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Clinical Study

Preoperative biomarkers of tumour vascularity are elevated in patients with glioblastoma multiforme

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ABSTRACT

We investigated the correlation between the circulating and imaging biomarkers of tumour vascularity, and examined whether they are prognostic of outcomes in patients with glioblastoma multiforme (GBM). Despite the increasing use of anti-angiogenic agents within neuro-oncology, there are still no validated biomarkers to monitor for a treatment response or relapse. The pre- and postoperative circulating endothelial cell (CEC) and progenitor cell (CEP) levels were assessed. Preoperative perfusion-weighted MRI (PWI) was also performed, and the relative cerebral blood volume (rCBV) histogram statistics of the contrast-enhancing tumour were analysed. A novel PWI parameter ($rCBV_{load}$) was developed to estimate the total volume of perfused tumour vessels, and it was hypothesised that this parameter would correlate with CEC and CEP concentrations. In total, 24 GBM patients were included. The mean preoperative CEC concentration was significantly higher in GBM patients than the controls ($p = 0.019$), and it then declined significantly postoperatively ($p = 0.009$). The preoperative CEP levels were significantly correlated with the median tumour rCBV (Spearman rank-order coefficient = 0.526; $p = 0.039$). Neither CEC nor CEP was correlated with the total tumour vessel volume, as measured by $rCBV_{load}$. None of the biomarkers that were investigated showed a significant correlation with progression-free or overall survival. We conclude that CEC are potentially useful biomarkers to monitor GBM patients during treatment. We found that CEC are increased in the presence of GBM, and that CEP levels appear to be proportional to tumour vascularity, as measured on PWI. However, in this study, none of the biomarkers of GBM vascularity were highly prognostic of patient outcomes.

1. Introduction

Glioblastoma multiforme (GBM) is the most common primary intracerebral malignancy and despite modern treatment regimens, survival remains poor. It is amongst the most vascularised of human cancers, and increased tumour vascularity has been shown to correlate with prognosis [1–4]. Despite the increasing clinical use of anti-angiogenic agents such as bevacizumab (Avastin; Genentech, San Francisco, CA, USA), a monoclonal antibody that inhibits vascular endothelial growth factor (VEGF), there are still no clinically validated biomarkers available to monitor the treatment response or relapse. The histological microvessel density has possible value as a predictor in cancer, however, its usefulness is limited by its invasive nature, its protracted processing time, and by its vulnerability to sampling error and interobserver variability [5,6]. Standard MRI using gadolinium contrast also has limited utility, because of the direct effects on tumour vascularity and the blood brain barrier of anti-angiogenic therapy, leading to the phenomenon of pseudo-response [7]. Therefore, there is a great need for, and interest in, the development of novel imaging and other minimally invasively biomarkers of GBM response to anti-angiogenic therapy.

As well as providing a physical barrier between the intra- and extravascular compartments, endothelial cells play an important role in blood vessel formation, coagulation, fibrinolysis, regulation of vascular tone, and the inflammatory process. Circulating endothelial cells (CEC) are those mature endothelial cells which have become detached and enter the bloodstream, whilst circulating endothelial progenitor cells (CEP) are mobilised from bone

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marrow in response to various angiogenic stimuli, including VEGF, granulocyte-macrophage colony-stimulating factor, and serum-derived endothelial factor-1. Previous attempts have been made to investigate the association of CEC and CEP levels with the presence of various cancers, but the reliability of these biomarkers has not yet been established. In preliminary reports, raised CEP levels have been reported to correlate with tumour grade and prognosis in glioma patients [8,9].

Perfusion-weighted MRI (PWI) is a technique for simultaneous quantification of cerebral blood flow and cerebral blood volume (CBV) [10–12]. Although the absolute quantification of cerebral blood flow and CBV can be problematic in brain tumour patients, it has been shown that the ratio of tumour CBV to normal-appearing white matter (NAWM) CBV, known as the relative CBV (rCBV), is reproducible and potentially allows for the differentiation of tumour grades [13,14]. Consistent with their marked vascular proliferation, GBM have been demonstrated to have regions of significantly elevated rCBV [15].

In this study, we aimed to investigate the relationship between CEC, CEP, rCBV and survival in a cohort of patients with GBM. We hypothesised that these biomarkers of vascularity would be associated with a poorer prognosis. We also postulated that the levels of CEC and CEP, as an indicator of increased tumour endothelial activity, would correlate with the total volume of perfused tumour vessels, as assessed by PWI. To do this, we needed to derive a novel PWI parameter representing the total volume of blood vessels within the tumour. We termed this $rCBV_{load}$, which was estimated by integrating the rCBV values within all voxels of the T1-weighted contrast-enhancing tumour volume.

2. Methods

2.1. Patient selection

The study participants were recruited prospectively from the Department of Neurosurgery at The Royal Melbourne Hospital, Victoria, Australia. The inclusion criteria were patients aged over 18 years, with a diagnosis of GBM confirmed on histopathology. The exclusion criteria were a history of other malignancy, infection or pregnancy. The study was conducted with institutional Human Research Ethics Committee approval, within the guidelines set by the National Health and Medical Research Council of Australia, and all participants consented to the study. Progression-free survival (PFS) was defined as the time from the initial histological diagnosis to that of recurrence or progression, based on the follow-up imaging. Overall survival (OS) was determined by the time from the initial histological diagnosis to death. The mortality data were obtained from the hospital medical records and the Australian Comprehensive Cancer Outcomes and Research database, a multi-institutional database of cancer patient demographics, treatment and clinical outcomes managed by BioGrid Australia (Parkville, VIC, Australia). It includes longitudinal data on multiple tumour types including brain, breast, lung, colorectal and haematological malignancies. The central nervous system (CNS) tumour database, from this group of datasets, prospectively enrols all patients with CNS tumours, who are treated at The Royal Melbourne Hospital and the co-located Melbourne Private Hospital.

2.2. CEC analyses

The patients consented preoperatively for the collection of both pre and postoperative blood samples. Blood samples were also taken from healthy volunteers without any known malignancy or pregnancy, to serve as controls. Peripheral blood was obtained via a venepuncture of the antecubital fossa and collected into ethylenediaminetetraacetic acid-containing tubes. The samples were stored at 4°C prior to the analysis of endothelial cell concentrations. Peripheral blood mononuclear cells (PBMC), including CEC and CEP, were isolated from peripheral blood samples via density gradient centrifugation using Lympholyte-H separation medium (Cedarlane Laboratories, Burlington, Ontario, Canada), as per the manufacturer's instructions, including multiple washings to remove platelets. Isolated PBMC were incubated with the Fc receptor blocking agent 2.4G2 rat anti-mouse FcγRII/III (FcR Blocking Reagent, Miltenyi Biotec, Bergisch Gladbach, Germany), and labelled with appropriate fluorochrome-labelled monoclonal antibodies for the identification of CEC and CEP by flow cytometry, as per the manufacturer's instructions. The anti-human mouse antibodies used were CD45-allophycocyanin (APC) and CD31-fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ, USA), and CD133/1-APC (Miltenyi Biotec). The cells were acquired using either a CyAn ADP (Beckman Coulter, Brea, CA, USA) or FACSCalibur (BD Biosciences) flow cytometer, and analysed with Kaluza 1.1 software (Beckman Coulter). Dead cells were excluded by propidium iodide uptake, and red cells and platelets were further excluded by size (Fig. 1). CEC were defined as CD45-negative/CD31-positive ($CD45^-/CD31^+$) events, whilst CEP were defined as CD31-positive/CD133-positive ($CD31^+/CD133^+$) events.

2.3. MRI

All patients underwent PWI as part of a standard brain tumour MRI protocol, on one of three different scanning machines at either 1.5T or 3T. Although the specific MRI protocols differed slightly between patients, they typically included T1-weighted pre- and postcontrast agent administration, fluid-attenuated inversion recovery, diffusion-weighted, and T2-weighted scans. Dynamic susceptibility-weighted MRI was used for PWI, whereby dynamic MRI were acquired during a bolus intravenous administration of contrast agent. A pragmatic attempt was made to acquire the data in accordance with previous recommendations [16], whilst keeping the total contrast agent dose within the recommended limits, and obtaining an acceptable slice coverage and image signal-to-noise ratio. To reduce the effect of contrast leakage on CBV calculations, a 5 mL bolus of gadolinium contrast agent (Magnevist; Bayer Healthcare, Leverkusen, Germany) was administered to the patient, 5 min prior to PWI acquisition, at a rate of 1 mL/s, followed by a 15 mL saline flush. For PWI, dynamic gradient-echo echo-planar images were then acquired every 1.5 to 3 s. After approximately 10–15 s of scanning, a 10 mL bolus of contrast agent was administered at a rate of 5 mL/s, followed by a 30 mL saline flush. Due to the differences in MRI system configurations, the relaxation time (1.2–3 s), echo time (30–60 ms), in-plane resolution (1.2–3 mm), slice thickness (4–7 mm), number of slices (14–20) and total acquisition times (70–100 s) were scanner dependent.

2.4. Postacquisition imaging analyses

All digital imaging and communications in medicine (DICOM) files were transferred to an external research computing station for postacquisition processing. Using Analyze 10.0 software (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN, USA), tumour regions of interest (ROI) were created from contrast-enhancing regions on T1-weighted sequences (Fig. 2A). Any cystic cavities and necrotic cores were not included in the tumour ROI. A further ROI was created from the T2-weighted NAWM of the centrum semi-ovale of the hemisphere contralateral to the tumour. ROI volumes (cm^3) were calculated by integrating