Microvessel count and cerebrospinal fluid basic fibroblast growth factor in children with brain tumours

Vincent W Li, Rebecca D Folketh, Hiroyuki Watanabe, Channing Yu, Maria Rupnick, Patrick Barnes, R Michael Scott, Peter Mcl. Black, Stephen E Sallan, Judah Folkman

Summary
Tumour growth is angiogenesis-dependent; brain tumours have more intense neovascularisation than other tumours and produce basic fibroblast growth factor, a potent angiogenic mediator. Because little is known about the release of basic fibroblast growth factor from brain tumours into extracellular fluids, we tested cerebrospinal fluid (CSF) from 26 children and young adults with brain tumours and 18 controls for basic fibroblast growth factor and for proliferative activity on cultured capillary endothelial cells. We also measured the density of microvessels in tumours by immunohistochemical staining.

Basic fibroblast growth factor was detected in the CSF of 62% (16 of 26) patients with brain tumours but in none of the controls. Specimens with basic fibroblast growth factor stimulated DNA synthesis of capillary endothelial cells in vitro. Endothelial proliferative activity was blocked by neutralising antibodies to basic fibroblast growth factor. Basic fibroblast growth factor correlated with mitogenic activity in CSF in vitro \( (p < 0.0001) \), and with density of microvessels in histological sections \((p < 0.005)\). A microvessel count of \( \geq 68 \) per 200 × field was associated with tumour recurrence \((p = 0.005) \) and with mortality \((p = 0.02) \).

Basic fibroblast growth factor in brain tumours may mediate angiogenesis as measured by microvessel density in histological sections, so has potential as both a marker for neoplasia and a target for tumour treatments. Furthermore, evaluation of cerebrospinal fluid basic fibroblast growth factor, along with microvessel quantitation in biopsied tumours, may provide improved prognostic information for the management of patients with brain tumours.

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Introduction
A feature of many brain tumours is the presence of neovascularisation. Angiogenesis is necessary for the growth of most solid tumours which, in an avascular state, are limited to a few cubic millimetres in volume \((< 10^6 \) cells). The switch to the angiogenic phenotype allows new capillaries to converge upon the tumour and growth to proceed at an exponential rate.

Basic fibroblast growth factor (bFGF) is one of the best characterised, most potent, and most widely distributed angiogenic molecules. This peptide stimulates vascular endothelial cell proliferation, and virtually all such cells either produce bFGF or have receptors for it. Immunohistochemical studies have detected bFGF in relatively high concentrations in brain tumours, especially in the microvasculature. Basic FGF gene expression has also been found in human brain-tumour cells. In addition, bFGF promotes the survival and differentiation of central nervous system neurones, and it increases in neural tissue after focal brain injury. However, while bFGF is angiogenic in vivo, little is known about how tumours release it, or about the relationship of its production to the degree of angiogenesis within a tumour.

Because angiogenic activity appears to be necessary for tumourigenesis, we sought biologically active bFGF in the cerebrospinal fluid (CSF) of children with brain tumours and correlated bFGF with measurements of tumour neovascularisation.

Patients and methods
Cerebrospinal fluid
Between July, 1991, and October, 1992, CSF was obtained from 26 children and young adults (3–20 years) who had brain tumours evaluated at the Dana-Farber Cancer Institute and Children’s Hospital, Boston. 17 patients were newly diagnosed and untreated; their ventricular CSF was obtained during initial surgery. The study was conducted according to institution-approved protocols, with informed consent. CSF samples were discarded portions of the CSF obtained at diagnosis or during management by neurosurgeons. Control specimens of CSF were collected from children with acute lymphoblastic leukaemia (9), acute myelogenous leukaemia (1), rhabdomyosarcoma (2), T-cell lymphoid lymphoma (1), and hydrocephalus (5).

Immunoassay
CSF samples were tested for bFGF in a previously described sandwich enzyme-linked immunoassay capable of detecting bFGF at \( \geq 30 \) pg/mL based on three monoclonal antibodies, each directed against a specific epitope of bFGF (Takeda Chemical Industries, Osaka, Japan).

Bovine capillary endothelial cells
Capillary endothelial cells isolated from bovine adrenal cortex were grown on Dulbecco’s Modified Eagle’s Medium (DMEM), (JRH
Table 1: Biochemical and histological markers of angiogenesis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>WHO grade</th>
<th>Newly diagnosed</th>
<th>bFGF in CSF (pg/mL)</th>
<th>Endothelial cell stimulation Index (DNA synthesis)</th>
<th>Neovascular grade</th>
<th>Microvessel density (200 x field)</th>
<th>Recurrence</th>
<th>Death</th>
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<tbody>
<tr>
<td>Pilocytic astrocytoma</td>
<td>I</td>
<td>+</td>
<td>1815</td>
<td>49.82</td>
<td>4+</td>
<td>95</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>1251</td>
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<td>3+</td>
<td>72</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<td>654</td>
<td>14.11</td>
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<td>62</td>
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<td>17.69</td>
<td>4+</td>
<td>83</td>
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<td>83</td>
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<td>5.97</td>
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<td>-</td>
<td>48</td>
<td>4.48</td>
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<tr>
<td>Anaplastic ependymoma</td>
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<td>N/A</td>
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<td>&lt;30</td>
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<td>3+</td>
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<td>-</td>
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<tr>
<td>Hypothalamic germinoma</td>
<td></td>
<td>+</td>
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<td>N/A</td>
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<td>-</td>
<td>-</td>
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<td>2+</td>
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<td>1+</td>
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<td>&lt;30</td>
<td>0.58</td>
<td>+</td>
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DNA Synthesis Assay

CSF specimens were tested in a modified bioassay. Endothelial cells in DMEM and 10% calf serum were plated onto gelatinised 48-well plates. The next day the medium was replaced with DMEM containing 2% calf serum, bovine serum albumin (5 mg/mL, Sigma, St Louis, Mo, USA), and thymidine (0.2 µg/mL, Sigma), 24 h later, test samples were added to each well to attain final CSF concentrations of 5% and 2-5%. DNA synthesis was assayed 21 h later by tritiated thymidine uptake (37 kBq/well, New England Nuclear, Boston, MA, USA). All values were expressed with reference to a control with only DMEM.

Acid phosphatase proliferation assay

A modification of the assay described by Connolly et al13 was used. Endothelial cells were starved by changing the medium to DMEM containing 4% calf serum but no bFGF; next day, cells were trypsinised and resuspended, then plated in 96-well, flat-bottomed tissue-culture plates (Costar, Cambridge, MA, USA) at 2500 cells per well; the day after, cells were challenged with CSF diluted to 5% and 2-5%. Human recombinant bFGF was used as a positive control. 72 h later, cells were washed with phosphate-buffered saline and exposed to a buffer solution containing 0.1 mmol sodium acetate (Curtin Matheson Scientific, Houston, TX, USA), 0.1% Triton X-100 (Bio-Rad, Hercules, CA, USA), and 10 mmol p-nitrophenyl phosphate (Sigma 104 phosphatase substrate, Sigma). After 2 h incubation, the colorimetric reaction was stopped with 1 N NaOH and optical density read at 410 nm with a rapid microplate reader (MR5000, Dynatech, Alexandria, VA, USA).

Neutralising antibodies

Samples were tested in the endothelial-cell proliferation assay described above. At the time of challenge, 10 µg of neutralising monoclonal anti-bFGF antibody 3H3 (Takeda) was added to each well.

Microvessel staining, grading, and counting

Slides of tumour samples obtained by biopsy or resection were evaluated microscopically without knowledge of the neuropathological diagnosis, the clinical course, or bFGF in CSF. From each case, one representative slide was selected. Tumours were classified according to World Health Organization nomenclature. From one paraffin block per case, one slide was stained for the endothelial cell markers, Factor VIII-related antigen, and/or CD34 (AMAC, Inc, Westbrook, ME, USA). Microvessel density, as highlighted by Factor VIII-related antigen and CD34 immunostaining, was assessed by the method of Weidner et al14 When present, extratumoural leptomeningeal blood vessels in the specimen were ignored. The area of highest neovascularisation was subjectively graded on a scale from 1 to 4. Individual microvessel counts were then made on a 200 x field (equivalent to 0.7386 mm² per field). Each count was expressed as stimulation index was expressed as a percentage of the DNA synthesis induced by 5 ng of human recombinant bFGF/mL (Takeda).

Figure 1: Correlation between endothelial DNA synthesis activity (stimulation index) and bFGF in CSF of patients with newly-diagnosed brain tumours

Spearman r = 0.87, p < 0.0001
Figure 2: Area with high density of microvessels in an anaplastic ependymoma stained with haematoxylin and eosin (top) and with Immunoperoxidase for CD34 (middle). Tissue section from a normal brain stained for CD34 is shown for comparison (bottom). Magnification ×500. Note the brown-staining microvessels highlighted by staining endothelial cells for CD34. Similar results were obtained in other cases stained for Factor VIII-related antigen.

the highest number of microvessels present within the field. The presence of a vascular lumen was not necessary for the identification of a microvessel. Glomeruloid clusters were counted as one microvessel because distinct endothelial cells and lumens could not be distinguished.

Gadolinium-enhanced magnetic resonance imaging
At the time of diagnosis, 25 of the 26 patients with brain tumours underwent routine magnetic resonance imaging (MRI) at 1.5 Tesla with gadolinium-DTPA (Magnevist, Berlex Imaging, Wayne, NJ, USA) at a dosage of 0.1 mmol/kg (maximum 10 mmol). These images were assessed for gadolinium enhancement, which indicates increased tumour vascularity and/or breakdown of the blood-brain barrier.

Clinical follow-up
Median follow-up was 14 months after CSF specimens were collected and 14.5 months after tissue biopsies were obtained. Recurrences were detected by neuroimaging or clinical deterioration. Deaths were ascertained by the review of medical records.

Analysis
Fisher's exact test and the Wilcoxon rank sum test were used to evaluate differences between case and control specimens with regard to bFGF and biological activity in endothelial cells. Kaplan-Meier survival curves were generated, and the log-rank test was used to evaluate time to recurrence in patients with bFGF in CSF or with increased tumour microvessel density. We defined increased microvessel density as ≥68 vessels per 200 × field, similar to our previous study with breast cancer, which showed an increase in the frequency of metastasis at this value.

Results
We detected bFGF in the CSF of 16 (62%) of 26 patients with brain tumours but not in control samples (table 1). CSF which contained bFGF stimulated endothelial-cell DNA synthesis to a level up to 49.8% of that induced by recombinant bFGF (5 ng/mL) and increased proliferation of endothelial cells at 72 hours (data not shown). Endothelial mitogenic activity in CSF from patients with brain tumours was significantly higher than that in CSF from controls (p≤0.0001). The median stimulation index was 11.6 for the 24 brain-tumour CSF samples tested and 1.35 for the 14 controls. Among newly diagnosed patients, bFGF was positively correlated with the degree of endothelial cell stimulation (Spearman r = 0.87, p≤0.0001; figure 1) and with tumour microvessel density (Spearman r = 0.64, p≤0.005). Factor VIII-related antigen or CD34 staining revealed dense areas of microvessels in brain tumour tissue, which were poorly visualised with haematoxylin and eosin staining and were not present in Factor VIII-stained or CD34-stained normal brain tissue (figure 2).

bFGF was present in all 6 specimens from patients with tumour microvessel density ≥68 vessels per 200 × field but in only 5 (45%) of 11 CSF specimens from patients with a microvessel density of <68. Addition of neutralising monoclonal anti-bFGF antibody 3H3 to CSF from patients with brain tumours inhibited endothelial cell proliferation by 55–82%. In 24 of 25 patients with brain tumours, magnetic resonance imaging with gadolinium-DTPA showed peritumoural enhancement consistent with increased neovascularisation and/or breakdown of the blood-brain barrier.

During a follow-up of 1 year to >2.5 years from the time of analysis of CSF for bFGF, we determined the time to recurrence or death for the 26 patients with brain tumours. 5 patients had a recurrence (2 with ependymoma and 1 each with glioblastoma multiforme, pineoblastoma, and medulloblastoma); 4 died. Among the 17 patients whose CSF bFGF and tumour microvessel counts were both available, there was a trend towards an association of earlier tumour recurrence with elevated bFGF in the cerebrospinal fluid (>30 pg/mL; p = 0.10; figure 3), and a statistically significant association of earlier tumour
the cerebrospinal fluid (30 pg/mL; Panel A) and for patients with recurrence with increased microvessel density (p = 0.005; figure 3). Table 2 summarises the results which suggest that bFGF is an angiogenic mediator in the CSF of patients with brain tumours.

All 5 patients with medulloblastoma had no detectable bFGF at the time of diagnosis. In fact, bFGF in patients with medulloblastoma was raised only in cases of tumour recurrence. No apparent correlation existed between cerebrospinal bFGF and histological tumour type or grade.

Discussion

These studies show that an angiogenic peptide, bFGF, is present in the cerebrospinal fluid of patients with brain tumours: 3 CSF samples from our cohort did not have bFGF, yet they stimulated endothelial-cell DNA synthesis. Furthermore, antibodies to bFGF did not completely block mitogenic activity of all samples of CSF. Other candidates for endothelial mitogens in the cerebrospinal fluid include acidic FGF or vascular endothelial growth factor (VPF/VEGF), a secreted peptide. We did not test for these peptides.

During preparation of this manuscript, it was reported that bFGF is present in CSF of patients with moyamoya disease, a progressive occlusion of the circle of Willis accompanied by development of transdural collateral circulation. Because such collateral vessels are rare in other occlusive cerebrovascular diseases, it was suggested that the abnormal bFGF may play a part in the pathogenesis of moyamoya disease. Thus, although CSF bFGF levels were not detectable in our control population but were in patients with brain tumours, bFGF cannot be considered a specific indicator of neoplastic disease.

It remains to be seen whether measurement of bFGF or other angiogenic peptides in CSF of patients with brain tumours will prove to be a prognostic indicator of component of capillary growth, had been detected in the cerebrospinal fluid of patients with brain tumours. The origin of bFGF in CSF in our patients is unknown. Although, bFGF does not contain a classic signal peptide sequence and is not considered to be a secreted growth factor, angiogenic tumour cells have been found to export this peptide. bFGF may also be mobilised from the extracellular matrix, because the peptide is known to be stored in the extracellular matrix, or it could be released by the new capillary endothelial cells recruited by the tumour. It has recently been recognised that the contribution of neovascularisation to tumour growth lies not only in perfusion of the tumour, but also in the paracrine effect of vascular endothelial cells on tumour cells. Thus, endothelial cells release growth factors which stimulate tumour cells. Dying or lysing tumour cells could also be a source of bFGF, but there is less experimental evidence in support of this. It is unlikely that bFGF is the only angiogenic peptide present in CSF of patients with brain tumours: 3 CSF samples from our cohort did not have bFGF, yet they stimulated endothelial-cell DNA synthesis. Furthermore, antibodies to bFGF did not completely block mitogenic activity of all samples of CSF. Other candidates for endothelial mitogens in the cerebrospinal fluid include acidic FGF or vascular endothelial growth factor (VPF/VEGF), a secreted peptide. We did not test for these peptides.

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**Table 2: Summary of results**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralising antibody studies (anti-bFGF 3H3)</td>
<td>Endothelial proliferative activity in vitro in brain tumour CSF is inhibited by 55-82%.</td>
<td>≤ 0.001</td>
<td>6</td>
</tr>
<tr>
<td>Tumour microvessel density (Factor VIII or CD34 stain)</td>
<td>Microvessel density (per 200 x field) correlates with bFGF level in CSF (Spearman r = 0.59)</td>
<td>≤ 0.05</td>
<td>17</td>
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<tr>
<td>Microvessel density of &gt; 68 (per 200 x field) is associated with tumour recurrence.</td>
<td>0.005</td>
<td>17</td>
<td></td>
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<tr>
<td>Microvessel density of &gt; 68 (per 200 x field) is associated with mortality.</td>
<td>0.02</td>
<td>17</td>
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</table>
recurrence or whether intensity of neovascularisation in brain tumours will predict risk of recurrence or will correlate with recurrence-free survival as it has in breast cancer. Other markers of malignancy have been identified in CSF, eg, elevated concentrations of beta-glucuronidase, polyamines, and carcinoembryonic antigen have correlated with extent of tumour extension to the meninges. Cytological examination of CSF detects recurrences in patients with previously diagnosed brain tumours. Cytological studies appear to have a prognostic value only insofar as they confirm the spread of tumour to the subarachnoid space—a relatively late step in progression of brain tumours. In contrast, elevated bFGF in CSF of newly-diagnosed brain-tumour patients was detected at initial presentation and foretold a poor clinical outcome in 27% of cases during our study interval. Thus, it is possible that measurement of bFGF and other angiogenic peptides in CSF, together with measurement of neovascularisation in the brain tumour itself, may be used in conjunction with other tumour markers as well as with diagnostic studies (such as magnetic resonance imaging), to improve the accuracy of prognosis of patients with brain tumours. Although this study focused on children and young adults with brain tumours, we have also found CSF bFGF and increased microvessels in biopsy specimens of adults with brain tumours (unpublished data).

Because angiogenic peptides are not only tumour markers, but are also mediators of tumour growth, it is conceivable that antibodies against these peptides may be useful in treatment. Significant suppression of tumour growth has been obtained in animals by treating them with neutralising antibodies against specific angiogenic peptides (bFGF and VEGF) which were the predominant mediators of angiogenesis for these tumours. The efficacy of other forms of antiangiogenic treatments for brain tumours may also depend on interfering with the mitogenic activity of angiogenic peptides on local capillary endothelial cells.

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References


