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Cytokine Networks and Survivin Peptide-Specific Cellular Immune Responses Predict Improved Survival in Patients With Glioblastoma Multiforme

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ABSTRACT

Purpose: We investigated serum cytokine and T-cell responses directed against tumour-associated antigens (TAAs) in association with survival of patients with glioblastoma multiforme (GBM).
Patients and Methods: Peripheral blood from 205 treatment-naïve patients with glioma (GBM = 145; non-GBM = 60) was obtained on the day of surgery to measure (i) circulating T-cells reacting to viral antigens and TAAs, in the presence or absence of cytokine conditioning with IL-2/IL-15/IL-21 or IL-2/IL-7, and (ii) serum cytokine levels (IL-4, IL-5, IL-6, TNF-α, IFN-γ and IL-17A). Patients were followed-up for at least 1000 days post-surgery. Survivin protein and gene expression in resected GBM tumour tissue were confirmed by immunohistochemistry and real-time polymerase chain reaction, respectively. Antigen-specific T-cell responses were gauged by ICS (intracellular cytokine production). Associations between patient survival and immunological reactivity patterns were analysed using univariate and multivariate statistics.
Results: Approximately 2% of patients with GBM and 18% of patients with non-GBM glioma, were alive beyond 1000 days of surgery. Univariate analysis indicated that the combination of three cytokines (IL-4/IL-5/IL-6, $p = .0022$; IFN-γ/TNF-α/IL-17A, $p = .0083$) but not a 'partial' combination of these cytokines, the IFN-γ immune response to EBV-EBNA-1 ($p < .0001$) as well as T-cell responses to the survivin$_{97-111}$ peptide ($p = .0152$) correlated with longer survival among patients with GBM. Multivariate analysis identified survivin$_{97-111}$-directed IFN-γ production with IL-2/IL-15/IL-21 conditioning ($p = .024$), and the combined presence of serum IFN-γ/TNF-α/IL-17a ($p = .003$) as independent predictors of survival.
Conclusion: Serum cytokine patterns and lymphocyte reactivity to survivin$_{97-111}$, particularly with IL-2, IL-15 and IL-21 conditioning may be instrumental in predicting survival among patients with GBM. This has implications for clinical follow-up of patients with GBM and the targeted development of immunotherapy for patients with CNS tumours.

1. Introduction

The World Health Organization (WHO) estimated that circa 2% of all human cancers occur in the central nervous system (CNS) [1]. The glioma WHO classification system includes (i) diffuse or anaplastic astrocytoma (isocitrate dehydrogenase (IDH)-wildtype/-mutant/not otherwise specified (NOS)); (ii) oligodendroglioma or anaplastic oligodendroglioma (IDH-mutant and 1p/19q-co-deletion/NOS); (iii) oligoastrocytoma or anaplastic oligoastrocytoma (NOS) and (iv) glioblastoma multiforme (GBM, IDH-wildtype/-mutant/NOS). GBM is the most common and aggressive clinical manifestation of glioma, with a 5-year survival rate of <3% compared to other primary gliomas, which have a 5-year survival rate of at least 50% [1, 2]. Patients diagnosed with primary GBM (IDH-wildtype) represent approximately 90% of cases, while those with secondary GBM (IDH-mutant) represent the

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remaining 10% [2]. The clinical outcome in patients with GBM remains poor despite advanced surgery, first-line temozolomide therapy and radiotherapy [3, 4], sometimes administered with adjunctive anti-vascular endothelial growth factor (VEGF) antibody (bevacizumab) immunotherapy [5].

Sporadic CNS inflammation has been attributed to GBM oncogenesis, with the involvement of pro-inflammatory and pleiotropic cytokines, e.g. interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin (IL) 6, IL-8, and IL-17A [6–8]. Non-specific inflammation may facilitate mutations in genes encoding proteins involved in the receptor tyrosine kinase pathway, including epidermal growth factor receptor (EGFR), as well as enzymes required for DNA repair and genetic stability [7]. The most frequent EGFR mutation associated with GBM is variant III of the receptor (EGFRvIII) [9, 10].

A key factor that contributes to the reduced survival of patients with GBM is the local immunosuppressive tumour microenvironment, including regulatory T-cell (Treg) induction and programmed cell death 1 (PD-1) expression [11–13]. Productive immune responses directed against tumour-associated antigens (TAA) i.e. EGFRvIII, survivin (encoded by baculoviral inhibitor of apoptosis repeat-containing 5, BIRC5 gene) or other antigenic targets are likely to be subdued. Production of immune-tolerising cytokines such as transforming growth factor beta (TGF-β), IL-10, IL-4 and IL-13, as well as the expression of the IL-13 receptor, which itself is a target for GBM immunotherapy [14] play a critical role in the balance of protective and pathological immune responses [15–17]. Temozolomide itself contributes to intratumoural immune suppression, affecting tumour-infiltrating lymphocyte (TIL) numbers as well as anti-tumour immune responses [18]. Adjunct immunotherapies e.g. tumour vaccination of patients with GBM [19] or the application of T cells expressing chimeric antigen receptors (CARs) targeting EGFRvIII (clinical trials identifier: NCT02209376), IL-13 Rα2 [14], or Her2 (clinical trials identifier: NCT02442297) are currenty tested in patients for safety and to improve treatment outcomes.

We studied immune responses in peripheral blood of patients using the determination of cytokines (by ELISA) and immune reactivity to specific target antigens defined by cytokine production to gain a better understanding of the global immune reactivity pattern in association with survival in patients with GBM.

2. Material and Methods

2.1. Patient Characteristics

The study was approved by the Regional Ethics Review Board (Regionala etikprövningsnämnden) at Karolinska Institutet, Stockholm (ethical permit number: 2013/576-31). 205 patients with glioma were selected to participate in the study, following written informed consent. The largest group comprised patients with GBM (WHO grade IV CNS tumour, n = 145), while patients with non-GBM glioma (n = 60) comprised individuals diagnosed with astrocytoma, oligodendroglioma/oligoastrocytoma or anaplastic oligoastrocytoma (WHO grades II-III CNS tumours) [2]. Venous blood for laboratory studies was drawn on the day of surgery and prior to initiation of cancer therapy. A description of the patient cohort is provided in Table 1.

Table 1
Summary of the clinical characteristics of patients with glioma included in this study. GBM: Glioblastoma multiforme.

Patient characteristics	Glioma				
	Histology		Grade		
	GBM	Non-GBM	IV	III	II
Sample size(N)	145	60	145	18	42
Age median(years)	63	40	63	38	42
Age range(years)	16–80	20–75	16–80	22–62	20–75
Sex(male/female)	99/46	38/22	99/46	10/8	28/14

2.2. Whole Blood Assay (WBA)

Venous blood from the patients with glioma was first diluted at a ratio of 1:1.5 with RPMI 1640 Glutamax medium (ThermoFisher Scientific, Carlsbad, CA) and supplemented with antibiotics (penicillin, 100 IU/ml and streptomycin, 100 μg/ml) (ThermoFisher Scientific, Carlsbad, CA). The diluted blood was then conditioned in following manner: i) without cytokines (RPMI only); ii) human IL-7 (10 ng/ml) and IL-2 (500 IU/ml) or iii) human IL-2 (1000 IU/ml), IL-15 (10 ng/ml) and IL-21 (10 ng/ml) (Prospec, Ness-Ziona, Israel) and added to 96-well microtiter plates containing a panel of tumour-associated antigens (TAA) and viral antigens (Supplementary Table S1). The survivin$_{97-111}$ peptide (TLGEFLKLDRERAKN) was tested separately since it induced superior immune reactivity in circulating lymphocytes and TIL in an initial screening test. The plates were incubated at 37 °C with 5% CO_2 for seven days. Incubation with medium alone was used as negative control while 5 μg/ml phytohaemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO), 30 ng/ml OKT3 (anti-human CD3 monoclonal antibody, Biolegend, CA) and 10 ng/ml SEA+SEB (Staphylococcal Enterotoxin A and B, Sigma-Aldrich, St. Louis, MO) were used separately as positive controls.

2.3. Blood Serum Preparation and Cytokine ELISA

For plasma preparation, a fraction of whole blood was layered onto Ficoll-Paque Plus solution (GE Healthcare, Uppsala, Sweden) and centrifuged at 1260 ×*g* for 10 min. The resulting layer of serum was removed and stored at −80 °C. Cytokines (IFN-γ, TNF-α, IL-17A, IL-4, IL-5 and IL-6) as well as WBA supernatants (IFN-γ production by circulating lymphocytes) were quantified with commercially available enzyme-linked immunosorbent assay (ELISA) kits (MABTECH, Stockholm, Sweden) according to the manufacturer's instructions.

2.4. Immunohistochemistry

Immunostaining for survivin was performed on 4 μm sections of formalin-fixed paraffin-embedded tissue using the Leica Bond-Max automated immunostaining system (Leica Biosystems AB, Kista, Sweden). For antigen retrieval, samples were incubated for 20 min at 100 °C with Bond Epitope Retrieval Solution 1 (Leica Biosystems AB, Kista, Sweden). Slides were stained for 30 min at room temperature with the survivin polyclonal antibody RB-9245 (Thermo scientific, Carlsbad, CA), diluted at 1:200. The percentage of positive cells was evaluated using a semi-quantitative score: 1+ <10%, 2+ = 10–20%, 3+ = 20–50% and 4+ >50% (the high-expression group refers to scores 4 and 3, while the 'low-expression' group includes scores 2 and 1).

2.5. Real-Time Polymerase Chain Reaction (RT-PCR)

Qualitative BIRC5 PCR: Total RNA was extracted from flash-frozen tumour specimens obtained during surgery. The tumour tissue was lysed using Qiazol tissue lysis reagent (Qiagen inc. Hilden, Germany) and total RNA was obtained by ethanol precipitation according to supplier's instructions. 5 μg total RNA was subjected to reverse transcription using an Oligo DT protocol of a RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific inc. Waltham, MA, USA) according to the manufacturer's instructions. 10 μl cDNA from each cDNA reaction was used to amplify the BIRC5 variant specific product in a 20 μl PCR reaction using BIRC5 commercially available transcript specific primers published previously [20] and according to the manufacturer's instructions (ThermoFisher Scientific, Carlsbad, CA). β-actin was used as positive control for housekeeping gene transcription. The PCR reaction was initiated at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Difference in cycle threshold (delta cycle threshold, ΔCt) for each sample refers to the difference between Ct values of BIRC5 and β-actin. Cut-off for values of Ct and ΔCt is lower than 35 and 10 respectively.

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