brain-tumours are permeable for serum constituents, because they exhibit fenestrations and numerous pinocytotic vesicles of the endothelium, widened intercellular junctions between the endothelial cells, dicontinuities of the endothelial basal lamina, and lack of enveloping astroglial feet (Long 1970; Hirano and Matsui 1975; Weller et al. 1977). According to Weller et al. (1977), however, only 20% of the vessels in human brain tumours are characterized by these morphological abnormalities. Possibly, these partial and variable changes may be correlated to the variable degree of BBB disturbances for serum proteins in gliomas as evident from our study. Corresponding observations have also been made in experimental gliomas (Groothius et al. 1982; Blasberg et al. 1983; Nishio et al. 1983) and in a metastatic brain tumour model (Blasberg et al. 1984), in which horseradish peroxidase and alpha-aminobutyric acid were used as tracers. In human brain tumours studies on the BBB changes have so far only been based on CT-enhancement or on preoperative applications of fluorescent sodium and radio-labelled albumin (Raimondi 1964; Ginsbourg et al. 1973). Only Lilja et al. (1981) and Burger et al. (1983) correlated the intravital appearance of brain tumours and the brain edema by CT-scanning to neuropathological examination. In an immunofluorescent study on brain tumour biopsies Szymaś and Hossmann (1984) recently observed, similar to our results, the interstitial occurrence of serum proteins in various anaplastic gliomas. These authors, however, did not apply their technique to grade II gliomas. A PAP study by Brett and Weller (1978) was focussed on the demonstration of immunoglobulin IgA, IgG, IgM, and albumin in the intracellular compartment of gliomas and showed, identical to our results, a staining of individual astrocytes beside adjacent astrocytes that remained unstained. Possibly, this phenomenon is a result of cellular up-take by reactive astrocytes that was ultrastructurally demonstrated by Raimondi (1964). Thus, this vital event can apparently be visualized by immunohistochemistry and may be helpful in the crucial differentiation between tumourous and reactive astrocytes.

With respect to human glioma biology, the knowledge about the existence of a blood-tissue barrier in human cerebral gliomas, as demonstrated in the present study, is probably of considerable concern for diagnostic and therapeutic procedures that are based on the systemic application of radio-labelled protein tracers such as iodinated albumin, IgG, or Fab-fragments of IgG (Long et al. 1963; Vick et al. 1977; Order 1982). Further work is necessary to determine the in- and outflux rate of serum proteins through the altered BBB in human gliomas.

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