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# Glioblastoma cell lines derived under serum-free conditions can be used as an *in vitro* model system to evaluate therapeutic response

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ABSTRACT

We provide evidence that six glioblastoma cell lines derived and maintained under serum-free conditions secrete VEGF and four also expressed $VEGF_{R2}$. Expression of $VEGF_{R2}$ was associated with reduced proliferation in response to anti-VEGF antibodies. Spontaneous loss of $VEGF_{R2}$ over passage was associated with loss of this anti-proliferative effect. Gain of expression of $VEGF_{R2}$ was not associated with the acquisition of responsiveness to anti-VEGF antibodies. Secretion of PDGF was absent in 5/6 of our cell lines and none of the cell lines had reduced proliferation in response to anti-PDGF antibodies suggesting that PDGF autocrine signalling was unlikely to be significant in tumour proliferation. These data are consistent with published clinical trials suggesting that glioblastoma cell lines derived under serum-free conditions have the potential for use in drug screening and individualising patient therapy.



## 1. Introduction

Glioblastoma (GBM) is the most common primary brain cancer in adults. It is highly aggressive, currently incurable and even with optimal management median survival is typically just 15 months [1]. Clinical trials of chemotherapy targeting VEGF or PDGF signalling in GBM patients have been widely reported and bevacizumab has recently been granted FDA approval for the treatment of recurrent GBM. However, there has been some controversy surrounding the basis for this rapid approval in the US, with doubts over the use of 6-month progression-free survival as the study end point rather than overall survival, which would perhaps be more appropriate when studying antiangiogenic agents [2]. The response to anti-VEGF therapy is variable, as some patients responded whilst others showed relentless disease progression [3,4]. This demonstrates the need for more research into the effects of anti-VEGF therapy. Clinical trials targeting PDGF signalling in GBM patients demonstrated little clinical response [5,6] despite promising pre-clinical data [7,8]. The underlying basis for this variability in response to anti-VEGF and anti-PDGF therapy is unknown.

We have used GBM cell lines derived under serum-free conditions from GBM patients (SF-GBM cell lines) which have been shown to be highly representative of the parent tumour at the molecular genetic level [9,10] to investigate VEGF and PDGF signalling. Previous research into VEGF signalling in GBM has focused on the role that inhibitors have on the vasculature. However, there is increasing evidence that VEGF receptors are present on some cancer cells such non-small-cell lung carcinoma, melanoma, prostate carcinoma, leukaemia, breast carcinoma [11] and glioblastoma [12]. In contrast PDGF signalling has been investigated in high passage serum-derived cancer cell lines. *In vitro* results have suggested a role of PDGF signalling in glioblastoma cell line proliferation using both a dominant negative mutant of PDGF [5] and the small molecule tyrosine kinase

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inhibitor imatinib [8], which both reduced glioblastoma cell line proliferation. These results were not clinically reproducible [6,13,14]. The variation in VEGF and PDGF activity within different primary glioblastoma cell lines may reflect the clinical pattern of response to treatment reported and provide a novel *in vitro* model that may provide insights into potential mechanisms of treatment failure. We examine SF-GBM cell lines from six GBM patients to determine if VEGF or PDGF growth factors have a role in cell line proliferation, and furthermore to assess the potential of cancer cell lines as a model for screening anti-cancer therapy.

## 2. Materials and methods

All materials were purchased from Sigma unless otherwise stated.

### 2.1. Cell culture and tumour formation assay

Anonymised tissue samples were obtained in accordance with local ethical guidelines from six patients with primary GBM (four female, two male aged 55–69 years). Tissue was mechanically minced in modified phosphate buffered saline (PBS) before enzymatic digestion. Single cells were then isolated by filtration through a 40 μm filter (Falcon, UK) and washed with 10 ml red blood cell lysis buffer. Live cells were quantified by trypan blue exclusion, seeded at standard density of 15,000 cells/cm$^2$ in defined media and allowed to form primary aggregates. These were collected and plated, without dissociation, onto extracellular matrix (ECM)-coated flasks (ECM 1:10 dilution, Sigma, UK) and allowed to form a primary monolayer. As the primary monolayer approached confluence cells were dissociated by incubation with accutase at room temperature and washed with PBS. The cell viability was assessed and cells were reseeded onto ECM-coated flasks at a density of 150 cells/cm$^2$ to generate the secondary monolayer. To generate subsequent monolayers cells were seeded at standard density 15,000 cells/cm$^2$ at each passage. Cells were cultured in 10 ml SF medium (phenol red-free Neurobasal A (Invitrogen, UK) with 20 mM L-glutamine and 1% (v/v) PSF solution with 20 ng/ml hEGF (Sigma, UK), 20 ng/ml hFGF (R&D systems, UK) and 20 ng/ml heparin, 2% (v/v) B27 (Invitrogen, UK) and 1% N2 (Invitrogen, UK) and incubated at 37.5 °C in 5% $CO_2$ as previously described [10]. For all experiments early passage cells between passage 1–4 or late passage cells between passage 10–20 were used. All experiments were conducted in normoxic conditions.

### 2.2. Enzyme-linked immunosorbent assay (ELISA)

Ten thousand cells per well were seeded into a 24-well plate coated with extra cellular matrix (ECM 1/10 dilution with Neurobasal A) with 0.5 ml of feeding medium, and cultured for 48 h (in the absence of EGF and FGF for $PDGF_A$ ELISA due to cross reactivity). ELISA was conducted using a Human VEGF ELISA Development Kit (Peprotech 900-K10) following the manufacturer's instructions. The $PDGF_A$ ELISA followed the same protocol. Antibodies used were Anti-Human $PDGF_A$ Antigen Polyclonal Antibody (Peprotech, 500-P46) and Biotinylated anti-Human $PDGF_A$A Polyclonal Antibody (Peprotech, 500-P46Bt).

### 2.3. Cell proliferation assay

Cell proliferation was assessed using CellTiter 97 Aqueous One Solution Cell Proliferation Assay (Promega) following the manufacturer's instructions. One thousand cells per well were seeded onto ECM-coated 96-well plates with 100 μl feeding medium per well. Cells were cultured for 7 days before the addition of 20 μl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reagent. The plate was then incubated for 150 min before the absorption at 490 nm was read on a universal microplate reader (ELx 800). Medium only readings were subtracted from wells containing cells. Inhibitory antibodies: Anti-Human VEGF (Peprotech, 500-P10) and Anti-Human $PDGF_A$ Antigen Affinity Purified Polyclonal Antibody (Peprotech, 500-P46) were used at concentrations indicated in the results. Growth factors $VEGF_{165}$ (Peprotech) and $PDGF_A$ (Peprotech) were used at concentrations indicated in the results.

### 2.4. Transcriptional profiling

RNA extraction and cDNA synthesis were performed as described previously [10]. The appearance of transcripts was assessed by reverse-transcriptase PCR (RT-PCR) using a Bio-Rad PCR thermocycler. Primer sets were designed using Beacon primer design based on the complete cDNA sequences deposited in GenBank and their specificity verified using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal positive control.

Primers:

| Gene | Accession no. | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|---|
| $PDGF_{R\alpha}$ | NM-06206 | 5′-TCCTCTGCCTGACATTGAC-3′ | 5′-CTCTTGATGAAGGTGGAACTG-3′ | 126 |
| $PDGF_A$ | NM-33023 | 5′-GCAGGAAGCAGGAATGTCTT-3′ | 5′-GGAAAAATACCGTCGCACTG-3′ | 312 |
| GAPDH | NM-002046 | 5′-CATCATCCCTGCCTCTAC-3′ | 5′-CTTCCTCTTGTGCTCTTG-3′ | 435 |
| $VEGF_{R1}$ | NM-002019 | 5′-GTGCTGCATCCTTGTTGAGA-3′ | 5′-TTTGGATGAGCAGTGTGAGC-3′ | 440 |
| $VEGF_{R2}$ | NM-002253 | 5′-TGCCTACCTCACCTGTTTCC-3′ | 5′-TGACTGATTCCTGCTGTGTTG-3′ | 85 |
| $VEGF_{R3}$ | NM-182925 | 5′-AAGGATCACGATCTCCATGC-3′ | 5′-GACAAGCACTGCCACAAGAA-3′ | 354 |
| $VEGF_A$ | NM-002019 | 5′-ACGAAGTGGTGAAGTTCATGG-3′ | 5′-CTGCATTCACATTTGTTGTGC-3′ | 280 |