Immunochemical Analysis of Glial Fibrillary Acidic Protein as a Tool to Assess Astroglial Reaction in Experimental C6 Glioma

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In experiments on Wistar rats with experimental C6 glioma, the immunohistochemical features of the astroglial reaction over 30 days after implantation were characterized. The formation of a glial border consisting of GFAP-positive reactive astrocytes at the periphery of C6 glioma was observed on postimplantation day 3 and until the death of the experimental animals. Reactive astrocytes encompassed not only the primary gliomal focus, but all tumor invasion foci. Quantitative assessment of astroglial reaction around glioma was carried out with immunofluorescent assay of glial fibrillary acidic protein (GFAP) on cerebral sections. The size of glioma and necrotic foci were analyzed morphometrically in parallel with enzyme immunoassay of serum GFAP. A correlation between morphometric indices of glioma and serum level of GFAP was found. It was concluded that serum concentration of GFAP correlated with the size of intracranial glioma, necrotic foci, and most strongly with the degree of reactive astrogliosis. Monitoring of the level of serum GFAP can serve as an additional diagnostic index reporting the state of intracranial glioma.

Key Words: *glial fibrillary acidic protein (GFAP); glioma C6; enzyme immunoassay; reactive astrogliosis*

Astrocyte is an important structural and functional unit of the blood-brain barrier [8,10]. These cells amounting to about $^1/_4$ all glial cells are the first to react to damage to the nervous tissue by active migration into the region of the pathological focus encompassing it with an astroglial border [14,20]. This universal defensive reaction accompanies almost all pathological processes in the brain. However, in some cases (specifically, during glioblastoma multiforme) reactive astrocytes do not limit the tumor, but even promote its growth due to enhanced expression of cell adhesion proteins stimulating invasion of glioma cells [11-13].

An important role in reactive activation of astroglial cells is played by glial fibrillary acidic protein (GFAP), a basic structural element of astrocytic intermediate filaments. It is currently accepted that GFAP stabilizes cytoskeleton of astrocytes by promoting interaction of filaments with nuclear and plasma membranes, thereby participating in the realization of structural-dynamic relations between the genetic apparatus and extranuclear structures [8-10,16,17].

During reactive activation of the astrocytes, the content of GFAP in these cells dramatically increases [4,5,7,8,15], which is accompanied by more intensive release of this protein into peripheral circulation. This peculiarity underlies the use of blood concentration of GFAP for evaluation of the degree of pathological

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alterations in CNS and even for prediction of various critical states [5,15,16].

Elevation of serum GFAP accompanying the development of low-differentiated tumor was previously erroneously explained by production of this protein by tumor cells [1,10]. Subsequent experiments showed that low-differentiated glioma cells *per se* do not contain GFAP [5,7,10,14,15]. Despite impressive number of papers examining the relationships between GFAP and morphometric indices of glioblastomas [5,7,8,15,16], there are no published reports on the dependence of serum GFAP on the intensity of astroglial reaction.

Our aim was to examine interrelations between the data on GFAP blood monitoring and morphometric indices of the peritumoral astroglial border obtained by immunofluorescent analysis during the development of experimental C6 glioma.

MATERIALS AND METHODS

The experiments were carried out on mature female Wistar rats initially weighing 200-220 g (n=80). C6 glioma cell were prepared for implantation as described elsewhere [2]. Glioma cells preparations (5×10^5 per rat) were implanted into the striatum under ketamine narcosis using a Narishige stereotaxic apparatus and Swanson rat brain atlas coordinates: Ap -1, L +3.0, V +4.5, and TBS -2.4 mm. Injection of about 10 µl cell preparation was performed at the rate of 3 µl/min with a microsyringe and an infusomat.

Histological examinations were performed on postimplantation days 3, 8, 15, 21, and 28. The rats were deeply narcotized and perfused transaortally with 4% paraformaldehyde. The brain was isolated and serial sections (40 µ) were prepared on a freezing microtome (Rechert). To perform general morphological analysis, the sections were stained by the Nissl method with 0.1% cresyl violet in 0.1 M acetate buffer (pH 3.3) and 0.1% toluidine in the same buffer.

For visualization of necrotic foci in the tumor, the sections were stained with combination of toluidine and 0.1% vanadium acid fuchsin by the method of I. V. Victorov [18].

Immunohistochemical assay of C6 glioma preparations was performed with monoclonal anti-GFAP antibodies (Department of Immunochemistry, V. P. Serbsky National Research Centre for Social and Forensic Psychiatry) [2,3]. The examination was carried out from postimplantation day 3 until animal death caused by intracranial dislocation. Immunoperoxidase and immunofluorescent development of the antigen was performed with secondary antispecies antibodies according to manufacturer's protocol with our modification [3]. The specimens were examined under a fluorescent microscope (Leica MS) and a TSC SP2 scanning laser confocal microscope (Leica MS) in N. K. Kol'tsov Institute of Developmental Biology (E. B. Tsitrin).

Morphometric analysis of glioma volume and necrotic foci was performed according to the previously published method [19] in our modification on sections stained after Nissl (measurement of glioma volume) with vanadium acid fuchsin and toluidine (measurement of the volume of necrotic foci) and then analyzed under a stereomicroscope equipped with a digital camera. The photos were digitized to measure the area of glioma or necrotic foci in mm^2. These values were multiplied by the thickness of the section and finally summed through all sections with the examined structure.

Detection of serum GFAP was performed with sandwich ELISA according to A. Voller *et al*. [2]. Preliminary, the blood was drawn from all rats with C6 glioma for GFAP assay performed in a test system based on monoclonal anti-GFAP antibodies, purified polyclonal anti-GFAP antibodies, and recombinant GFAP as the standard agent. The test system sensitivity limit with recombinant GFAP as the standard was 0.4 ng/ml.

The data were processed statistically using MS Excel software, functions of descriptive statistics, and correlation analysis.

RESULTS

Morphometry of growing glioma made it possible to quantitatively assess the volume of glioma at different terms after implantation and to characterize the dynamics of necrotic foci (Fig. 1).

Two weeks after implantation, the tumor volume increased almost 20-fold (Table 1). On postimplantation day 30, the volume of C6 glioma became 883.3±17.7 mm^3 (15.2% brain volume). Since the injected volume of the implant was about 10 mm^3, the volume of intracranial C6 glioma increased by more than 80 times over 4 weeks. The overall data of glioma development attest to high take of the tumor (about 100%) and rapid progress of this type of tumor after its stereotaxic intracranial injection to Wistar rats. The data on individual tumor volumetric indices indicate pronounced reproducibility of this experimental glioma model.

Analysis of necrotic alterations revealed an increase in mortality rate of glioma cells due to hypoxia, which progressed in parallel to tumor growth. After detection of the necrotic foci, their absolute volume increased by almost 10-fold from 0.6 mm^3 on postimplantation day 3 to 61 mm^3 on days 29-30.

On postimplantation day 3, the relative necrotic volume was 3.1% of glioma volume. Probably, at the

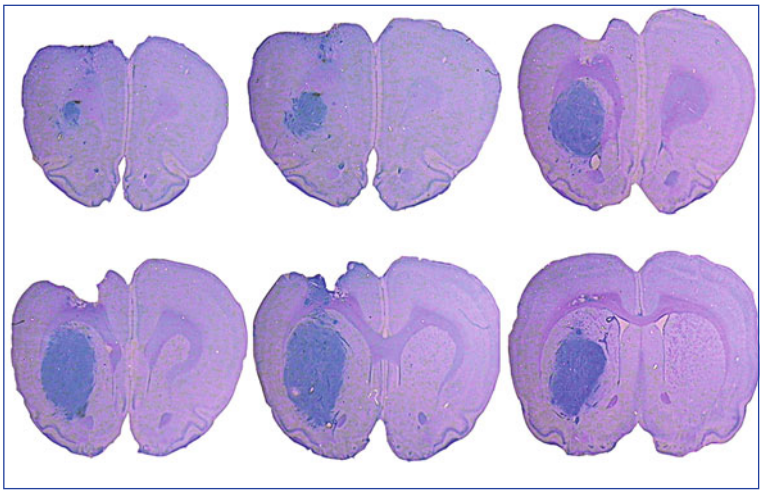

Fig. 1. Serial sections of cerebral glioma on postimplantation day 17 (tumor volume 426 mm^3). Nissl staining (×5).

early stage of tumor development, high necrotic index results from the death of a certain part of glioma cells immediately after implantation. The volume of necrotic foci was 2.5% of glioma volume on postimplantation days 14-15 and attained the maximum (about 7% glioma volume) on day 21 (Table 1).

Quantitative assessment of the volume of astrocytic border during tumor development was carried out on a significant statistical sample (n=21, Fig. 2). To this end, 8 sections were selected from the serial ones containing glioma cells that characterized various regions of the tumor (two "poles", the middle part, and the "epicenter"). These sections were incubated with rabbit polyclonal anti-GFAP antibodies and then with secondary goat anti-rabbit antibodies (Alexa Fluor 488, Invitrogen). The automatic panoramic photo of fluorescent picture was obtained at small magnification (×4) with the help of motorized microscope stage under a light microscope (Leica DM). Panoramic photomontage of astrocytic border was accomplished with a Leica Microsystems software. Photoshop SC software (Adobe) was employed in semiautomatic mode to delineate the regions of fluorescent GFAP-positive cells and to calculate the area of the astrocytic border.

TABLE 1. Morphometric Indices of C6 Glioma (M±m)

Postimplantation day	Volume, mm^3		Relative necrotic volume (%e of glioma volume)	Volume of astrocytic border, mm^3	Relative volume of astrocytic border (% of glioma volume)
	glioma	necrotic focus			
3	14.8±1.2 (n=6)	0.6±0.4 (n=6)	3.1±1.7 (n=6)	1.8±0.2 (n=4)	13.7±1.8 (n=4)
7-8	44.7±1.4 (n=9)	2.2±0.9 (n=9)	5.1±2.2 (n=9)	15.5±0.6 (n=4)	36.2±1.6 (n=4)
14-15	195.1±12.9 (n=9)	4.9±1.5 (n=9)	2.5±0.8 (n=9)	38.4±2.5 (n=4)	19.2±0.9 (n=4)
16-17	326.9±10.0 (n=11)	15.0±5.6 (n=11)	4.6±1.7 (n=11)		
20-21	426.8±21.3 (n=11)	33.9±7.8 (n=11)	7.1±1.6 (n=11)	56.6±3.8 (n=4)	13.5±0.9 (n=4)
24-25	664.9±13.9 (n=5)	40.0±10.5 (n=5)	6.1±1.6 (n=5)		
29-30	883.3±17.7 (n=8)	61.8±9.0 (n=8)	7.1±1.1 (n=8)	40.7±2.3 (n=5)	4.5±0.2 (n=5)

Note. n is number of rats.

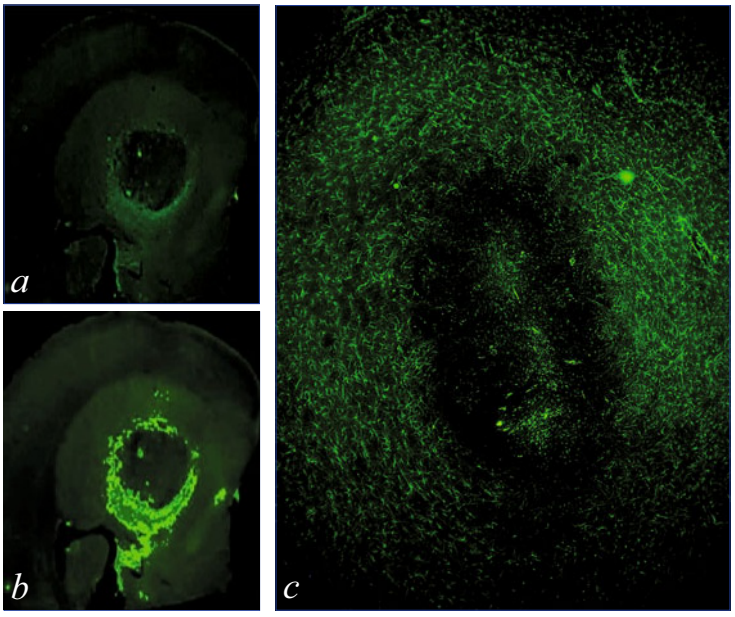

Fig. 2. Measurement of astrocytic border volume with immunofluorescent visualization with polyclonal antibodies against rabbit GFAP and secondary goat antirabbit antibodies (Alexa Fluor 488, Invitrogen). *a*) and *b*) glioma on postimplantation day 14 (×5); *b*) semiautomatic detection of the color whose intensity corresponded to fluorescence of the astroglial border in order to calculate the area of the outlined ring; *c*) glioma on postimplantation day 8 (×200).

The volume of this border was calculated as the sum of products of all areas by section thickness.

In peritumoral region, reactive astrocytes were detected on postimplantation day 3. The astrocytic border was formed by postimplantation day 7 and persisted until the terminal stage of glioma development on day 30 (Fig. 3). At the end of the experiment (postimplantation day 30), the tumor invaded almost the entire hemisphere; in that period, intensive astroglial reaction was observed in the narrow band of intact tissue along the edge of cerebral hemisphere (Fig. 3, *f*).

GFAP-positivity of glioma cells was not observed at any terms of tumor development, which agrees with the data obtained by other researchers [14,20]. The volume of astrocytic border increased to postimplantation day 21 (56.6 mm^3), thereafter the number of the reactive astrocytes decreased. On postimplantation day 30, the volume of astrocytic border was only 40.7 mm^3 (Table 1). Relative to glioma volume, the volume of astrocytic border attained the maximum on days 7-8 (36%, Table 1). At later terms, the relative volume of astrocytic border decreased to 19.2 and 13.5% on days 14-15 and 20-21, respectively (Table 1), which can be explained by dramatic increase of the tumor. On day 30 of tumor development, the relative volume of astrocytic border decreased to 4.5%.

Simultaneously with histological and immunofluorescent analysis, we measured serum concentration of GFAP.

The control level of serum GFAP was determined in intact and healthy donor rats (n=17) by repeated testing of serum samples. In our test-system, this level was 1.8±0.1 ng/ml.

Enzyme immunoassay of GFAP in 80 serum samples drawn from rats with experimental glioma showed that elevation of GFAP concentration occurred as early as on postimplantation day 3 and remained elevated during the entire period of the experiment (Table 2). On days 7-8, serum concentration of GFAP in the experimental rats surpassed the control level by more than 4 times (p<0.05). It attained the maximum on postimplantation day 21 (p<0.05) and then somewhat decreased despite glioma growth (Table 2).

Pearson correlation analysis revealed certain interrelations between serum concentration of GFAP and the examined morphometric indices (Table 3).

As expected, the age of glioma strongly correlated with the volume of tumor and necrotic foci. Weaker correlation was revealed between glioma age and the

TABLE 2. EIA Data on GFAP in Rats with Glioma

Specimen for EIA		Number of rats	GFAP concentration, ng/ml
Intact rats		17	1.8±0.1
Glioma in entire sample		83	12.1±0.4*
Glioma, postimplantation days	3	6	6.8±0.4
	7-8	17	9.9±0.6*
	14-15	17	11.5±1.3*
	16-17	11	12.5±0.6*
	20-21	11	15.2±1.4*
	24-25	13	14.1±0.4*
	29-30	8	13.7±0.6*

Note. *p<0.05 compared to the control.

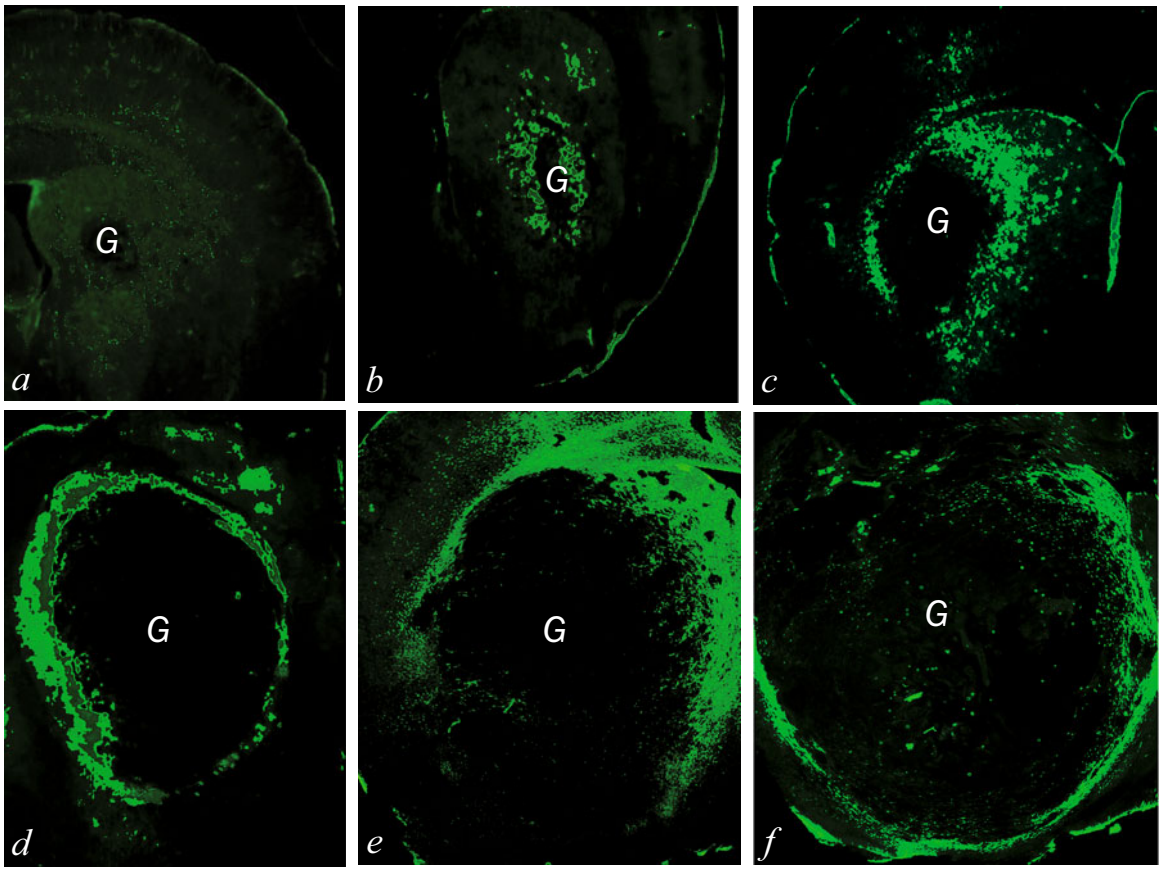

Fig. 3. Dynamics of the development of astrocytic border around C6 glioma (×4). Postimplantation days 3 (*a*), 7 (*b*), 14 (*c*), 21 (*d*), 25 (*e*), and 30 (*f*). Visualization was performed with polyclonal antibodies raised against rabbit GFAP and the secondary goat antirabbit antibodies (Alexa Fluor 488, Invitrogen). The astrocytic border was contrasted with Photoshop SC (Adobe) software. G: glioma.

volume of astrocytic border or GFAP concentration. Probably, the latter observation is related to the declining trend for GFAP serum concentration and the volume of astrocytic border at the later terms of tumor development.

The same trend explains the fact that GFAP concentration in peripheral blood most strongly correlated with morphometric indices of the astrocytic border. Despite the popular view that the intensity of GFAP elimination is predominantly determined by the degree of necrotic alterations in the tumor tissue [7,15], the correlation coefficient between serum GFAP concentration and necrotic volume was minimum. Thus, the above morphometric data and correlation analysis showed that the level of GFAP released into the blood is mainly determined by the degree of astroglial reaction in the parenchyma against the tumor.

Interrelation between serum GFAP concentration and the tumor size in CNS was recently reported [5-7,15,16]. These studies stemmed from the finding that patients with stage IV glioblastoma had higher level of GFAP in comparison with patients with more differentiated tumors. The authors juxtaposed the values of serum GFAP with the tumor size assessed by mag-

TABLE 3. Correlation Coefficients between Morphometric Indices of C6 Glioma and Serum GFAP Concentration

Index	Postimplantation day	Glioma volume	Necrotic volume	Astrocytic border volume
Glioma volume	0.96**			
Necrotic volume	0.7**	0.74**		
Volume of astrocytic border	0.65*	0.64*	0.52*	
GFAP	0.5*	0.46*	0.34*	0.62*

Note. *p<0.05, **p<0.01.

netic resonance tomography and obtained a positive correlation coefficient. In their study, some glioblastomas containing predominantly non-differentiated astrocytes did not express GFAP or expressed other GFAP isoforms similar to those observed in anaplastic astrocytoma [16]. Here we studied the mechanisms determining elevated serum concentration of GFAP in the presence of low-differentiated tumor that does not produce intermediate filament protein. To this end, we carried out morphometric analysis of the tumor and juxtaposed the indices with serum GFAP concentration.

Correlation analysis showed that the size of glioma and intensity of necrotic death of glioma cells are closely related to the degree of reactive activation of astrocytes and the release of GFAP into the bloodstream. The intensity of astroglial reaction of the parenchyma against the tumor is the major factor affecting the level of GFAP elimination into the circulation. In addition, high level of this antigen in the blood could result from increased permeability of the endothelial barrier in the glioma–brain direction. Of particular importance are the cases with both dramatic increase or decrease of GFAP concentration, since the latter process can indicate that the developing glioblastoma attained the maximum size and "exhausted" the compensatory potential of reactive astrocytes.

Thus, glial border formed by GFAP-positive reactive astrocytes appeared on day 3 after impanation of glioma cells and surrounded the tumor during the entire life of the experimental rats (up to 30 days after implantation). Reactive astrocytes encompassed all foci of tumor invasive growth. Implantation of glioma cells into the brain and subsequent development of experimental glioma were accompanied by significant elevation of serum GFAP concentration. Although this concentration positively correlated with glioma volume in accordance to glioma age, it correlated most strongly with the volume of astrocytic border, the correlation coefficient being 0.62.

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