The Relevance of Cell Proliferation, Vascular Endothelial Growth Factor, and Basic Fibroblast Growth Factor Production to Angiogenesis and Tumorigenicity in Human Glioma Cell Lines

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ABSTRACT

Tumor growth is partially dependent on angiogenesis, a process that relies on angiogenic factors. Tumorigenicity of cancer cells is thought to be associated with the production of various angiogenic factors that stimulate or inhibit the rate of endothelial cell migration and proliferation. However, the relative importance of specific individual factors originally studied in cancer cell lines has yet to be determined in vivo. In this study, we examined seven human glioma cell lines for dynamic changes of two major angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and for doubling time and tumorigenicity in nude mice. Various correlation studies demonstrated that in these glioma cell lines, VEGF expression correlated well with RBC density in tumor sections (r² = 0.804) and with average tumor weight (r² = 0.987). In contrast, bFGF expression in the observed glioma cell lines did not correlate with tumorigenicity (r² = 0.001) or with VEGF expression (r² = 0.255). Furthermore, there was no correlation between doubling time and tumorigenicity in these cell lines (r² = 0.160). Taken together, these results suggest that VEGF plays a major role in glioma formation and that down-regulation of VEGF, rather than bFGF, would be a more effective choice for glioma gene therapy.

INTRODUCTION

With the exception of its activity during the ovulation cycle, angiogenesis (new blood vessel formation from preexisting vasculature) is quiescent in healthy adult mammals. Thus, angiogenesis in mature mammals is considered to be a pathologic event that occurs in tissue injury, rheumatoid arthritis, and tumor growth and metastasis (1–4). Angiogenesis is known to be driven and maintained by positive and negative factors (1). Although more than 10 positive factors are known to be involved in angiogenesis, the majority of studies have focused on VEGF and bFGF (5–7). This is because the expression of both of these cytokines is ubiquitous throughout the body. Both cytokines are present in endothelial cells (8, 9) and are up-regulated in the vast majority of human tumors (10, 11). Their expression levels correlate with cancer progression in the various tumor stages (11–14) of many types of cancer, including brain astrocytomas (15, 16). Previous studies have shown that inoculation of tumor cells transformed by bFGF or VEGF leads to the induction of vascularity and growth of the xenograft tumor mass (17, 18). Likewise, down-regulation of the bFGF and VEGF genes by antibodies or antisense sequences has been shown to inhibit cancer cell tumorigenicity and metastasis (19–23). In addition, a synergistic effect between exogenous bFGF and VEGF expression has been observed (17, 24–26), and combined administration of bFGF with VEGF has been shown to greatly stimulate blood circulation (27).

Recent studies investigating tumor progression suggest that VEGF is more important than bFGF in the induction of angiogenesis. This notion is supported by the fact that bFGF receptors are expressed in virtually all cells, whereas VEGF receptors (Flk/KDR and Flt-1) are located exclusively on endothelial cell (28). Furthermore, bFGF is a membrane-bound protein, and its paracrine action is dependent on the expression of other factors such as bFGF binding protein (29). In addition, VEGF is capable of greater angiogenic regulation because three of the five VEGF isoforms are secreted from the VEGF-producing cells, whereas bFGF remains membrane bound (30). Finally, loss of even a single VEGF allele causes deficits in blood vessel formation and is lethal in embryonic development (31), suggesting that VEGF is essential for blood vessel formation and that its concentration is important for induction of angiogenesis.

These previous findings led to the development of our hypothesis that tumorigenicity of cancer cell lines with different genetic backgrounds may partially result from their ability to manufacture major angiogenic inducers and/or to eliminate such host defensive machinery as angiogenic inhibitors. Furthermore, if endogenously expressed bFGF plays a role in the up-regulation of VEGF expression in glioma cells, then VEGF expression levels must correlate with bFGF levels.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; SFM, serum-free medium.
To address the above hypotheses, we examined seven human glioma cell lines for their doubling time, production of bFGF and VEGF, tumorigenicity, and ability to attract host endothelial cells in vivo. Finally, we performed various correlation studies with the gathered data. Our results demonstrated that endogenous production of VEGF, but not bFGF, correlates with angiogenesis and tumorigenicity in these glioma cell lines. We also found that endogenous bFGF levels do not correlate with VEGF levels. In addition, the rate of cellular proliferation of these seven glioma cell lines did not correlate with their tumorigenicity in nude mice.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Seven glioma cell lines were used. Three lines, A-172, U-251 MG, and U-87 MG, were obtained from the American Type Culture Collection (Manas- sas, VA). Cell lines EFC-2, NG-1, and LG-11 were derived from surgical specimens of human glioma and established in the Department of Neuro-Oncology at the University of Texas M. D. Anderson Cancer Center (32). HF U-251 MG was obtained from the Department of Neurosurgery at the M. D. Anderson Cancer Center. All seven cell lines were cultured in a 1:1 mixture of DMEM and Ham's F-12, supplemented with 10% FCS in a humidified atmosphere containing 5% CO₂ at 37°C.

Protein Extraction from Cells and Western Analysis. Cells were washed briefly with PBS and lysed with buffer (150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 20 mM Tris, pH 7.6). To 1 ml of lysis buffer, 1 μl of leupeptin (10 mg/ml), 1 μl of DTT (1 mM), and 10 μl of phenylmethylsulfonyl fluoride (100 mM) were added just before the cells were lysed. The lysed cells were collected, passed five times through a 23 gauge needle with a syringe, and incubated on ice for 1 h. Cells were then centrifuged at 22,000 × g at 4°C for 20 min. After centrifugation, the supernatant was divided into aliquots and stored at –80°C before electrophoretic separation on a 12% SDS-PAGE gel. Proteins on the gel were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The blot was equilibrated with Blotto (50 mM Tris, pH 7.5, 0.9% NaCl, 3% nonfat dry milk, and 0.05% Tween 20) for 4–5 h at room temperature with gentle shaking. The membrane was probed overnight at 4°C with Blotto (50 mM Tris, pH 7.5, 0.9% NaCl, 3% nonfat dry milk, and 0.05% Tween 20) for 4–5 h at room temperature with gentle shaking. The membrane was probed overnight at 4°C with 1:1000 diluted VEGF (Ab-2) polyclonal rabbit IgG (Oncogene Science, Inc., Cambridge, MA) or FGF-2 (147) rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After the overnight incubation, the membrane was washed with Blotto three times for 20 min each time and then incubated with 1:1000 diluted goat antirabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, England). The bFGF or VEGF signal was visualized through the enhanced chemiluminescence (ECL) protocol (Amersham Pharmacia Biotech).

Conditioned Medium Preparation for ELISA. To quantitate secreted and endogenous bFGF and VEGF, human bFGF or VEGF ELISAs were performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). To prepare the conditioned medium for collection of secreted protein, cells were seeded overnight in 6-well plates with 2 ml of medium. The next day, the cells were washed twice with 2 ml of SFM and then preconditioned for 6 h at 37°C in 2 ml of medium containing 2% serum. After aspiration of the preconditioned medium, the cells were incubated in 1 ml of medium containing 2% serum for 24 h at 37°C. The media from three replicas were collected in separate tubes containing 1 μl of 100 mM phenylmethylsulfonyl fluoride. The medium was then centrifuged at 4,000 rpm for 3 min, and the supernatant (conditioned medium) was collected, divided into 210-μl aliquots, and stored at –20°C.

![Fig. 1 Growth curve (a) and doubling time (b) of glioma cells. For every cell line, a total of 18 wells on three six-well plates were seeded with 30,000 cells/well on day 1. Cells from three of the 18 wells were counted daily starting on day 2 for a total of 6 days. Doubling time of each cell line was calculated by plotting logarithmic average cell numbers against their day of harvesting. Two points corresponding to 100,000 cells (log 5) and 200,000 cells (log 5.3) were located on each of the curves. Subsequently, two other points on the horizontal axis (day of harvesting) were located by drawing vertical lines from the two points on the curve toward the horizontal axis. Using distance on the axis of day of harvesting, the doubling time was calculated. Note that A-172 and U-87 MG cells had similar doubling times, as did LG-11 and NG-1 cells.](https://cancerreres.aacrjournals.org/0200 american association for cancer research.
until further analysis. The cells were harvested and counted immediately after the medium was collected. Because of differences in seeding efficiency among experiments and variations in proliferation rates among cell lines, the VEGF expression level was evaluated by final unit cell numbers (pg of VEGF/10⁵ cells/24 h). Cells in the other three replicas were subjected to total protein extraction with 1 ml of protein extraction buffer as described for the Western analysis.

**Tumor Cell Implantation and Growth in Nude Mice.**

To compare tumorigenicity in our glioma cell lines, exponentially growing cells were harvested by a brief washing with PBS followed by a brief exposure to 0.25% trypsin/0.02% EDTA. The trypsin was neutralized by the culture medium, and the cells were washed once with SFM. Five million cells from each cell line were suspended in 100 ml of SFM in the presence of 1/3 antibiotics and antimycotics (Life Technologies Inc., Grand Island, NY) and 100 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA), which has been shown to enhance tumor growth in nude mice (33, 34). The mixture was inoculated s.c. into the flanks of 6–8-week-old athymic female nude mice (Harlan Sprague Dawley, Inc. Minneapolis, MN). For each cell line, a set of three animals was implanted with two tumors, one in each flank, and each set was caged together. Tumor size was measured once a week with a caliper beginning the second week after injection. The tumor volume (mm³) was estimated by the formula \( V = \frac{1}{2} L \times W^2 \times 0.52 \), where \( L \) is length and \( W \) is width. Four weeks after inoculation, all mice were sacrificed. Tumors were dissected and weighed individually before they were fixed in 10% formalin.

**Histology.** H&E-stained sections of the glioma cell-generated tumors along with control (the injected Matrigel mass) were examined for their pathological features, including tumor cell occupancy in Matrigel, tumor morphology, and mitotic figures (the number of cells in mitosis among cells in microscopic views). The number of RBCs in necrosis-free areas of tumor sections was counted under a microscope. RBC density in tumor sections was taken to indirectly evaluate blood vessel ingrowth, because efficiency of the host nutritional supply to tumors may be related not only to the number of microvessels but also to the size of the vessels in which RBCs are confirmed.

**Immunohistochemistry.** The paraffin-embedded sections were subjected to deparaffinization in xylene, rehydration in graded series of ethanol, and rinsing with distilled water. For detection of VEGF and expression of the vascular marker factor VIII, two different primary antibodies and procedures were used...
**Table 1** Accumulation of bFGF and VEGF production by glioma cell lines in 5 days, quantitated by ELISA

Thirty replicas were plated for each cell line on day 0 by seeding 6000 cells with 3 ml of medium containing 10% serum in one well of six-well plate. Starting on day 1, three replicas from each cell line were taken each day for preparation of the conditioned medium for VEGF quantitation. Cells were counted at the same time (see “Materials and Methods”). Another three replicas were also preconditioned and conditioned, but their cultured supernatants were discarded, and only cells were used for extraction of cellular protein for quantitation of bFGF. For ELISA analysis of bFGF, 1 μg of total cellular protein was mixed with SFM to meet the requirement of a final assay volume of 200 μl/well. Because of very low levels of bFGF in LG-11 and NG-1 cells, total protein for the assay was increased to 5 μg. The accumulated amount of bFGF in this table was obtained from the fifth day of cell extract. Because cells do not tolerate 5 days of conditioning in the medium containing 2% serum, the amount of accumulated VEGF was a sum of five individual days. These data also enabled us to determine the amount of VEGF or bFGF produced per cell (see Fig. 3).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>bFGF (pg)</th>
<th>VEGF (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-172</td>
<td>21,956</td>
<td>148</td>
</tr>
<tr>
<td>EFC-2</td>
<td>4,162</td>
<td>814</td>
</tr>
<tr>
<td>LG-11</td>
<td>304</td>
<td>1,049</td>
</tr>
<tr>
<td>NG-1</td>
<td>103</td>
<td>524</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>2,200</td>
<td>137</td>
</tr>
<tr>
<td>HF U-251 MG</td>
<td>821</td>
<td>8,754</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>7,557</td>
<td>9,675</td>
</tr>
</tbody>
</table>

According to Super Sensitive immunodetection system protocols (BioGenex, San Ramon, CA). To unmask antigen VEGF, the rehydrated sections were microwaved with antigen retrieval solution AR 10 in the Tender Cooker (Nordic Ware, Minneapolis, MN) for 15 min. The sections were incubated with the optimized epitope (LYL) specific VEGF polyclonal antibody for 30 min at room temperature followed by washing for 5 min with distilled water. The sections were then incubated with MultiLink (a biotinated secondary antibody) for 30 min, followed by being washed in PBS for 5 min. The sections were further incubated with Label, an alkaline phosphatase-conjugated streptavidin, for 20 min at room temperature. After several rinses, the Fast Red TR (substrate) was applied on the sections until an acceptable intensity of color had been reached.

To retrieve factor VIII, the rehydrated sections were digested by 0.1% pepsin in 0.01 HCl solution for 20 min and subsequently incubated with 3% H2O2 for 10 min to block any endogenous peroxidase activity. The sections were incubated with anti-factor VIII primary antibody for 2 h at room temperature. After application of MultiLink and Label, the avidin and biotinylated horseradish peroxidase macromolecular complexes were visualized by substrate AEC chromogen. Negative control sections were performed without primary antibodies. All sections were counterstained with Mayer's hematoxylin and mounted in aqueous Kaiser medium. All reactions were carried out in a moist chamber at room temperature except where stated otherwise. The staining was visualized as red-brown precipitates using a light microscope.

**RESULTS**

**Glioma Cell Doubling Times.** Many factors contribute to the uncontrolled growth of cancer cells, including a high proliferation rate. To examine any possible association of glioma cell tumorigenicity with cell proliferation rate, doubling times of the seven glioma cell lines were examined. The growth curves (Fig. 1a) show the greatest proliferation by HF U-251 MG cells. The doubling time of HF U-251 MG cells (21.1 h) was determined to be approximately 5 h less than that of U-251 MG cells and half that of the EFC-2 cells (46 h). Cell lines A-172 and U-87 MG exhibited similar doubling times: 30.7 and 29.8 h, respectively. LG-11 and NG-1 cell lines exhibited doubling times of 35.5 and 37.4 h, respectively (Fig. 1b).

**Expression of Basic FGF and VEGF.** In our initial experiments, we used ELISA to examine bFGF and secreted VEGF levels in conditioned media and in cell lysates prepared from three glioma cell lines. We discovered that unless cells were detached from the cultured surface, more than 99% of total bFGF was maintained within cells (data not shown). Based on those observations, we examined bFGF expression solely from cell lysates. Three bFGF isoforms were detected in five of the seven cell lines studied (Fig. 2). bFGF was expressed at high levels in A-172, EFC-2, and U-87 MG cells, at moderate levels in U-251 MG and HF U-251 MG cells, and at low levels in LG-11 and NG-1 cells. Unless the blot was extensively exposed to X-ray film, bFGF bands from 50 μg of LG-11 cellular protein were not visible by Western blot analysis. In addition to the intrinsic bFGF expression differences among the seven cell lines, bFGF isoforms were not translated in equal amounts from the three initiation codons (Fig. 2). For example, the 22-kDa species was the major product in U-251 MG and HF U-251 MG cells, whereas the 18-kDa species was the major product in EFC-2 cells. These data suggest that the three translation initiation codons in bFGF mRNA are used differently among the cell lines. bFGF ELISAs were then performed on the cell extracts, and the results were consistent with the Western blot analysis (for more detailed data, see below).

Our preliminary data indicated that approximately 80–90% of the VEGF detected by ELISA constituted the immediately secreted fraction. Western blot analysis with 50 μg of cellular protein for VEGF expression detected two cell-associated VEGF isoforms with high molecular mass (42 and 50 kDa), but it was not sensitive enough to detect the other three VEGF isoforms. With Western analysis of lyophilized conditioned media, however, these isoforms were identified, and their molecular masses were 17.7, 19.1, and 21.5 kDa (data not shown). With the exception of U-87 MG cells, the total amount of VEGF precipitated from 1 ml of conditioned medium (see “Materials and Methods”) was not visible by Western blot analysis. As a result, we evaluated VEGF expression from the conditioned media only by ELISA. Among seven glioma cell lines, the highest levels of secreted VEGF were found in U-87 MG cells (392 pg/10⁵ cells in 24 h) followed by EFC-2 (179 pg), LG-11 (160 pg), NG-1 (134 pg), HF U-251 MG (129 pg), A-172 (82 pg), and U-251 MG cells (24 pg).

**Accumulation of VEGF and bFGF.** Because angiogenic factors may require chronic and sustained exposure to stimulate angiogenesis and tumorigenicity, examining the cumulative production of bFGF and VEGF over time may be more important than a one-time sampling of these factors. Under our experimental conditions, both VEGF and bFGF production increased daily, but the rate of increase was cell line specific. The
Fig. 4  Comparison of glioma cell line tumorigenicity. In the presence of 1× antibiotics and antimycotics, a mixture of 100 μl each of Matrigel and SFM containing 5 × 10⁶ cells was inoculated s.c. into each side of 6–8-week-old athymic female nude mice. Tumor sizes were measured weekly beginning the second week after injection. Tumor size from week 0 was the volume of the injected cells with the Matrigel. The size was theoretically calculated according to the syringe used for injection and the formula \( V = L \times πr^2 \). Results are presented as average tumor volume over time (a). Matrigel control is not plotted because its flat shape is not considered as tumor in our experiments. The average tumor weight is expressed in grams (b). The final tumors were photographed immediately after dissection. Their relative sizes are shown against the same scale ruler (c).
HF U-251 MG and U-87 MG cells produced a significant amount of VEGF after 3 days of culture starting from 6000 cells, whereas the other five cell lines demonstrated a significantly lower amount of VEGF (Fig. 3). Total VEGF accumulation in 5 days in U-87 MG and HF U-251 MG cells reached 9675 and 8753 pg, respectively (Table 1). Although the doubling time of A-172 and U-87 MG cells was similar (Fig. 1b), the 5-day VEGF accumulation in A-172 cells was approximately 65 times less than the amount detected in U-87 MG cells. The cumulative amount of VEGF in U-87 MG cells was approximately 9, 12, 18, and 70 times greater than that in LG-11, EFC-2, NG-1, and U-251 MG cells, respectively (Table 1).

The daily cumulative bFGF level in the seven cell lines quantitated by ELISA is presented in Fig. 3b. bFGF levels were highest in A-172 cells, followed by U-87 MG, EFC-2, U-251 MG, HF U-251 MG, LG-11, and NG-1 cells. These results are consistent with those obtained from the Western blot analysis (Fig. 2). Because bFGF is not secreted, the amount of bFGF measured on the fifth day was considered to be the total accumulation for 5 days. The A-172 cells expressed 21,956 pg of bFGF, which was about three times higher than in U-87 MG cells, and 5, 10, 27, 72, and 213 times higher than in EFC-2, U-251 MG, HF U-251 MG, LG-11, and NG-1 cells, respectively (Table 1). As shown in Fig. 1b, the doubling time of HF U-251 MG cells was about 4 h shorter than U-251 MG cells. However, the bFGF expression level was about three times lower in HF U-251 MG cells than in U-251 MG cells. These results suggest that the production of bFGF in these cell lines does not correlate with their proliferation rates.

**Tumorigenicity and Induction of Angiogenesis.** Tumor growth curves (Fig. 4a) demonstrate that U-87 MG and HF U-251 MG cells generated larger tumors in nude mice than the other five glioma cell lines. At the fourth week after injection, some U-87 MG and HF U-251 MG tumors had grown to a size beyond the limitation set forth by the institutional protocol so that all of the animals had to be sacrificed. The size of the tumors created by the other five cell lines remained unchanged or even decreased during 4 weeks of growth in the nude mice (Fig. 4a). The dissected final tumors and their average weights are presented in Fig. 4, b and c. Within 4 weeks, HF U-251 MG and U-87 MG cells generated an average tumor mass about 10 times greater than that of U-251 MG tumors and about 25–45 times greater than those of the other four gliomas.

In H&E-stained tumor sections prepared from A-172, EFC-2, LG-11, and NG-1 tumors, cells were sparse in Matrigel and appeared not to be dividing. The penetrating capillaries in these tumors were small, and the RBC counts were lower than those found in U-251 MG tumors (Table 2 and Fig. 5). In contrast, in HF U-251 MG and U-87 MG tumors, many RBCs were confined to larger blood vessels distributed across the sections. Also, necrosis occurred in the center of these two types of tumors.

To determine whether differences in angiogenesis in vivo corresponded to diverse productivity of the growth factors expressed by these cell lines in vitro, tumor sections were analyzed by immunohistochemistry. Tumor cells were stained positively after incubation with antibody against VEGF. Because the intensity of VEGF signal in A-172, EFC-2, LG-11, and NG-1 tumor sections was low and expression was similar among all cell lines tested, we chose EFC-2 staining as a representative tumor section. VEGF levels were apparently higher in U87 MG and HF U-251 MG tumors (Fig. 6, panels 1 and 2) than U-251 MG and EFC-2 tumors (Fig. 6, panels 3 and 4). Angiogenesis also occurred in all tumors as indicated by factor VIII staining. Larger and denser blood vessels were found in U-87 MG and HF U-251 MG tumor sections (Fig. 6, panels 5 and 6) than those in U-251 MG and EFC-2 tumors (Fig. 6, panels 7 and 8). The other three types of tumor staining for factor VIII are similar to EFC-2 tumor (pictures not shown). These data suggest a positive correlation between VEGF expression and angiogenesis in vivo.

**Correlation Analyses.** With seven sets of experimental data gathered, multiple possible combinations were tested for their correlative value. Our first correlation analysis indicated that glioma cell doubling time did not correlate with tumorigenicity (r² = 0.160, in Table 3). We then determined that high levels of endogenous bFGF expression did not correlate with VEGF expression (r² = 0.255). Daily bFGF levels correlated neither with angiogenesis (r² = 0.007), which was expressed by RBC density in tumor sections, nor with tumorigenicity (r² = 0.001), irrespective of cell culture density. As expected, angiogenesis correlated with tumorigenicity (r² = 0.836). This number was similar to the correlation factor obtained by analysis of the accumulated VEGF expression versus RBC density in tumor sections (r² = 0.804). Finally, the accumulated VEGF was found to be the only other variable that correlated with tumorigenicity (r² = 0.987).

**Table 2  Pathological description of H&E-stained tumor sections generated from human glioma cell lines in mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell occupancy</th>
<th>Cell morphology</th>
<th>AMP</th>
<th>RBC (no./mm²)</th>
<th>Area examined (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel</td>
<td>Few</td>
<td>Uniformly pink</td>
<td>3.7</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>A-172</td>
<td>More than above</td>
<td>Atrophy &amp; malnutrition</td>
<td>5.9</td>
<td>38.4</td>
<td></td>
</tr>
<tr>
<td>EFC 2</td>
<td>More than A-172</td>
<td>Atrophy &amp; homochromatic</td>
<td>72.4</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>LG-11</td>
<td>Some</td>
<td>Atrophy &amp; homochromatic</td>
<td>47.7</td>
<td>21.1</td>
<td></td>
</tr>
<tr>
<td>NG-1</td>
<td>Some</td>
<td>Atrophy &amp; malnutrition</td>
<td>78.1</td>
<td>24.3</td>
<td></td>
</tr>
<tr>
<td>U-251 MG</td>
<td>About 85%</td>
<td>Active division &amp; aplasia</td>
<td>3.1</td>
<td>165.5</td>
<td></td>
</tr>
<tr>
<td>U-87 MG</td>
<td>About 100%</td>
<td>Very active division &amp; obvious aplasia</td>
<td>10.8</td>
<td>259.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.7</td>
<td>456.5</td>
<td></td>
</tr>
</tbody>
</table>

a AMF, Average mitotic figures.
b Areas examined for counting red blood cells varied as tumor and section size.
c Among six tumors, one tumor was occupied by about 70% tumor cells.

r² = 0.255, in Table 3. We then determined that high levels of endogenous bFGF expression did not correlate with VEGF expression (r² = 0.255). Daily bFGF levels correlated neither with angiogenesis (r² = 0.007), which was expressed by RBC density in tumor sections, nor with tumorigenicity (r² = 0.001), irrespective of cell culture density. As expected, angiogenesis correlated with tumorigenicity (r² = 0.836). This number was similar to the correlation factor obtained by analysis of the accumulated VEGF expression versus RBC density in tumor sections (r² = 0.804). Finally, the accumulated VEGF was found to be the only other variable that correlated with tumorigenicity (r² = 0.987).
Fig. 5  Representative tumor sections (× 100). Morphology of glioma cells in nude mice co-injected with Matrigel is shown. A few RBCs were found in the Matrigel mass, which is known to contain a short-term supply of many angiogenic factors (33, 34). Arrows, RBC clusters confined in large blood vessels. Clearly, more and larger clusters were found in HF U-251 MG and U-87 MG tumor sections than in any other tumor sections, suggesting that more active angiogenesis occurred in these two tumors.
Fig. 6 Immunohistochemistry analyses of glioma sections by antibodies against VEGF and factor VIII. Magnification is × 400 for VEGF (panels 1–4) and × 200 for factor VIII (panels 5–8). VEGF is stained in red. Factor VIII is stained dark brown. Pictures demonstrate clear correlation among level of VEGF expression, blood vessel size, and blood vessel density in tumor. VEGF is highly expressed in U87 MG and HF U-251 MG tumors (panels 1 and 2), and large blood vessel density is evident (panels 5 and 6). U-251 MG and EFC-2 tumors expressed less VEGF (panels 3 and 4) and produced smaller blood vessels (panels 7 and 8).
DISCUSSION

Artificial manipulation of cells in vitro can alter the gene expression profile in cellular ecosystem (35). It has proved to be more reliable for establishing the relative importance of certain genes in tumorigenesis by correlation analyses of patient samples (35–37) and established cell lines (37–39). The genetic diversity of in vitro cell lines parallels that of individual patient cases. It allows correlation analyses that compare tumorigenicity with quantitative gene expression to relate to comparisons of malignancy with quantitative tumor markers among biopsy specimens. For example, by analyzing gene expression in four cell lines using the chorioallantoic membrane assay, Park et al. (40) found that coexpression of uPA and u-PAR was required for tumorigenicity in gastric cancer cells, whereas concurrent expression of matrix metalloproteinases 2 and 9 was not necessary. Interestingly, results from an in vitro assay involving the coculture of endothelial cells with glioma cells and antibodies against VEGF, bFGF, and IL-8 revealed two pathways: a VEGF/bFGF pathway and an IL-8 pathway of endothelial cell tubular morphogenesis in two separate glioma cell lines (39). Our previous observations revealed that U-251 MG has a lower tumor take rate and forms significantly smaller tumors than those formed by U-87 MG cells.4 We hypothesized that the differences in tumorigenicity among the different cell lines were associated with the expression levels of bFGF and/or VEGF, two potent angiogenic factors. It was reported that the onset time of tumor cell-induced angiogenesis was found to be 2–3 days after inoculation. The increase of tumor blood vessel formation was statistically significant 4–7 days after inoculation (41, 42). Therefore, the production in the first week of bFGF and VEGF by the inoculated cancer cells is critical for tumorigenesis. Because daily monitoring of the product production from the inoculated tumor cell mass is difficult, we used in vitro product accumulation data to correlate with tumorigenicity of the glioma cell lines. To avoid any density effect on bFGF and VEGF expression (43–46), the same culture density and inoculation volume were applied in our study.

We found that bFGF levels were not related to angiogenesis and tumorigenicity of the glioma cells that we studied (Table 3). Similar conclusions were drawn in several other studies, including work by Landriscina et al. (47), who found no correlation between bFGF levels and the stage of human colorectal cancer in 35 patients. Likewise, in human medullar ade

4Unpublished data.

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