

Immunohistochemical Study of CD68 and CR3/43 in Astrocytic Gliomas

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Abstract: Diffuse and high-grade astrocytomas are invasive neoplasms which grow diffusely into the brain parenchyma. Microglia has been termed the brain's immune system, although its specific role remains uncertain. Objective of this study was to assess in a series of astrocytic neoplasms, the expression of a macrophage marker CD 68 and Major Histocompatibility Complex Class II CR3/43. We examined 10 pilocytic astrocytomas, 13 diffuse astrocytomas and 17 anaplastic astrocytomas. For macrophages we used the CD68 monoclonal mouse antibody. For assessing the presence of MHC Class II complexes we used the specific monoclonal antibody CR3/43. CD68-positive mononuclear cells were observed in perivascular and hypoxic areas, within neoplastic tissue, inside and contiguous to vessel wall. CR3/43 positive complexes were detected in mononuclear elongated elements with amoeboid extensions strictly attached to endothelial cells, or contiguous to perinecrotic areas within neoplastic tissue. We suggest an active involvement of macrophage/microglia infiltrates in neovascularization and malignancy in astrocytomas. Macrophage infiltration and major histocompatibility complex class II complexes reactivity in gliomas could also suggest the occurrence of immune surveillance with a preliminary host's immune response. In addition, macrophages could promote angiogenesis mechanisms and induction of tumor growth.

Keywords: CD68, Astrocytoma, CR3/43, Immunesurveillance, Immunohistochemistry, Gliomas, Macrophages, MHC Class II, Microglia, Neoplasms.

INTRODUCTION

Treatment of cerebral gliomas has classically consisted of surgery followed by adjuvant therapy such as radiation therapy, and chemotherapy. Despite recent improvements in multimodal approaches, the prognosis for patients affected by diffuse astrocytomas remains disappointing. Neoplastic cells proliferate infiltrating extensively the brain parenchyma yielding ineffective the adopted treatments.

Microglia are parenchymal cells capable of antigen presentation to T-cells that patrol the central nervous system (CNS). This process of tumor antigen presentation to T-cells occurs within the context of major histocompatibility complex (MHC) class II molecules, which can be expressed on the surface membrane of microglia and perivascular cells. Previous studies have demonstrated a valid correlation of the monocyte/macrophages with angiogenesis in human tumors [1-2]. Tumor associated macrophage/microglia (TAMs) play an important role in the secretion of growth factors, cytokines and matrix metalloproteinases which represent the key angiogenic effector cells capable of modulating new vessel formation [1-3]. In astrocytomas, the number of intra-

tumor microglia/macrophages is higher than in peritumoral area and normal brain, and microglia increase in number with grade of malignancy [4]. However, an accurate role of microglia in astrocytomas progression remains unclear, and a direct comparison between microglia/macrophage activation in tumors and normal CNS has not been well studied. Activated microglia release reactive oxygen intermediates and proteinases and become capable of phagocytosis, MHC expression, and lymphocytes activation [5].

CD68 is a 110kDa transmembrane glycoprotein expressed by human monocytes and macrophages. CD68 can be used for identifying a population of cells being of mononuclear phagocyte origin, and for assessing the number of macrophages infiltrating a neoplasm. CR3/43 is the MHC Class II specific monoclonal antibody (mAb). CR3/43 is directed against the β -chain of all products of the MHC class II gene subregions HLA-DR, HLA-DQ and HLA-DP for microglial cells after antigen presentation [6].

In this study we have investigated, in a series of astrocytic tumors by immunostaining with an antibody against the macrophage-specific marker CD68. Furthermore, we also have evaluated astrocytomas potential to engage in antigen presentation based on their expression of MHC class II antigens by immunostaining with CR3/43 antibody.

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MATERIALS AND METHODS

We investigated 40 astrocytic tumors specimens, consisting of 10 pilocytic astrocytomas (25%), 13 diffuse astrocytomas (32.5%) and 17 anaplastic astrocytomas (42.5%). Histological grading of the tumors specimens were performed according to the WHO classification. Pilocytic astrocytomas were defined as circumscribed tumors composed of bipolar and fusiform cells, showing a biphasic patterns with pilocytic and loosely structured microcystic components. Diffuse astrocytomas presented moderate cell density with hyperchromatic and irregular nuclei and low mitotic activity. Anaplastic astrocytomas showed areas of anaplasia consisting of increased cellularity, pleomorphism, nuclear atypia and mitotic activity.

Immunohistochemical Analysis

Paraffin-sections of 5 µm thickness were deparaffinized, rehydrated and after washing in phosphate buffered saline (PBS), were stained by standard methods using the streptavidin biotin complex (StreptABComplex-HRP Duet, Dako, Carpenteria, CA) to localise the antibody bound to antigen. Predigestion with 0.025% protease (Pronase E: Sigma, St. Louis, MO.) for 6 min at 37°C was used for immunostainings. Endogenous peroxidase was blocked by incubation for 30 min in 1% H₂O₂. The solution of 3,3'-diaminobenzidine as the final chromogen was then used and all immunostained sections were lightly counterstained with haematoxylin. For macrophages immunoreaction we used the CD68 monoclonal mouse antibody (Dako, Carpenteria, CA) at dilution 1:100 in

PBS. For assessing the presence of MHC Class II complexes we used the specific monoclonal antibody CR3/43 (Cat. M-0775, DAKO, Carpenteria, CA) at dilution 1:100 in PBS, directed against HLA-DR, -DP and -DQ subregions. As negative control we used paraffin sections from the same tissues without application of the primary antibody. Immunohistochemical labeling for each antibody was graded on a scale of 0-3 grades according to the following assessment: no detectable labeling (grade 0), weak labeling (grade 1), moderate labeling (grade 2), marked labeling with local and/or widespread reactivity (grade 3). The intensity of reactivity was then reported as a range from the lowest to the highest immunoreactivity observed in each case in different sampling areas of the tumor.

RESULTS

Pilocytic Astrocytomas

In Table 1 are summarized the characteristics of each case of pilocytic astrocytoma on the basis of age, sex, location of the tumour and immunoreaction to CD68 and CR3/43.

Of the patients studied, six (60%) cases were woman and four (40%) were man. Patients mean age was 14.9 years with a range of 3-44 years. Preoperative symptoms were headache in all patients, vomiting in five and jacksonian seizures in one case. The neurological findings consisted of visual deficit in three patients. There was no operative mortality and no recurrence has resulted so far in follow-up observations.

Table 1: Characteristics of Each Case of Pilocytic Astrocytoma on the Basis of Age, Sex, Tumour Location and Immunohistochemical Expression of CD68 and CR3/43

Patient no.	Age (yr)/sex	Location	CD68	CR3/43
1	44/F	Right Temporal	Weak	Weak
2	14/F	Right Cerebellar	No detectable	No detectable
3	23/M	Ipothalamic	No detectable	No detectable
4	5/M	Vermian	No detectable	No detectable
5	4/F	Left Cerebellar	No detectable	No detectable
6	11/M	Vermian	No detectable	No detectable
7	17/F	Right Cerebellar	No detectable	No detectable
8	3/M	Right Cerebellar	No detectable	No detectable
9	7/F	Right Cerebellar	No detectable	No detectable
10	21/F	Ipothalamic	No detectable	No detectable

CD68

Weak labeling immunoreaction to CD 68 was demonstrated only in a case (Table 1). The CD68 immunoreaction was detected inside of large mononuclear irregular cells mainly distributed close to microcystic degeneration areas. Microcystic degeneration areas were surrounded by CD68 positive glioma-infiltrating macrophages.

CR3/43

Immunopositive cells labeling CR3/43 antibody were evidenced only in a single case with weak reactivity (Table 1). The presence of MCH class II complexes was demonstrated by the presence of elongated elements with amoeboid and radiate extensions. The CR3/43 more intensely immunopositive cells were distributed neighboring neoplastic elements.

Diffuse Astrocytomas

In Table 2 are summarized the characteristics of each case of diffuse astrocytoma on the basis of age, sex, location of the tumour and immune-reaction to CD68 and CR3/43.

Of the patients studied, seven (53.8%) were woman and six (46.1%) were man. Patients mean age was 49.1 years with a range of 33-69 years. Preoperative symptoms were headache in six cases, Jacksonian

seizures in three cases, temporal seizures and psychological changes in one case respectively. In a case the patient was admitted with intracranial hypertension signs. The neurological findings consisted of hemiparesis in five patients and Babinski sign in four. There was no operative mortality and in two cases a recurrence was disclosed in follow-up observations.

CD68

CD68-positive cells were documented in eleven cases (Table 2). CD68 immune-reaction was detected inside of large mononuclear and round-ovoidal shape cells, mainly distributed around the tumor elements, as scattered single cells or groups of cells. CD68 positive cells were especially detected in perivascular areas, where, in some cases, appeared to circumscribe and go through large vessels (Figures 1a and 1b).

CR3/43

Immunopositive cells labeling CR3/43 antibody were evidenced in twelve cases (Table 2). The positive MHC Class II complexes were found arranged as isolated cells or as cluster of cells with various shape often localized adjacent to perivascular area, at the edge of vascular channels. Neoplastic cells, localized close to endothelial cells which circumscribe and seem to invade large vessels with amoeboid elongated CR3/43 positive elements that are markedly collected around. Frequently microglial cell showed abnormally long and stringy cytoplasmic processes. Structural

Table 2: Characteristics of Each Case of Diffuse Astrocytoma on the Basis of Age, Sex, Tumour Location and Immunoreaction to CD68 and CR3/43

Patient no.	Age (yr)/sex	Location	CD68	CR3/43
1	43/M	Left Temporo-insular	Weak	No detectable
2	34/M	Right Fronto-temporal	No detectable	Weak
3	43/F	IV Ventricle	Moderate	Weak
4	47/M	Left Temporal	Moderate	Moderate
5	36/F	Right Temporo-parietal	Moderate	Moderate
6	65/M	Left Parietal	Weak	Moderate
7	33/F	Right Temporo-parietal	No detectable	Weak
8	47/F	Right Temporo-parietal	Moderate	Marked
9	50/F	Right Frontal	Moderate	Marked
10	49/F	Left Temporal	Moderate	Moderate
11	65/M	Left Temporo-parietal	Moderate	Moderate
12	69/M	Right Parietal	Moderate	Moderate
13	58/F	Right Temporal	Moderate	Marked

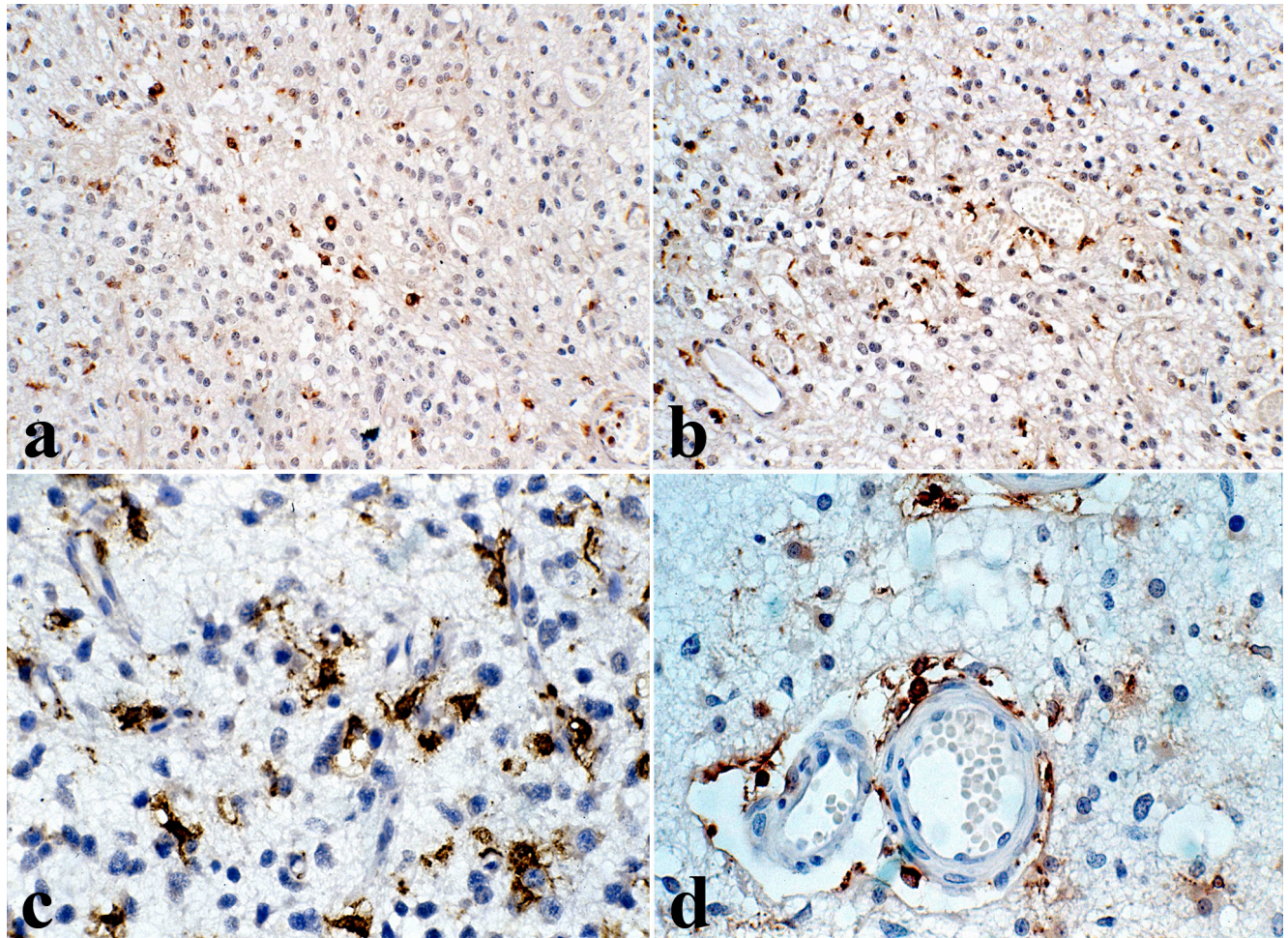


Figure 1: CD68 and CR3/43 immunoreactive cells in diffuse astrocytomas. **a.** Minimal immunoreactivity within neoplastic parenchyma and in perivascular areas. Magnification x 20. Grade 1. **b.** Marked CD68 immunoreactivity detected inside of mononuclear, round-oval shape cells, around tumor cells, in perivascular areas. They appear to circumscribe and go through large vessels. Magnification x 20. Grade 3. **c.** CR3/43 immune-reactive cells more intensely distributed bordering vascular channel within neoplastic tissue, surrounding neoplastic cells. Structural abnormalities in microglial cell shape, such as atypical, tortuous processes are also evidenced. Magnification x 40. Grade 2. **d.** The presence of MHC Class II complexes is represented by elongated elements with amoeboid, tortuous extensions radiate in all directions. CR3/43 positive cells with various shape and often localized adjacent to perivascular area. Neoplastic cells localized close to endothelial cells, that circumscribe and seem to invade large vessels with amoeboid elongated CR3/43 positive elements that are markedly collected around. Magnification x 40. Grade 2.

abnormalities in microglial cell shape, such as atypical, tortuous processes are also evidenced (Figures 1c and 1d).

Anaplastic Astrocytomas

In Table 3 are summarized the characteristics of each case of anaplastic astrocytoma on the basis of age, sex, location of the tumour and immune-reaction to CD68 and CR3/43.

Of the patients studied, thirteen (76.4%) patients were man and four (23.5%) were woman. Patients mean age was 59.7 years with a range of 43-75 years. Preoperative symptoms were headache in ten patients, Jacksonian seizures in five, temporal seizures in two

and psychological changes in one case. The neurological findings consisted of hemiparesis in nine patients, Babinski sign in seven and visual deficits in one case. There was no operative mortality and in four cases a recurrence was disclosed in follow-up observations. Three patients deceased in the follow-up observations.

CD68

CD68-immune-positive positive cells were documented in all patients (Table 3). CD68 immune-reaction was detected inside of large mononuclear and round-oval cells distributed around the neoplastic cells, which appear directed towards microglia (Figure

Table 3: Characteristics of Each Case of Anaplastic Astrocytoma on the Basis of Age, Sex, Tumour Location and Immunoreaction to CD68 and CR3/43

Patient no.	Age (yr)/sex	Location	CD68	CR3/43
1	73/F	Right Cerebellar	Weak	Weak
2	65/M	Left Frontal	Moderate	Moderate
3	57/M	Right Temporal	Moderate	Marked
4	65/M	Left Temporal	Moderate	Weak
5	67/M	Right Temporo-insular	Weak	Weak
6	43/M	Left Frontal	Marked	Moderate
7	50/M	Right Parietal	Marked	Marked
8	47/M	Right Temporal	Marked	Marked
9	57/F	Right Fronto-temporal	Moderate	Weak
10	69/F	Left Parietal	Marked	Moderate
11	75/M	Left Parietal	Marked	Moderate
12	61/M	Right Temporal	Weak	Weak
13	53/M	Right Fronto-temporal	Marked	Moderate
14	44/M	Right Frontal	Moderate	Marked
15	56/M	Left Fronto-temporal	Marked	Marked
16	64/F	Righth Frontal	Marked	Moderate
17	70/M	Right Temporal	Marked	Moderate

2a). We also observed the presence of large immune-reactive cells that passed through vessel wall, neighboring, in some cases, an adjacent necrotic area, with few positive elements in neoplastic area (Figure 2b).

CR3/43

Immune-positive cells labeling CR3/43 antibody were evidenced in all cases (Table 3). The MHC Class II complexes were found arranged as isolated cells or in group of cells with various aspects. There were some CR3/43-positive-cells close to perivascular area, demarcating neoplastic elements (Figure 2c). MHC Class II molecular complexes were represented by elongated elements with amoeboid extensions around large vascular channel especially in neo-angiogenic areas. In particular cases this feature was also noted with numerous strongly reactive cells contiguous to endothelial cells, inside a large vessel, that leading through the vessel wall (Figure 2d).

DISCUSSION

Macrophages/microglial cells constitute the first line of cellular defense against a variety of stressors to the CNS, participating in the regulation of innate immune

responses in human gliomas [7]. Under pathological conditions, the activated microglia are characterized morphologically by a gradual transition from a quiescent stellate form to a macrophage-like morphology, which is accompanied by upregulation of surface antigens and the formation of cell clusters.

In the tumor micro-environment, macrophages are the largest population among tumor infiltrating immune cells. TAMs, attracted by tumor, release pro-angiogenic cytokines and growth factors which facilitate tumor growth through activation of cell kinetic and motility [3]. Macrophages can exert a dual influence on blood vessel formation. On the one hand macrophages produce proangiogenic molecules on the other hand they can express anti-angiogenic molecules and damage the integrity of blood vessels. In general the pro-angiogenic functions of TAMs prevail [3]. Recently, various substances that promote angiogenesis have been shown to be expressed by macrophage hypoxic conditions, such as VEGF, TNF- α , bFGF, CXCL8, interleukin-1, interleukin-6, interleukin-8 [8]. Therefore, macrophages recruited in situ represent an indirect pathway of amplification of angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. In experimental *in vitro* studies, glioma cell lines seem produce high levels of a monocyte-

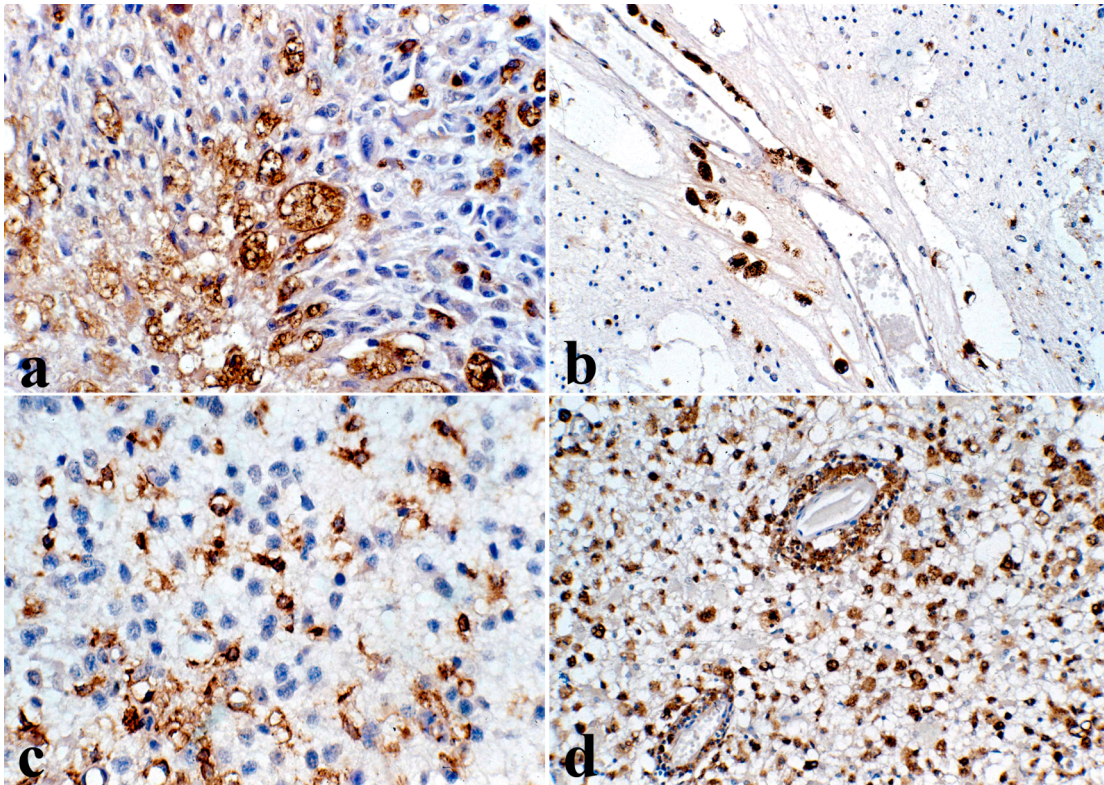


Figure 2: CD68 and CR3/43 positive cells in anaplastic astrocytomas. **a.** Large mononuclear CD68 immune-positive cells, of round-ovoidal shape distributed around the neoplastic cells, which appear directed towards microglia. Magnification x 40. Grade 3. **b.** Large positive cells passed through vessel wall, bordering an adjacent necrotic area, only few positive elements in neoplastic area. Magnification x 20. Grade 2. **c.** CR3/43-positive cells arranged with various aspects of activation. CR3/43-positive cells close to perivascular areas, circumscribing neoplastic cells. Magnification x 40. Grade 3. **d.** CR3/43 reactive cells observed inside large vessels, leading through vessel wall and distributed within neoplastic tissue. Magnification x 20. Grade 2.

macrophages-derived cytokine, IL-8, that induces formation of tube-like structures by human microvascular endothelial cells [9]. We previously demonstrated significant increase in IL-8 protein level in astrocytic cultures treated with PGE2. The ability of PGE2 to increase IL-8 expression in glioma cells has a significant biological impact on tumorigenesis, as shown by increased growth and reduced apoptosis in PGE2-treated cells [10].

CD68 is a monoclonal mouse antibody that labels human monocytes and macrophages. The antigen recognized by CD68 is absent from resting microglia but readily detectable in phagocytic microglia, perivascular cells and brain macrophages [11]. Nishie *et al.*, showed an association between CD68-positive macrophage/microglia infiltration and neovascularization in high-grade gliomas [12]. Ahmed *et al.*, described the orientation of individual tumor cells as they enter blood vessels, and observed that tumor cells seem to be attracted to macrophages [13]. Strojnik *et al.* suggested that CD68 positivity may be also associated with gliomas progression. The authors emphasize that some biological properties are shared

by macrophages and astrocytoma cells, such as phagocytosis and production of antigenic factors [14]. In our study we have evidenced the presence of CD68 positive cells neighboring of neoplastic cells, contiguous to necrotic and hypoxic areas within neoplastic tissue. We have, also, observed the presence of TAMs that passed through vessel wall also in low-grade astrocytomas (Figure 1b). In anaplastic cases, neoplastic cells seem to be guided towards microglia. Macrophage infiltration could be closely associated with neovascularization and malignancy in human gliomas. Macrophages produce, also, several matrix-metalloproteases which cause the extracellular matrix (ECM) degradation and the blood-brain barrier breakdown. In this view Markovic lately provided *in vitro* the evidence about tumorigenic effects of microglia, like in facilitation of tumor invasion through proteolysis of the extracellular matrix [15]. Pollard *et al.* recently proposed a model by which macrophages promote tumor invasion through their proteolytic activity and subsequent breakdown of the basal membrane (BM) around the pre-invasive tumors, thereby enhancing the ability of tumor cells to escape into the surrounding stroma [3]. In our previous study, we

have demonstrated, in a series of paediatric glioblastoma, the presence of laminin, fibronectin and type IV collagen in hyper-plastic vessels, in and around vascular channel, in vascular walls and at level of BM associated with endothelial glomerulus-like proliferations [16]. LM within the BM can bind to both endothelial cells and tumor cells and is involved in angiogenesis and tumor growth. We can hypothesized that in pilocytic astrocytoma, the ECM integrity cause a reduced macrophagic/microglial migration. In this view, an incompetent control of interactions occurs between microglial adhesive molecules and ECM substrates. In low-grade astrocytomas is achievable that low macrophage/microglial recruitment is also correlated with a lower vascular neo-angiogenesis. This relationship may influence microglial morphology, blocking microglial migration and phagocytosis. To support these findings, a recent study showed an increase of CD68 positive cells which correlated with trends toward worse event-free survival. Consequently, the elements of the tumor environment, such as actively proliferating monocytic or microglial cells, may contribute to tumor growths in these neoplasms [17].

The brain has been described as an immunologically privileged organ as consequence of the BBB, of lack of conventional lymphatics, of low MHC expression and low T-cell trafficking. At later stages of tumor growth, when massive tissue damage with destruction of the BBB and tumor cell necrosis with antigen drainage to the periphery occurs, brain tumors become accessible to the peripheral immune system.

Macrophage/microglia is essential for host defense. In recent years it has been demonstrated that macrophage/microglia are able for antigen presentation. The process of antigen presentation to T-cells occurs within the context of MHC class II molecules, as expressed on the surface of microglial cells [18]. An ongoing debate is whether the immune system can recognize the presence of a tumor. Actually the capacity of microglial cells for antigen presentation could be exerted under tumor control. Indeed glioma may escape from the host's immune response, not only by suppressing T-cell function, but also by reducing the antigen-presenting capability of microglia. Activated microglia show direct cytotoxicity towards tumor cells and/or elicit tumor destructive reactions through alterations of the tumor microvasculature [1].

CR3/43 is a sensitive marker for human microglia at different stage of immunological activation. MHC class II complexes are represented by elongated elements

with amoeboid extensions probably referable to immunological-activated phenomena [19]. The immune-histochemical expression of CR3/43 positive cells could indicate an active involvement in the process of immunologic reaction to the tumor. Tran *et al.* found a reduced expression of MCH class II antigens by microglia/macrophages in highly cellular tumor areas, in contrast they found large number of positive macrophage/microglia in tumor areas showing little immunoreactivity, suggesting downregulation of MHC class II molecules [11]. Rossi *et al.* suggested that this down-regulation of microglial immune competence and the expression of MHC class antigens could represent synergistic mechanisms by which diffusely growing astrocytic gliomas elude CNS immune defense [20]. Komoara demonstrated a relationship between the number of infiltrating microglia/macrophages and the grade of histological malignancy [21]. Abnormal evidenced immunoreactivity to CR3/43 in grade III astrocytomas suggests the role in recognizing and starting immune response against neoplastic tissue. The presence of macrophage/microglia infiltrates and MHC class II complexes in glioma demonstrates the occurrence of immune surveillance with a preliminary host's immune response. An aberrant HLA-DR, HLA-DQ and HLA-DP expression with an allied loss of immune-competent MHC class II-expressing microglia could also occur. Alterations of the antigenic phenotype of the malignant cells could explain change in MCH class II expression. We found CR3/43 positive cells along large vessels, with numerous strongly reactive cells contiguous to endothelial cells that leading through the vessel wall, indicating a probable intimate relationship between macrophage/microglia infiltrates and extracellular matrix. These aspects could indicate that regulation of expression and distribution of ECM molecules influence the degree of microglial activation and migration.

Our findings support the view that microglia accumulation in gliomas reflects participation of these cells in a variety of processes concerning immune defense, proliferation and migration. Microglia might able to deposit extracellular matrix components that facilitate gliomas invasion or they could digest extracellular matrix components that binder tumor cell motility. The present study could also suggest that TAMs appear as attractive candidate of novel therapeutic strategies. Inhibition of their recruitment and/or of their survival at the tumor site and of their positive effects on angiogenesis is amenable of therapeutic treatment. We propose moreover, that

these novel prognostic marker should be evaluated and standardized in larger populations of patients to investigate their clinical value for the management of patients with astrocytic tumors and their application to the development of individualized therapeutic regimens. In addition, a control of factors from glioma and enhancement of the depressed antigen-presenting function of APC in the brain are required to develop immunotherapy in glioma. Therefore, for the data that ECM, *via* integrin signaling, is able to regulate gene expression and consequent behavior in microglial cells, very much work is needed to clarify the precise roles. A better understanding of the regulation and function of microglia may help to establish more efficacious novel therapies for malignant brain tumor management.

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