Enhanced neutrophil activity is associated with shorter time to tumor progression in glioblastoma patients

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ABSTRACT
Glioblastoma multiforme (GBM) is a highly malignant tumor with a poor outcome that is often positive for human cytomegalovirus (HCMV). GBM patients often have excessive numbers of neutrophils and macrophages near and within the tumor. Here, we characterized the cytokine patterns in the blood of GBM patients with and without Valganciclovir treatment. Furthermore, we determined whether neutrophil activation is related to HCMV status and patient outcome. Blood samples for analyses of cytokines and growth factors were collected from 42 GBM patients at the time of diagnosis (n = 42) and at weeks 12 and 24 after surgery. Blood neutrophils of 28 GBM patients were examined for CD11b expression. The levels of pro- and anti-inflammatory cytokines and chemokines—including interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-17A, transforming growth factor (TGF)-β, interferon-γ, interferon-α, tumor necrosis factor α, and monocyte chemotactrant protein (MCP)-1—were analyzed with a bead-based flow cytometry assay. During the first six months after surgery, neutrophil activity was increased in 12 patients and was unchanged or decreased in 16. Patients with increased neutrophil activity had enhanced IL-12p70, high grade HCMV and a shorter time to tumor progression (TTP) than patients without or decreased neutrophil activity (median TTP; 5.4 vs. 12 months, 95% confidence interval; 1.6–10 vs. 0.1–6, hazard ratio = 3 vs. 0.4, p = 0.004). The levels of IL-12p70 were significantly decreased in Valganciclovir treated patients (n = 22, T 12W vs. T 24W, p = 0.03). In conclusion, our findings suggest that neutrophil activation is an early sign of tumor progression in GBM patients.

Abbreviations: GBM, glioblastoma; HCMV, human cytomegalovirus; IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; NK, natural killer; OS, overall survival; TGF, transforming growth factor; TTP, time to tumor progression; VIGAS, Efficacy and Safety of Valcyte as an Add-on Therapy in Patients with Malignant Glioblastoma and Cytomegalovirus Infection

Introduction
Glioblastomas multiforme (GBM) is the most frequent and malignant brain tumor in adults. In Sweden, GBM is diagnosed in 300–500 people annually. Despite a tremendous increase in knowledge concerning the molecular and biological characteristics of GBM, the prognosis remains dismal.1 The median survival time is 12–15 months and less than 3% of patients survive more than 5 years.2-4 Numerous studies have reported that GBM patients are immunosuppressed, as exemplified by decreased function of natural killer (NK) cells and T cells and high peripheral release of both TGF-β and prostaglandins.5-8 GBMs produce high levels of TGF-β, a potent immunosuppressive cytokine that may inhibit the infiltration of immune cells and prevent an efficient antitumor response.

Despite their immunosuppressive state, patients with GBM often have an increase in circulating neutrophils.9 An increased number of neutrophils in the margins of these tumors has been linked to increased malignancy.9,10 Infiltrated neutrophils can release elastase, which can damage surrounding tissue and may promote tumor invasion.10 In addition, glioma cells have been reported to produce survival factors for neutrophils, such as interleukin (IL)-6 and IL-8, resulting in a delay in their spontaneous apoptosis.11

Increasing evidence suggests that HCMV is linked to several forms of cancer.12,13 An active HCMV infection has been reported in 90–100% of GBM,14-16 medulloblastomas,17 neuroblastomas,18 colon cancers,19 breast cancers,20-23 and prostatic carcinomas.24 HCMV is a common virus that causes life-long latency after a primary infection and is carried by 60–90% of the world’s population.25 The role of HCMV in cancer development is unclear and under debate. HCMV is considered to be an onco-modulatory rather than an oncogenic virus. It encodes numerous viral proteins that can dysregulate pivotal cellular functions that are important in oncogenesis, including cellular...
differentiation, cell cycle control and proliferation, induced oncogene expression, epigenetic functions, induced migration and angiogenesis, DNA repair mechanisms, and inhibition of apoptosis.\textsuperscript{13,26-30} Through HCMV’s unique ability to avoid detection and elimination by the immune system, virus-infected tumor cells may be difficult to eliminate. We and others have reported that 90–100% of GBMs are infected by HCMV, and we found that the level of HCMV infection in these tumors is a prognostic factor for patient survival.\textsuperscript{15}

In a randomized, double-blind study aiming to evaluate the safety and efficacy of antiviral treatment of HCMV in GBM patients (the VIGAS study), we found that the median overall survival (OS) was similar in Valganciclovir treated and placebo groups (17.9 vs. 17.4 months, \( p = 0.430 \)). Patients could take Valganciclovir for compassionate use after the study phase and explorative analyses showed prolonged survival in patients who were treated with Valganciclovir for more than 6 months (OS 24.1 months).\textsuperscript{31} Several GBM patients were thereafter prescribed Valganciclovir as add on to standard therapy at our hospital. In a retrospective study of the outcome of these patients, we found that GBM patients who received anti-HCMV treatment in addition to standard therapy had significantly prolonged survival, especially those who had received continuous Valganciclovir treatment (56.4 months vs. 13.5 months, \( P < 0.001 \)).\textsuperscript{31,32} These observations suggest that HCMV may play an important role in the pathogenesis of GBM and that this virus may serve as a unique target for GBM therapy, which needs to be addressed in a randomized trial.

HCMV infection of neutrophils results in activation of these cells and increased resistance to apoptosis.\textsuperscript{33} Cytokines such as IL-6, IL-8, interferon-\(\gamma\), and TNF-\(\alpha\) are induced by HCMV infection and may contribute to neutrophil activation and survival.\textsuperscript{34} In this study, we assessed the activation state of peripheral blood neutrophils in HCMV-positive GBM patients included in the VIGAS trial. We measured cytokine and chemokine levels before and during antitumor treatment and antiviral treatment and searched for correlations between neutrophil activation and cytokine levels as well as to TTP and OS.

Results

Neutrophil activation is associated with shorter TTP. Activation and recruitment of neutrophils to the tumor site have been reported in patients with glioblastoma and may correlate with greater malignancy.\textsuperscript{3} In addition; the number of circulating neutrophils is often increased in GBM patients.

We found that the number of neutrophils was significantly decreased over time in Valcyte treated patients (T0 vs. T12W; \( p = 0.01 \), T0 vs. T24W; \( p = 0.0004 \), T12W vs. T24W; \( p = 0.04 \)). In the placebo cohort, the number of neutrophils was also significantly decreased from T0 to T24W (\( p = 0.0002 \)). However, the decreased number of neutrophils in the placebo cohort was not statistically significant at T0 vs. T12W (\( p = 0.16 \)) and T12 vs. T24W (\( p = 0.14 \)) (Fig. S1). Information regarding neutrophil count was not available from four patients in the placebo cohort.

Previously, we showed that HCMV infection activates neutrophils.\textsuperscript{32} Here, we characterized the activation status of peripheral neutrophils in 28 of 42 GBM patients in the VIGAS trial as measured by the induced surface expression of CD11b, at T = 0, 3, and 6 months as well as in healthy controls (\( n = 6 \)). All GBM patients were diagnosed with an HCMV infection in their tumor, as required for inclusion in the VIGAS study. However, as previously reported, 29% (12/42) of them were HCMV IgG negative, as shown by enzyme-linked immunosorbent assay. Nevertheless, blood T cells from 85% of these patients were reactive against HCMV (immediate early and pp65 peptides), and their blood T cells were positive for HCMV DNA.\textsuperscript{35}

Over time, neutrophil activity was enhanced in 12 patients and was unchanged or decreased in 16 patients (Group I; GI with enhanced neutrophil activity: T0 vs. T24W: \( p < 0.0001 \) and T12W vs. T24W: \( p < 0.0001 \), Group II; GII with unchanged or decreased neutrophil activity: T12W vs. T24W: \( p = 0.014 \), Fig. 1A). Patients with enhanced neutrophil activity (Group I) had significantly shorter TTP than patients with unchanged or decreased neutrophil activity (Group II) (median TTP: 5.4 vs. 12 months, 95% confidence interval (CI); 1.6–10 vs. 0.1–0.6, hazard ratio= 3 vs. 0.4, \( p = 0.004 \)) (Fig. 1B). During the first 6 months after the first surgery, 10 (36%) of 28 patients had a tumor recurrence, and 7 (70%) had high neutrophil activity at the time of recurrence. OS tended to be shorter in patients with enhanced neutrophil activity but did not reach statistical significance in this small patient cohort (16.4 vs. 19.5 months, respectively; 95% CI; 0.9–5.6 vs. 0.2–1, hazard ratio = 2 vs. 0.4, \( p = 0.07 \)) (Fig. 1C). All patients received antiviral treatment (Valganciclovir) except for two patients in Group I and three patients in Group II who received placebo treatment. We noted a high grade of HCMV infection (grade 3 and 4) in brain tumor tissues from all patients in Group I but in only 69% of patients in Group II (Fig. 1D, \( p = 0.047 \)). This finding suggested enhanced neutrophil activity in patients with higher HCMV activity. However, none of the neutrophils examined for HCMV infection expressed pp65, as shown by immunofluorescence staining (data not shown). Due to the small number of patients without antiviral treatment, we could not evaluate the effect of Valganciclovir treatment on neutrophil activity in this cohort.

Despite their higher neutrophil activity, GBM patients are considered to suffer from a general immunosuppression characterized by high levels of the strong immunosuppressive cytokine TGF-\(\beta\), among others. Interestingly, we found that the levels of IL-12p70, which has been shown to activate neutrophils,\textsuperscript{35} was significantly increased only in Group I patients who had enhanced neutrophil activation (T0 vs. T24W, \( p = 0.002 \), Fig. 2A).

The levels of the neutrophil attractants IL-8 and MCP-1 were significantly elevated over time in patients in both Group I and Group II compared with healthy controls (HC) (IL-8: GI: T0 vs. HC, \( p = 0.0009 \); T12W vs. HC, \( p = 0.008 \) and T24W vs. HC, \( p = 0.0009 \) and GII: T0 vs. HC, \( p < 0.0001 \); T12W vs. HC, \( p = 0.0002 \) and T24W vs. HC, \( p = 0.0007 \)) (Fig. 2B) (MCP-1, GI: T0 vs. HC, \( p = 0.0002 \); T12W vs. HC, \( p = 0.0002 \); T24W vs. HC, \( p = 0.0002 \) and GII: T0 vs. HC, \( p = 0.0002 \); T12W vs. HC, \( p = 0.0002 \) and T24W vs. HC, \( p = 0.0005 \)) (Fig. 2C). However, the level of IL-6 and IL-8 were deceased at 12 weeks (\( p = 0.02 \) and \( p = 0.04 \), respectively, Fig. 2B) only in Group II
who had decreased or unchanged neutrophil activity (Fig. 2B, D). The levels of MCP-1 did not differ between GI and GII groups (Fig. 2C and Fig. S2). Other examined factors did not differ between the two groups (Fig. 2E, F and Fig. S2).

All 42 GBM patients in the VIGAS study were examined for plasma levels of cytokines and growth factors at baseline (T0, n = 42) and after antitumor treatment for 12 weeks (T12, n = 37) and 24 weeks (T24, n = 35). Specifically, we measured 12 pro- or anti-inflammatory cytokines that are vital in regulating the immune response: IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-17A, interferon (IFN)-α, IFNγ, TNF-α, TGF-β, and MCP-1. The median levels of IFNγ IL-2, IL-10, and IL-17A were equal with zero both in GBM patients and HC (Fig. 3A–F). The median levels of IL-1β, IL-6, IL-8, IL-12p70, IFN-α TNF-α, MCP-1, and TGF-β (but not IFNγ, IL-2, IL-10, and IL-17A) were higher at baseline in GBM patients than in healthy controls (Fig. 3A–F). The median levels of all examined cytokines except for IL-8, MCP-1, TGF-β were below detection levels in healthy controls. During the 24 weeks of antitumor treatment, GBM patients had significantly decreased levels of MCP-1 (T0 vs. T12, p = 0.006; T0 vs. T24, p < 0.0001, T12 vs. T24, p = 0.04), and IL-6 (T0 vs. T12, p = 0.04) and increased levels of IL-12p70 (T0 vs. T12, p = 0.04) (Fig. 3B–C, E–F). The levels of IL-1β, IL-2, IL-8, IL-10, IL-17A, IFNγ, and TNF-α were unchanged during follow-up (Fig. 3A, D).

Among the VIGAS patients, 22 patients were randomized to receive antiviral treatment (Valganciclovir) and 20 patients to placebo (in the intention-to-treat protocol) during the 24-week study phase. The cytokine profiles in these two groups did not differ (Fig. 4A, D, F, G and Fig. S3), except for IL-12p70 and IL-8. IL-12p70 levels decreased significantly in Valganciclovir-treated patients (T12 vs. T24, p = 0.03, Fig. 4E) but increased significantly in placebo patients (T0 vs. T24, p = 0.004) (Fig. 4C). The level of IL-8 decreased only in the placebo group at 12 weeks (T0 vs. T12, p = 0.008) (Fig. 4B). All GBM patients had short term corticosteroid treatment, high dose pre- and directly post-surgery. The postoperative corticosteroid intake was thereafter rapidly decreased to a minimum effective dose to control cerebral edema. Cytokine data collected from placebo and Valganciclovir treated patients at base line, 12 weeks and 24 weeks post-surgery did not demonstrate any major differences...
over time except for what is described above, suggesting a limited effect by corticosteroid treatment (data not shown).

Discussion

The results of this study further support the potential role of neutrophils in the pathogenesis of GBM. We found that activation of neutrophils was associated with higher plasma levels of IL-12p70 and a shorter TTP; patients with high neutrophil activity had a significantly shorter TTP and also tended to have shorter OS. Of 10 patients who had a tumor recurrence during the first 6 months, 7 (70%) had high neutrophil activity at the time of recurrence. Thus, activation of peripheral neutrophils may be an early sign of tumor progression and may be related to a more aggressive tumor. In either case, activation of neutrophils may be a useful biomarker of disease progression and should be further investigated for potential clinical impact.

Despite the activation of neutrophils, GBM patients often have a sustained general immunosuppression characterized by a decreased function of cytotoxic T cells and NK cells. These patients can also be lymphopenic, and their peripheral T cells may be reduced in number as a result of their disease and its treatment. Tumor cells can also influence the immune system by secreting cytokines, such as TGF-β and IL-10, that inhibit T cells, dendritic cells and NK cells—thereby enhancing the ability of the virus to promote its own survival. In GBM patients, we assessed the levels of 12 pro-inflammatory and immunosuppressive cytokines and chemokines that can modulate the immune response, including factors that activate neutrophils. We found that GBM patients (n = 42) had elevated levels of IL-1β, IL-6, IL-8, IL-12 p70, TNF-α, IFN-α, TGF-β, and MCP-1. The levels of IFNγ, IL-2, IL-10, and IL-17A were similar in GBM patients and healthy controls. Several of these factors are affected by HCMV infection, and most GBMs are positive for HCMV. Only levels of TGF-β1, MCP-1, and IL-6 were significantly decreased during antitumor treatment, and only IL-12p70 during anti-viral treatment.

IL-1, IL-6, IL-8, IL-12p70, and MCP-1 can induce the migration, activation, and respiratory burst of neutrophils. Among the examined cytokines in all GBM patients (n = 42) included in the VIGAS study, only IL-12p70 levels were increased in patients at 12 weeks after surgery. The level of IL-12p70 increased over time in the GI group, which may explain higher neutrophil activity in this group. Neutrophils have been described as an important source of different cytokines including IL-12 p70. This cytokine can be released during infections previous studies have reported induction of
IL-12 during Herpes Simplex virus infection.\textsuperscript{43-46} In our study, all patients with high neutrophil activity ($n = 12$) had high-grade HCMV infection in their GBMs, while 69% of patients without high neutrophil activity had high grade infection.

In theory, higher HCMV activity can directly affect and activate neutrophils,\textsuperscript{33} or induce neutrophil-activating factors such as IL-12p70. In support of this notion, IL-12p70 levels were lower in Valganciclovir-treated patients than in placebo-treated patients; Valganciclovir-treated patients would be expected to have less HCMV activity and subsequently less inflammation at the tumor site. Secretion of high levels of IL-12p70 by APCs and activated neutrophils may further increase neutrophil activity and migration to the tumor site and thereby promote tumor progression in the absence of an activation of an

\begin{figure}
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\caption{Median levels of 12 cytokines/chemokines, and inflammatory factors in plasma of all GBM patients over time and in healthy controls (HC) (3A–F). The median levels of IL-1β, IL-6, IL-8, IL-12p70, IFN-α, TNF-α, MCP-1, and TGF-β (but not IFNγ, IL-2, IL-10, and IL-17A) were higher at baseline in GBM patients than in healthy controls (Fig. 3A–F). During the 24 weeks of antitumor treatment, GBM patients had significantly decreased levels of IL-6 (T0 vs. T12, $p = 0.04$) (B) and increased levels of IL-12p70 (T0 vs. T12, $p = 0.04$) (C), significantly decreased levels of MCP-1 (T0 vs. T12, $p = 0.008$; T0 vs. T24, $p = 0.03$) (E) and TGF-β (T0 vs. T12, $p = 0.0006$ and T0 vs. T24, $p < 0.0001$, T12W vs. T24W, $p = 0.04$) (F).}
\end{figure}
Figure 4. Median levels of 12 examined pro-inflammatory cytokines and chemokines in the plasma of GBM patients treated with valganciclovir (V) or placebo (P) over time (A–I). The cytokine profiles in these two groups did not differ (Fig. 4A, D, F, G, H, I), except for IL-12p70 and IL-8. IL-12p70 levels decreased significantly in valganciclovir-treated patients (T12 vs. T24, p = 0.03) (E) but increased significantly in placebo patients (T0 vs. T24, p = 0.004) (C). The level of IL-8 decreased only in the placebo group at 12 weeks (T0 vs. T12, p = 0.008) (B).
Materials and methods

Patient samples. Forty-two consecutive GBM patients were enrolled in a randomized, double-blind study (VIGAS) to evaluate the safety and efficacy of antiviral treatment of HCMV in HCMV-positive GBM patients (ethical permission 2006/755–31). The study was registered at the Swedish Medical Agency (Eudra number 2006–002022-29) and at ClinicalTrials.Gov (Identifier NCT00400322). Inclusion criteria were age >18 years, diagnosis of GBM, HCMV infection in the tumor, and successful radical surgery (estimated tumor removal >90%). After surgery, patients received standard chemotherapy (temozolomide) and were randomized to receive oral anti-HCMV treatment (Valganciclovir) or placebo. Valganciclovir was given twice daily (900 mg/day) for 3 weeks and then twice daily (450 mg/day) for 21 weeks. Study patients in whom standard treatment failed were allowed to receive prescribed Valganciclovir. All patients were monitored for tumor progression and OS time.

Blood and plasma samples were collected once from six age matched healthy subjects and three times from patients: at the time of diagnosis (n = 42) and at 3 (n = 37) and 6 months (n = 35) after diagnosis. Plasma samples were stored at –85°C until further use. Neutrophils were purified from fresh blood collected once from six healthy subjects and three times from patients: at the time of diagnosis (n = 28) and at 12 (n = 28) and 24 (n = 26) weeks after diagnosis.

Seralogical screening for HCMV-IgG and HCMV-IgM. Plasma was examined for HCMV IgG and IgM with Enzygnost anti-HCMV-IgG and HCMV-IgM serological assay kit; OWBK15, Dade Behring, Germany) according to the manufacturer’s instructions. IgG and IgM results have been reported elsewhere.28

Isolation of human peripheral blood neutrophils. Neutrophils were isolated according to the Böyum method.48 Briefly, 25 mL of whole blood was layered on 20 mL of Polymorphpre and 5 mL of Lymphoprep (both from Medinor, Stockholm, Sweden; Lymphoprep: Cat. 1114547 and Polymorphpre: Cat. 1114683) and centrifuged for 40 min at 480 g at room temperature. The polymophonuclear lymphocytes (neutrophils) rich band was collected and contaminating erythrocytes were removed by hypotonic lysis. Cells were washed twice in Krebs-Ringer’s phosphate buffer (KRG) without Ca²⁺ and Mg²⁺ and suspended in KRG.

Measurements of surface expression levels of CD11b on neutrophils. One million cells in 100 µL of KRG were incubated with or without N-formyl-methionyl-leucyl-phenylalana nine (fmlp, final concentration, 0.1 M) for 10 min at 37°C and then with or without 5 µL of anti-CD11b antibodies, IgG1 isotype control (both from DakoCytomation, Denmark, anti-CD11b antibodies: Cat. R0841and IgG1 antibodies, Cat. XV0928) for 30 min on ice. Cells were washed in KRG, suspended in 0.1% parformaldehyde, and analyzed by flow cytometry (FACSCalibur, BD Pharmingen) and CellQuest software. Gates were set to exclude contaminating lymphocytes, monocytes, erythrocytes, and cellular debris. Percent CD11b up-regulation on blood neutrophils was calculated by subtraction of mean channel values for fmlp activated neutrophils from mean channel values for neutrophils without activation, divided with mean channel values for unactivated neutrophils.

Quantification of cytokines in plasma samples. Sera from all patients and six healthy controls were thawed on ice, and the levels of IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17, IL-10, TNF-α, IFN-α IFNy, MCP-1, and TGF-β were measured with FlowCytomix (Bender MedSystems), a custom bead-based flow cytometry assay (FACSCalibur, BD Pharmingen), and CellQuest software. The data were analyzed with FlowCytomixPro 1.0 Software (Bender MedSystems).

Antigenemia assay. Purified blood neutrophils were counted and spotted on slides with a Cyto spun centrifuge. Cells were fixed in a solution of saccharose, formalin, and phosphate-buffered saline (Substrate Department, Karolinska University Hospital) for 10 min; permeabilized in detergent solution consisting of saccharose, NP40, fetal calf serum, and phosphate-buffered saline (Substrate Department, Karolinska University Hospital) for 5 min; and washed in phosphate-buffered saline. The cells
were incubated with antibodies against HCMV pp65 (Argene, France, Cat. 11-002). HCMV pp65 was detected with immuno-fluorescently labeled goat anti-mouse antibodies (DakoCytomation, Denmark, Cat. F0313). Human fibroblasts infected with HCMV strain AD169 were used as positive control. All slides were analyzed by fluorescence microscopy.

Immunohistochemical staining. Paraffin-embedded brain tumor tissues from all patients were stained for HCMV immediate-early protein (Millipore, Stockholm, Sweden, Cat. 810R) and graded (1+; 2+; 3+; 4+) according to the estimated number of tumor cells expressing immediate-early protein, as described.\(^\text{13,20}\)

Statistical analyses. OS and TTP were analyzed with Cox regression models; the covariates were enhanced neutrophil activity (Group I) or decreased/unchanged neutrophil activity (Group II). All patients who were alive or did not have progression at the time of investigation were censored in the analysis (August 23, 2012). The results are presented as hazard ratios with 95% confidence intervals. Survival graphs were created with the Kaplan–Meier method. The median times to end points were estimated from the Kaplan–Meier curves, with 95% CIs. Values are expressed as medians, and statistical significance was tested by one-way ANOVA, Wilcoxon matched pairs and Mann–Whitney U test and Kruskal–Wallis test. \(P < 0.05\) was considered significant.

Disclosure of potential conflicts of interest

C.S.N. has received speaker’s fees and sponsored travel costs to present data on the indirect molecular effects of HCMV at scientific meetings over 6 years ago.

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