Journal of Neuroimmunology 278 (2015) 271–276

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

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

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ARTICLE INFO

Article history:
Received 29 September 2014
Received in revised form 13 November 2014
Accepted 14 November 2014

Keywords:
Glial tumor
Complement system
Mannose Binding Lectin (MBL)
C1q
Factor B
Glioblastoma Multiforme

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.

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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http://dx.doi.org/10.1016/j.jneuroim.2014.11.016
0165-5728/

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.

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

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.

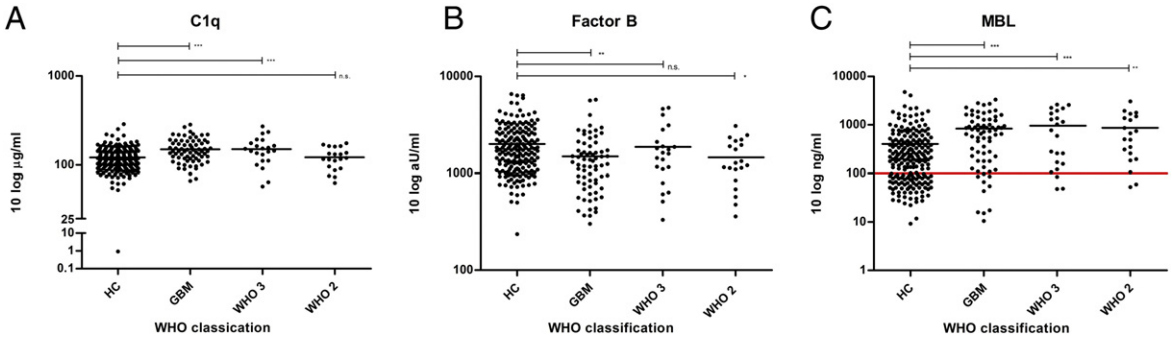

Fig. 1. Mean sera levels of C1q (A), factor B (B) and MBL (C) of glial tumor patients compared to age and sex matched controls. The *, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.0001$. n.s., not significant.

3. Results

3.1. Altered serum level of C1q, factor B and MBL in glial tumor patients

In order to evaluate quantitative differences in levels of the recognition molecules of the three complement pathways (CP, AP, LP) between glial tumor patients and healthy controls, we performed ELISA for C1q, MBL and factor B on sera of patients and controls. We measured total protein concentrations of the sera of patients and controls and observed small differences. Therefore we corrected the measured levels of C1q, MBL and factor B for total protein content in each sample.

The mean concentrations of C1q in glioma patients, 145 ± 4 µg/ml (mean, SEM, N = 113), was significantly increased as compared to controls 121 ± 2 µg/ml (Mean, SEM, N = 208). For Factor B a significantly decreased concentration in patients was noted, 1556 ± 100 aU/ml, as compared to the controls 1996 ± 85 aU/ml. Fig. 1A and B shows the scatter plots of the corrected C1q and factor B concentrations in healthy controls and glial tumor patients when divided into the WHO classification for gliomas.

Evaluation of the MBL serum levels in patients with glial tumor versus healthy controls revealed elevated MBL concentrations in the glial tumor group 870 ± 75 ng/ml versus 404 ± 41 ng/ml, ($p < 0.0001$). When divided into subgroups according to the WHO classification for gliomas, all groups showed a significant increase in mean MBL (Fig. 1c). The frequency of MBL deficiency, defined as <100 ng/ml, in our control group (33%), corresponds with the normal distribution in the western population (Minchinton et al., 2002). The frequency of MBL deficiency was strongly decreased in the GBM group versus the control group (14% vs. 33%; $p = 0.006$), as shown in Table 2 and Fig. 1c.

3.2. Complement deposition in tumor tissue

The observed changes in C1q, factor B and MBL serum levels may reflect the involvement of these proteins in the tumor process. Therefore we performed immunohistochemistry on tumor tissue in order to identify the deposition of C1q, factor B and MBL at the protein level.

Interestingly, C1q was highly present in tumor tissue as shown in Fig. 2, independent of CD45 positive leukocytic infiltration (Fig. 2c), and suggesting deposition on tumor cells. Factor B and MBL were also present in tumor tissue (Fig. 3), but show relatively lower staining intensities as compared to C1q.

The three initiating pathways of the complement system converge at the level of proteolytic cleavage of C3 that, ultimately, may lead to full-blown activation of the complement cascade and to the formation of the C5b-9 complex. Consequently, the presence of C3 in tumor tissue is essential for the continuation of the complement cascade. Fig. 4 shows the diffuse staining pattern of C3, which is abundantly present in both necrotic and non-necrotic areas of GBM tumor tissue. In addition, the C5b-9 complex was detected on individual cells in GBM tumor tissue (Fig. 5), which indicates that complement activation up to the C5b-9 complex locally has taken place.

Table 2
Frequency distribution of MBL deficiency in GBM patients (14%) compared to healthy controls (33%). χ^2 test, $p = 0.002$.

Frequency of MBL deficiency			
	MBL		Total
	Non-deficient	Deficient	
Control group	139	69	208
GBM patients	61	10	71
Total	200	79	279

3.3. Complement levels are not overtly associated with patient survival

In order to identify the possible influence of preoperative MBL, C1q or factor B serum concentrations on GBM survival, survival curves were plotted for subdivisions of these groups (C1q, factor B 25th and 75th percentiles, MBL sufficient and deficient). Kaplan Meier curves did not show significant survival difference in the three groups, as depicted in Fig. 6.

4. Discussion

Glioblastoma Multiforme is a primary brain tumor, which shows an aggressive clinical course despite debulking surgery and chemoradiation treatment. This malignant process is characterized by its infiltrative nature, hyper vascularization, presence of necrosis and impairment of the blood–brain barrier. These characteristics could in theory be aided or facilitated by the immunological response to the tumor process. Components of the adaptive immune system have been identified in gliobastoma patients whereas only little is known about the contribution of innate immunity.

Under healthy conditions, the blood–brain barrier restricts the influx of blood borne cells and proteins into the brain parenchyma. Immune surveillance and differentiation between “self” and “non-self” in the brain are under normal conditions provided by resident cells, including microglia and astrocytes. These glial cells, and also neurons can locally produce complement factors and regulatory proteins (Veerhuis et al., 1996, 1999). The complement cascade is mainly responsible for the clearance of micro-organisms and senescent or ‘altered’ cells in the healthy brain and during embryonal development in synapse pruning and development of neural networks. In disease conditions, complement activation is evident in acute brain injury (infection, trauma, stroke) and also in neurodegenerative diseases (including Alzheimer's disease) associated with chronic inflammatory processes.(Veerhuis et al., 2011) In this study we investigated the involvement of the three complement cascade initiating pathways and its consequences in terms of complement pathway continuation in GBM by determining preoperative serum levels and tissue localizations of C1q, MBL, factor B, as well as of C3 and C5b-9.

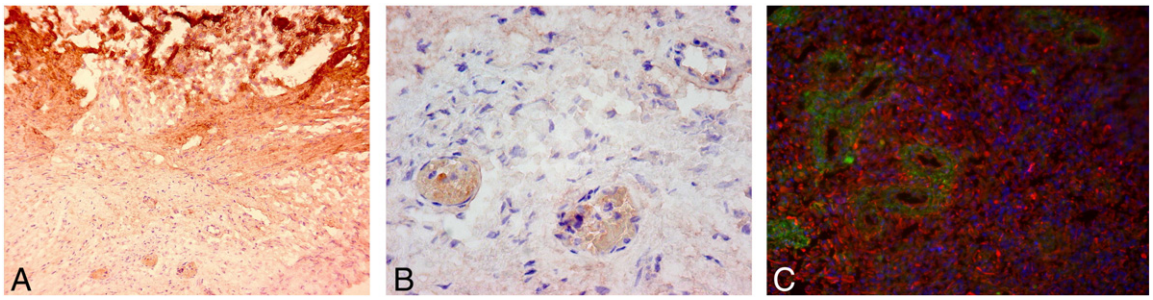

Fig. 2. Immunohistochemical staining of GBM slides for C1q using a standard IHC protocol: primary antibody was diluted 1000×, slides were developed using DAB. Pictures taken at 16× (A) and 40× (B) magnifications. C: Immunofluorescence staining of GBM tissue for C1q (1:1000 red) using a double staining protocol together with CD45 (1:250 green). Pictures were taken at 16× magnification.

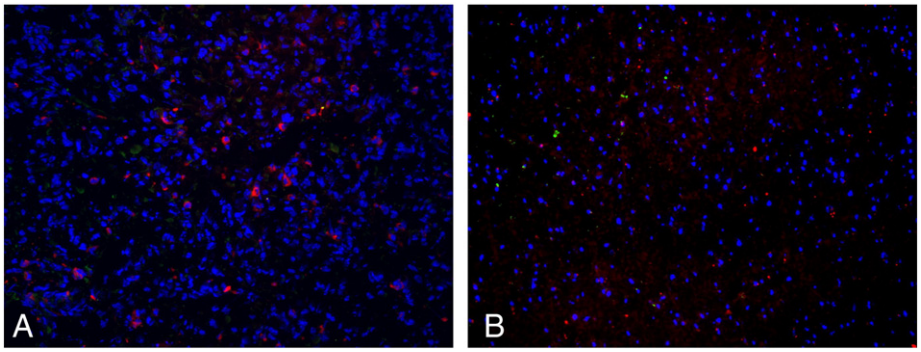

Fig. 3. Immunofluorescence staining of GBM tissue for factor B and MBL using a double-staining protocol together with astrocyte marker Glial fibrillary acidic protein (GFAP). A: MBL (1:200 red) GFAP (1:250 green), 16× magnification. B: Factor B (1:200 red) GFAP (1:250 green), 16× magnification.

Genetic variants in the exons and or promoter region of the MBL2 gene are associated with decreased concentrations. Very low concentrations of functional Mannose Binding-Lectin (<100 ng/ml) are considered to be a reflection of homozygous mutations in the MBL2 gene. In general accordance with large epidemiologic studies, our age and gender matched control subjects show a frequency of MBL deficiency of 33%. Interestingly, we found MBL deficiency in only 14% of the glioblastoma subjects (Table 2), which could suggest a possible cancer

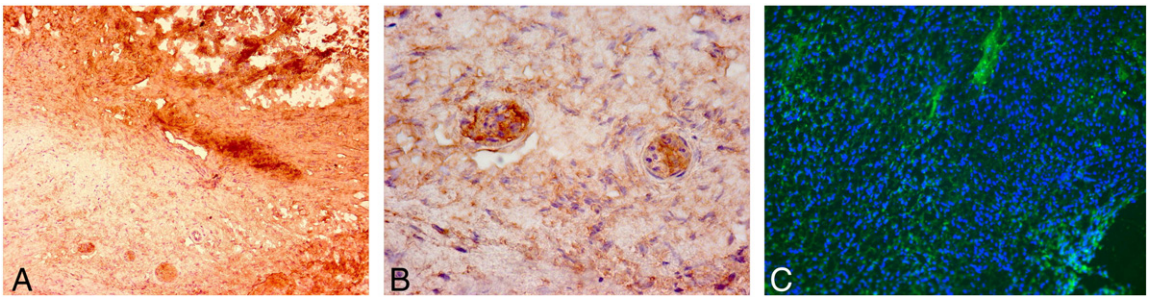

Fig. 4. Immunohistochemical staining of GBM slides for C3c using a standard IHC protocol: primary antibody was diluted 1000×, slides were developed using DAB. Pictures taken at 16× (A) and 40× (B) magnifications. C: Immunofluorescence staining of GBM tissue for C3c (1:1000 green). Picture taken at 16× magnification.

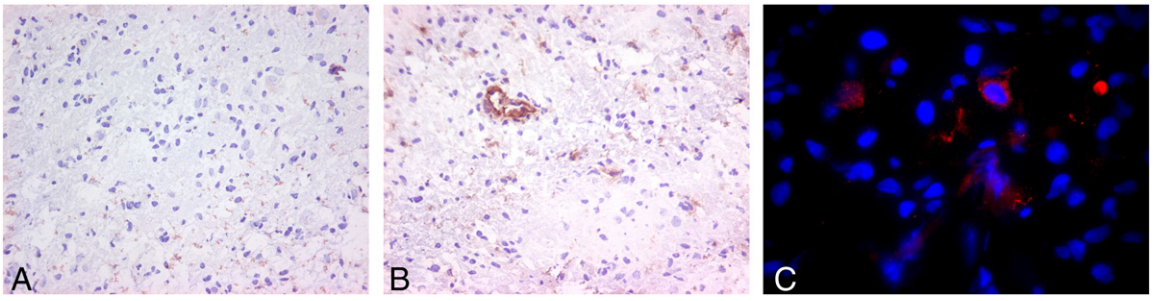

Fig. 5. Immunohistochemical staining of GBM slides for the C5b-9 complex using a standard IHC protocol: primary antibody was diluted 250×, slides were developed using DAB. Pictures taken at 16× (A) and 40× (B) magnifications. C: Immunofluorescence staining of GBM tissue for C5b-9 complex (1:250 red). Picture taken at 66× magnification.

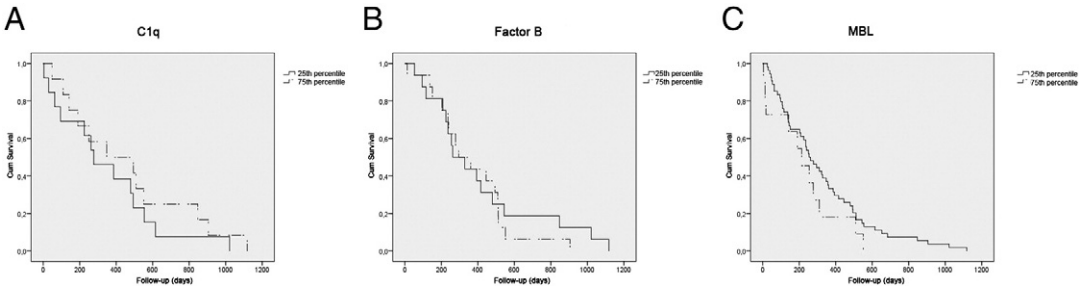

Fig. 6. Kaplan Meier curves. C1q 25th versus 75th percentile did not significantly differ for survival ($p = 0384$) as 25th and 75th percentiles of factor B ($p = 0.661$) and MBL deficient versus sufficient ($p = 0.188$).

protective effect of MBL deficiency. On the contrary, MBL deficiency predisposes to stomach cancer (Baccarelli et al., 2006). MBL serum concentration was found to be increased in glioblastoma patients which either could be a reflection primarily of the tumor process, or a response to surgery or inflammation elsewhere., This increase has also been observed for colorectal and pancreatic cancer patients (Ytting et al., 2004; Rong et al., 2010). The exact implication of the presence of MBL in cancer biology remains yet to be understood. MBL deficiency did not significantly correlate with GBM survival, when MBL deficient and MBL sufficient subjects were compared, suggesting that MBL deficiency does not influence tumor progression, but tumor initiation. Future in-vivo research will assess the possible protective role of MBL deficiency and the impact of the increase of MBL serum concentration.

Further focusing on the classical pathway, the initiating component of this pathway C1q, circulates in a complex with C1r and C1s to form C1. We observed an increase in C1q serum concentration in comparison to our healthy controls, which can be attributed to reactive upregulation by the tumor process as observed in infectious diseases as active tuberculosis and bacterial meningitis (Cai et al., 2014; Mook-Kanamori et al., 2014).

Immunohistochemical staining shows the abundant presence of C1q in the vicinity of both malignant cells and necrotic debris in sections of GBM tissue. The presence of necrotic tissue, one of the main pathological characteristics and diagnostic features of GBM, could contribute to the C1q consumption (Jensen, 2009). C1q binding on necrotic or late apoptotic tumor cells induces complement activation and therefore deposition of specific complement components, which can act as opsonins and facilitate phagocytosis (Trouw et al., 2008; Gullstrand et al., 2009; Nauta et al., 2002; Martin et al., 2012). The presence of C1q in tumor tissue could either be beneficial or detrimental regarding tumor growth. C1q is able to induce apoptosis in malignant cells through activation of the tumor suppressor WOX1 as is shown for prostate cancer (Hong et al., 2009).

Preoperative low C1q concentration (25th percentile) was not significantly related to survival as compared to the 75th percentile. C1q deposition, either indirect or direct, can be attended with deposition of factor H, a soluble AP regulation protein (Yin et al., 2007; Kang et al., 2012). Factor H blocks AP C3-convertase and, as cofactor for factor I, it potentiates degradation C3b and C4b. However, a positive regulator of the alternative pathway, properdin, is also known to bind to late apoptotic or necrotic cells (Xu et al., 2008). In this perspective, we also observed factor B deposition in tumor slices. This deposition of factor B could contribute to the degradation of the ECM and therefore promote tumor invasion and migration (Andrades et al., 1996). However, tumor bearing factor B deficient mice did not show different tumor growth compared to wild-type controls (Markiewski et al., 2008). Nevertheless, our data suggests that factor B is actively consumed by the glial tumor process, which might imply a further activation of the complement cascade through the formation of AP C3-convertase. The three initiating pathways converge at the level of proteolytic cleavage of C3 into C3a and C3b after the formation of C3 convertases. Therefore it is important to investigate the presence of C3 in order to determine whether the continuation of the complement cascade is facilitated. Our GBM sections showed the profound presence of C3 deposition compared to sections of post-mortem healthy brain tissue. This deposited C3 initiated further complement activation as evidenced by deposition of the C5b-9 complex.

In conclusion, we show that serum concentration of critical components of the classical, lectin and alternative complement pathways are significantly altered in glial tumor patients, suggesting the involvement of the innate immune system in glial tumor pathology. In addition, the presence of C3 deposition and more specifically the deposition of the C5b-9 complex, suggests that glioblastoma tumor localized complement activation. Serum concentrations of these three critical proteins of initiation pathways of the complement system were not correlated with survival. However, the frequency of MBL deficiency was strongly reduced in GBM patients, which may implicate a protective effect against developing the disease. Future in-vivo research will have to reveal the exact influence of complement involvement on glial tumor progression as well as the possible consequence of MBL deficiency.

Acknowledgements

We thank J. Kloezeman, A. Kleijn and E.W. Levarht for their laboratory assistance. This project was financially supported by the Department of Neurosurgery, Erasmus MC, Rotterdam. L.T. is supported by a ZON-MW Vidi grant and by a fellowship from Janssen Biologics.

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