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Complement activation in Glioblastoma Multiforme pathophysiology: Evidence from serum levels and presence of complement activation products in tumor tissue

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ABSTRACT

Inflammation plays a key role in the pathophysiology of Glioblastoma Multiforme (GBM). Here we focus on the contribution of the so far largely ignored complement system.
ELISA and immunohistochemistry were combined to assess levels and localization of critical components of the initiation- and effector pathways of the complement cascade in sera and tumor tissue from GBM patients and matched controls.
Serum levels of factor-B were decreased in GBM patients whereas C1q levels were increased. C1q and factor-B deposited in the tumor tissue. Deposition of C3 and C5b-9 suggests local complement activation. MBL deficiency, based on serum levels, was significantly less frequent among GBM patients compared to controls (14% vs. 33%). Therefore low levels of MBL may protect against the initiation/progression of GBM.
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1. Introduction

Gliomas are the most common type of primary brain tumors in adults. Glioblastoma Multiforme (GBM) accounts for over 51% of all gliomas, and newly diagnosed GBMs show an overall survival of 17–30% at one year, and only 3–5% at two years (Adamson et al., 2009). Despite the increasing extent of surgical resections using state-of-the art preoperative, intraoperative neuroimaging and monitoring techniques and advances in radiotherapy and chemotherapy, the prognosis for GBM patients remains dismal (Li et al., 2009). The etiology of GBM still remains largely unknown but probably involves genetic, immunologic, hormonal as well as environmental factors (Kanu et al., 2009).

Several studies of human cancers have established that chronic and insidious inflammation promotes the process of carcinogenesis and exacerbates the growth of existing tumors (Balkwill and Mantovani, 2001; Wiemann and Starnes, 1994). Components of the adaptive immune system have been identified in GBM patients, indicating involvement of immune activation in the pathology of GBM. Surprisingly, very little is known about the contribution of innate immunity in GBM patients (Bach et al., 2009).

The human complement system is a major contributor to both adaptive and innate immunity, and forms a functional bridge between the innate and adaptive immune response (Ricklin et al., 2010). The complement system comprises around thirty fluid phase as well as membrane-associated proteins. Apart from being the first line of defense against microbial invasion it is also thought to additionally take part in a range of diverse processes including synapse maturation and phagocytosis of cellular debris.

Three pathways of complement activation have been recognized; the lectin pathway (LP), the classical pathway (CP) and the alternative pathway (AP). These pathways are activated via their recognition molecules, C1q, Mannose Binding Lectin and C3-H_2O. Genetic variants, both common Single Nucleotide Polymorphisms and mutations have been identified in the complement genes. Some of the variants in C1q and MBL can have interesting consequences. Genetic variants in both C1q and MBL, can increase the susceptibility to specific infections (Turner, 2003). Although being very rare, mutations in either one of the three genes encoding for C1q is almost always accompanied by the presence of Systemic Lupus Erythematosus. Certain SNPs can result in complete MBL deficiency with a frequency up to 40% in healthy Caucasian adults and are associated with cardiovascular disease (Minchinton et al., 2002; Mead et al., 1997; Madsen et al., 1994; Skattum et al., 2011). Increased serum levels and activity are seen for MBL in colorectal cancer and newly diagnosed acute myeloid leukemia patients (Ytting et al., 2004).

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Complement activation can promote carcinogenesis and facilitate the fundamental requirements of the malignant cell, as it was shown to sustain proliferative signaling, angiogenesis, resistance to apoptosis, and also to modulate anti-tumor immunity and activate invasion and migration (Rutkowski et al., 2010; Hanahan and Weinberg, 2011). Therefore, the activated complement cascade is thought to exert great influence on tumor progression and survival of tumor patients. Consequently, high levels of complement activation proteins may be beneficial for the tumor (Markiewski et al., 2008; Markiewski and Lambris, 2009a,b). In sharp contrast, several studies have reported on the possibility to eradicate tumors by increasing complement activation on tumor cells by i.e. inhibiting endogenous complement inhibitors through application of monoclonal antibodies (Beurskens et al., 2012; Teeling et al., 2004).

Genetic status and consequently serum levels of key proteins of the activation pathways have, as illustrated above, successfully been related to a variety of diseases. However, the precise involvement of the complement cascade on glial tumor pathophysiology remains, yet, unexplained. In the present study, the degree to which complement is activated and which activation pathways of complement activation are involved in GBM was studied in different stages of glial tumor progression. Deposition of C1q and C3 was observed in tumor tissue, suggesting a role for complement in GBM pathogenesis. Remarkably, a lower frequency of innate MBL deficiency in GBM patients was observed in this study, suggesting a protective effect on tumor initiation/progression.

2. Material and methods

2.1. Patient samples

Tissue and serum samples were obtained during elective debulking surgery of susceptible primary glial tumor patients at the Erasmus Medical Centre, Rotterdam, The Netherlands. Tumor tissue samples were immediately snap-frozen in liquid N_2 and together with sera stored at −80 °C until further analysis. Sample collection and handling was performed with informed consent from patients as approved by the institutional review board of the Erasmus Medical Center, Rotterdam, The Netherlands.

Of the 131 patients who were operated with suspected intracranial glial tumor disease, histopathological analysis confirmed 113 glial tumors of which 71 patients with WHO grade IV Glioblastoma Multiforme, 22 WHO grade III, and 20 WHO grade II. The grade III and grade II glial tumors comprised of (anaplastic) oligodendroglioma, (anaplastic) astrocytoma and (anaplastic) oligoastrocytoma. All were included for further analysis of preoperative serum concentrations of C1q, MBL and factor B. See Table 1 for patient demographics.

2.2. Immunohistochemical- and immunofluorescence-stainings

Immunohistochemistry was performed using cryopreserved −80 °C serial tissue sections (6 μm). Tissue sections were fixed with cold acetone (10 min) and rehydrated using phosphate-buffered saline (PBS). Sections were then incubated with peroxidase blocking reagent (DAKO; Glostrup, Denmark) and subsequently with 1:10 normal Goat serum (Sigma-Aldrich, St. Louis, USA). After rinsing with PBS, sections were incubated 1 h at RT with polyclonal antibodies diluted in PBS containing 1% BSA (PBS–BSA) specifically staining human MBL (Mannose Binding Lectin; 1:250 DAKO) C1q (1:1000 DAKO), factor B (Santa Cruz Biotechnology, Santa Cruz, USA), C3c (1:500 DAKO) and C5b-9 complex (1:200 Santa Cruz Biotechnology, Santa Cruz, USA) followed by washing with PBS. Peroxidase conjugated anti rabbit/mouse HRP (Envision Kit, DAKO) was used as a secondary antibody for all tissue sections. Sections were developed with diaminobenzidinetetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin to allow morphological analysis. The specificity of the antibody staining was confirmed by omitting the primary antibody.

For immunofluorescence staining the acetone fixated sections were incubated with PBS–BSA for 30 min and washed in PBS. Primary antibody incubation (1 h) was followed by washing and incubation (30 min) of the tissue sections with anti-mouse Alexa 488 IgG (Invitrogen, USA; 1:250) or anti-rabbit Alexa 568 IgG (Invitrogen, Carlsbad, USA; 1:500) and nuclei were stained with DAPI (Vector Laboratories, Burlingame USA). All incubation steps were performed using PBS–BSA as a buffer at room temperature.

2.3. ELISA for MBL and C1q

Enzyme-linked immunosorbent assays (ELISA) for the detection of human MBL and C1q were developed and used as previously described (Castellano et al., 2004; Roos et al., 2001; Worthley et al., 2006). The Sandwich ELISA for factor B was performed by coating goat anti human Factor B (Quidel, San Diego, USA) overnight at room temperature. Following blocking with PBS–BSA for 1 h at 37 °C plates were washed and a standard of Normal Human Serum (NHS) or the patient samples diluted in PBS–BSA–0.05% Tween were incubated for 1 h at 37 °C. Following additional washing the plates were incubated with mouse anti-factor B (Quidel) followed by goat anti-mouse IgG-HRP (DAKO) and assay development using the substrate ABTS.

2.4. Total protein analysis and correction

Total protein was measured for each serum protein sample of both glial tumor patients and healthy controls in quadruple using a BCA assay (Pierce, Rockford, USA). To correct for small total protein discrepancies between healthy controls and glial tumor patients, protein levels of C1q, MBL and factor B were divided by the corresponding total protein concentration for each sample and normalized to the mean protein content of sera of the healthy controls.

2.5. Survival analysis

Survival curves from date of operation were plotted for the 25th vs. 75th percentile of the corrected factor B and C1q serum concentrations. For MBL, the survival curves were drawn for MBL deficient vs. MBL sufficient patients. In this analysis only GBM patients were taken into account who did not meet exclusion criteria. Exclusion criteria comprised of post-operative death defined as death < 1 month after surgery, recurrent/secondary glioblastoma, and unnatural and unknown cause of death. Of the initially included 71 patients 52 patients remained for survival analysis.

2.6. Statistical analysis

Differences between Kaplan–Meier survival curves were calculated by the log-rank (Mantel–Cox) test. Protein levels of the serum samples from separate WHO classifications were individually compared to the Healthy controls by Student's t-test. MBL deficiency in glial tumor grading compared to healthy controls was analyzed using Chi (Li et al., 2009) test. All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago) or Graphpad Prism 5.00 (Graphpad Inc.) software.

Table 1
Patient demographics. * indicates significant difference ($p < 0001$; Student's t-test) compared to mean age healthy controls.

Patient demographics					
	Healthy controls	Glial tumor	GBM	WHO III	WHO II
N	209	113	71	22	20
Mean age	44	52	58*	42	43
Male/female	103/106	61/52	38/33	14/8	9/11

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