Increased Levels of Tissue Endostatin in Human Malignant Gliomas[1]

Takashi Morimoto, Masaru Aoyagi,[2] Masashi Tamaki, Yoshikazu Yoshino, Hisae Hori, Lian Duan, Takeo Yano, Masao Shibata, Kikuo Ohno, Kimiyoshi Hirakawa, and Noriko Yamaguchi

Department of Neurosurgery, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519 [T. M., M. A., M. T., Y. Y., L. D., K. O., K. H.]; Department of Molecular Pathogenesis, Division of Adult Disease, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 101-0062 [H. H., N. Y.]; and Medical and Biological Laboratories Co., Ltd., Nagano 396-0002 [T. Y., M. S.], Japan

ABSTRACT

Purpose: **Malignant gliomas are typically angiogenic and express greater amounts of angiogenic factors. We examined glioma tissues for their expression of an endogenous inhibitor of angiogenesis, endostatin, a COOH-terminal fragment of collagen XVIII.**

Experimental Design: **We examined frozen tissues from 51 patients with astrocytic tumors (grade 2, 13; grade 3, 9; and grade 4, 29). Frozen tissues were subjected to immunoblot analysis and immunohistochemistry for endostatin. Tumor vascular density was determined by calculating the percentage of tumor capillary vessel areas/tissue section area. Tissue concentrations of vascular endothelial growth factor and basic fibroblast growth factor were examined by enzyme immunoassay.**

Results: **The levels of endostatin protein estimated by immunoblotting were significantly higher in grade 4 than lower-grade glioma tissues. The immunoreactive bands for endostatin were identified as the fragment derived from noncollagenous domain 1 of collagen XVIII, a peptide 15 residues longer than endostatin toward the NH_2-terminal end, by NH_2-terminal amino acid sequencing. In addition to an intense immunoreactivity for endostatin in tumor blood vessels, sections from malignant gliomas showed widely distributed immunoreactivity around tumor cells near the hyperplastic microvessels. The tumor vascular density and the levels of vascular endothelial growth factor in grade 4 glioma tissues were significantly higher than grade 2 and grade 3 gliomas, whereas the levels of basic fibroblast growth factor were the same.**

Conclusions: **The results indicate a positive correlation between the levels of tissue endostatin and malignancy grades in gliomas. The endostatin may be released near the tumor blood vessels with hyperplasia to counteract angiogenic stimuli in malignant gliomas.**

INTRODUCTION

Gliomas are the most common form of primary brain tumors, with high-grade gliomas constituting the most serious as well as the most common group (1, 2). Malignant gliomas are typically angiogenic and characteristically show endothelial cell hyperplasia (3, 4). Recently, several angiogenic factors have been demonstrated in human gliomas, including VEGF,[3] basic FGF, and hepatocyte growth factor/scatter factor (5). Particularly, recent evidence has shown that the VEGF concentration in glioblastoma tissues is significantly higher than in low-grade glioma tissues (5, 6). Angiogenesis of tumors might be the result of a net balance between the positive and negative regulators of neovascularization (7, 8). Indeed, several human tumors generate angiogenesis inhibitors (9). These negative regulators include recently identified endogenous angiogenesis inhibitors angiostatin (8) and endostatin (7), which are generated similarly by proteolysis from large precursor proteins. Endostatin is a COOH-terminal globular domain of collagen XVIII that localizes mainly in the perivascular layer around blood vessels (7, 10) and may be generated by proteolytic cleavage by MMPs, elastase, or cathepsins (11–13). Because malignant gliomas, especially glioblastomas, are rich in angiogenesis with high levels of expression of angiogenic factors, we hypothesized that glioblastoma tissues also express high levels of endostatin. Ratel *et al.* (14) showed recently the presence of an antibody for endostatin in tissues and serum from a patient with multiple glioblastomas. In contrast, Strik *et al.* (15) reported a negative correlation in gliomas between endostatin positivity and malignancy grades by immunohistochemistry. In the present study, we examined human glioma tissues of various grades for the presence of the endostatin protein by immunoblot and immunohistochemistry and found the direct evidence that high levels of endostatin are contained in brain malignant gliomas.

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[2] To whom requests for reprints should be addressed, at Department of Neurosurgery, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5262; Fax: 81-3-5803-0140; E-mail: aoyagi.nsrg@tmd.ac.jp.

[3] The abbreviations used are: VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; NC1, noncollagenous domain 1.

MATERIALS AND METHODS

Tumor Tissue Preparation. Tumor specimens were obtained from 51 patients with astrocytic tumors who underwent surgery during the past 3.5 years at the University Hospital of the Tokyo Medical and Dental University. Informed consent was obtained from the patients and/or guardians during this study. The patients included 29 females and 22 males with a mean patient age of 46.5 ± 15.5 (SD) years. Immediately after surgical removal, the tumor samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h at 4°C and embedded in paraffin. Tumor samples were also embedded and frozen in OCT compound (Miles, Elkhart, IN). H&E staining was routinely performed, and the tumors were classified according to the malignancy criteria of the WHO. Tumor tissues were also frozen immediately in liquid nitrogen and stored at −80°C.

Western Blot Analysis. Frozen tumor tissues were homogenized in neutral buffer containing 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), 1 mM pepstatin A (Sigma), and 2 mg/ml aprotinin (Sigma) with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The tissue extract supernatants were mixed with SDS loading buffer containing DTT and then boiled for 5 min. Equal amounts of protein, 100 μg/lane, were loaded on 12.5% SDS-polyacrylamide gels and electrophoresed at 20 mA. The samples were transferred from the gels to Immobilon P membranes (Millipore, Tokyo, Japan) at 1.5 mA/cm^2 for 1 h. After blocking with 5% nonfat dry milk, the membranes were incubated with monoclonal antibodies against endostatin, followed by alkaline phosphatase-conjugated goat antimouse or antirat antibody (ICN, Aurora, OH). Endostatin-related proteins were visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate solution (Roche, Mannheim, Germany). Purified mouse monoclonal antibody to human endostatin (1D1) was generated by immunizing mice with recombinant human endostatin.[4] The hybridoma culture medium producing antibodies against mouse endostatin by immunized rats were generous gifts from Shigei Laboratories (Okayama, Japan). The recombinant human endostatin was purified from the conditioning medium of 293-EBNA cells stably transfected with a human endostatin gene as described previously by Yamaguchi *et al.* (16). The recombinant human endostatin was applied to the gels and used as a standard. The protein expression levels of the endostatin in tumor tissues were quantified by densitometric scanning with an NIH image program for the Macintosh.

Amino Acid Sequencing. Tumor tissue lysates were dialyzed against 0.02 M Tris-HCl (pH 7.5), 0.15 M NaCl and applied to a Hi-trap heparin column (Pharmacia, Piscataway, NJ) equilibrated in the same buffer. The proteins were eluted by a linear NaCl gradient concentration from 0.15 to 0.8 M and then dialyzed against PBS. The proteins were separated by SDS-PAGE using 5–20% gradient gels and then blotted onto Immobilon PSQ membranes (Millipore) for NH_2-terminal sequencing. An Applied 477 Pulsed Liquid Sequencer (Applied Biosystems, Foster City, CA) was used for automated Edman degradation, and the cleaved amino acid derivatives were analyzed using an Applied Biosystems 120 A Analyzer.

Immunohistochemistry. Seven-μm cryosections were autoclaved at 121°C for 20 min in 10 mM citrate buffer (pH 6.0). After treatment with 0.1% trypsin, the sections were incubated with mouse monoclonal antibodies against human endostatin (1D1) or rabbit polyclonal antibody against human von Willebrand factor (Dako, Kyoto, Japan). Immunohistochemical detection was carried out by a labeled streptavidin-biotin method with an LSAB kit (Dako). The sections were finally developed with diaminobenzidine and counterstained with hematoxylin. We substituted nonimmune serum or IgG for the primary antibodies and confirmed the specificity in every immunohistochemical test.

Tumor Blood Vessel Density. Tumor blood vessel density was determined by counting the number of capillary blood vessels or calculating the areas of tumor capillary vessels/high power field in sections stained with antibodies against factor VIII-related antigens. Vessel density was expressed as the mean number of vessels/field (0.25 mm^2) or the mean percentage of vessel areas/field from three highly vascularized areas.

Immunoassay for VEGF and Basic FGF. Concentrations of VEGF and basic FGF in the tumor tissue lysates were measured with enzyme immunoassay kits for human VEGF (R&D Systems, Minneapolis, MN) and basic FGF (Cytimmune, College Park, MD). The assays were performed in a blinded fashion.

Statistical Analysis. All values were calculated as mean ± SD. The differences in the mean values among various grades of gliomas were compared by unpaired *t* test. Correlation between tumor blood vessel densities and endostatin concentrations in human glioma tissues was analyzed using Pearson's correlation test.

RESULTS AND DISCUSSION

Increased Levels of Tissue Endostatin in Human Malignant Gliomas. The astrocytic tumors were classified according to the WHO criteria for malignancy. Thirteen patients were classified as grade 2 (astrocytomas), 9 patients as grade 3 (anaplastic astrocytomas), and 29 patients as grade 4 (glioblastomas). We examined the glioma tissues for the presence of endostatin by Western blot. Immunoreactive bands corresponding to endostatin were observed strongly in the grade 4 glioma tissues but were all very weak in the grade 2 gliomas (Fig. 1*A*). The size of the immunoreactive band corresponding to endostatin in glioma tissues was M_r 25,000 and was greater than that of recombinant human endostatin, *i.e.*, M_r 21,000–22,000. To confirm the identification of the immunoreactive band as endostatin, we performed NH_2-terminal amino acid sequencing. The NH_2-terminal sequences of M_r 25,000 immunoreactive bands was found to be SYVXXGPA, which was identified as the fragment derived from NC1 of collagen XVIII, NC1-25, a peptide 15 residues longer than endostatin toward the NH_2-terminal end (17).

The NC1 domain of collagen XVIII reportedly consists of an NH_2-terminal association region (−50 residues), a central protease-sensitive hinge region (−70 residues), and a COOH-terminal stable endostatin domain (−180 residues; Ref. 18).

[4] T. Yano, unpublished data.

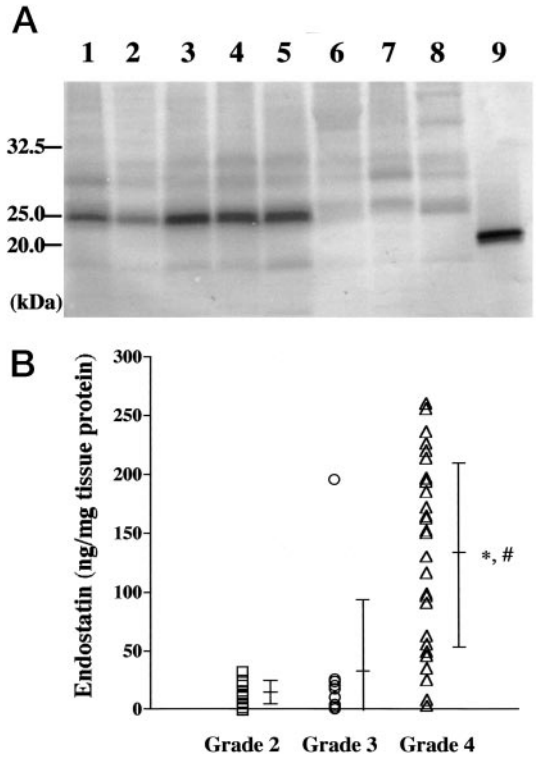

Fig. 1 *A,* Western blotting for endostatin in human glioma tissue extracts. *Lanes 1–4,* glioblastoma (grade 4); *Lane 5,* anaplastic astrocytoma (grade 3); *Lanes 6–8,* astrocytoma (grade 2); *Lane 9,* 20 ng of recombinant human endostatin. Samples were electrophoresed in a 12.5% SDS-polyacrylamide gel, transferred to a membrane, and detected with an antibody against endostatin (1D1). Immunoreactive bands are seen strongly at M_r 25,000 in tissues from several malignant gliomas but are weak in grade 2 gliomas. *B,* concentrations of endostatin in glioma tissues of various grades. The levels of endostatin protein were estimated semiquantitatively by densitometric scanning of the M_r 25,000 immunoreactive bands and comparing with those of recombinant human endostatin as a standard. □, astrocytoma (grade 2); ○, anaplastic astrocytoma (grade 3); △, glioblastoma (grade 4). Data are also expressed as means; *bars,* SD. *, $P < 0.001$ *versus* grade 2; #, $P < 0.02$ *versus* grade 3.

Wen *et al.* (11) and Felbor *et al.* (12) showed that elastase and cathepsin L generate endostatin from murine NC1. However, the murine endostatin cleavage site is not conserved in human collagen XVIII (12). Ferreras *et al.* (13) showed that proteinases including cathepsins and MMPs process human NC1, but the derived fragments are longer than the cathepsin L-generated murine fragment defined as endostatin. Their data are consistent with our results and those of Ständker *et al.* (19) and Sasaki *et al.* (18), who identified slightly larger peptides containing the endostatin domain in human plasma. Yamaguchi *et al.* (16) have shown that flag-containing endostatins and even NC1 are as effective inhibitors of endothelial cell migration as endostatin. Taken together, the M_r 25,000 immunoreactive band for endostatin that we identified from human malignant glioma tissues could have an inhibitory effect on angiogenesis.

Previously, serum or plasma endostatin concentrations were reported for patients with various types of cancers (20–24). Feldman *et al.* (23) have shown that serum levels of endostatin are elevated in patients with renal cell carcinoma and soft tissue sarcomas (24), whereas other authors reported circulating levels similar to healthy controls in patients with squamous cell vulvar carcinoma (20) and hepatocellular carcinoma (22). The inconsistency in these results may be attributable to the limitation of the enzyme immunoassays used for the detection of circulating levels of the endostatin. The enzyme immunoassays may not accurately estimate the endostatin concentration because of the reaction of endostatin antibodies with collagen XVIII-related antigens other than endostatin. Ratel *et al.* (14) showed recently the presence of an endostatin antibody in tissues and serum from a patient with multiple glioblastomas, suggesting the presence of endostatin in glioblastoma tissues. In the present study, the levels of endostatin protein expression in glioma tissues were estimated semiquantitatively by densitometric scanning of the immunoreactive bands in comparison with that of recombinant human endostatin as a standard. The amounts of endostatin protein contained in grades 2, 3, and 4 glioma tissues were 12.3 ± 9.83, 33.2 ± 61.7, and 132.9 ± 79.2 ng/mg tissue protein, respectively. The levels of endostatin protein were significantly higher in grade 4 than grade 2 and grade 3 glioma tissues (Fig. 1*B*; $P < 0.001$ *versus* grade 2 and $P < 0.02$ *versus* grade 3).

Immunoreactivity for Endostatin Localizes around Tumor Blood Vessels in Malignant Gliomas. We performed immunohistochemistry to examine the localization of endostatin in glioma tissues (Fig. 2). Positive immunoreactivity for endostatin was observed on tumor blood vessels in all grades of glioma tissue. The endostatin immunoreactivities in glioma tissues were almost the same as those for factor VIII-related antigens, being confined to the perivascular zone of blood vessels and not seen on tumor cells, especially on grade 2 glioma tissues (Fig. 2*A*). Sections from glioblastoma tissues with high amounts of endostatin as demonstrated by immunoblot showed that the immunoreactivity for endostatin was distributed widely around tumor blood vessels with hyperplasia. The immunoreactivity for endostatin was seen even around tumor cells near the perivascular zone and seemed to be localized extracellularly at a higher magnification, whereas the immunoreactivity for factor VIII-related antigens was confined to the tumor blood vessels (Fig. 2, *B–D*).

Our results do not agree with those of Strik *et al.* (15), who reported a negative correlation between endostatin positivity and malignancy grades in gliomas by immunohistochemistry. The findings of immunohistochemistry by Strik *et al.* (15) are quite different from those in the present study. The immunoreactivity for endostatin shown by Strik *et al.* (15) was observed even on macrophages and lymphocytes and was not seen on tumor capillary vessels. The difference may be attributable to the specificity of the antibody used in the study. Furthermore, immunohistochemistry alone could not accurately estimate the levels of endostatin in tumor tissues, because endostatin antibodies could react with endostatin as well as collagen XVIII. In fact, the present study showed that the positive immunoreactivity for endostatin on tumor microvessels of grade 2 gliomas was quite similar with that for collagen XVIII (10). In addition to immunohistochemistry, we performed Western blot analysis, and the result was confirmed by NH_2-terminal amino acid sequencing. Our results indicate a positive correlation between

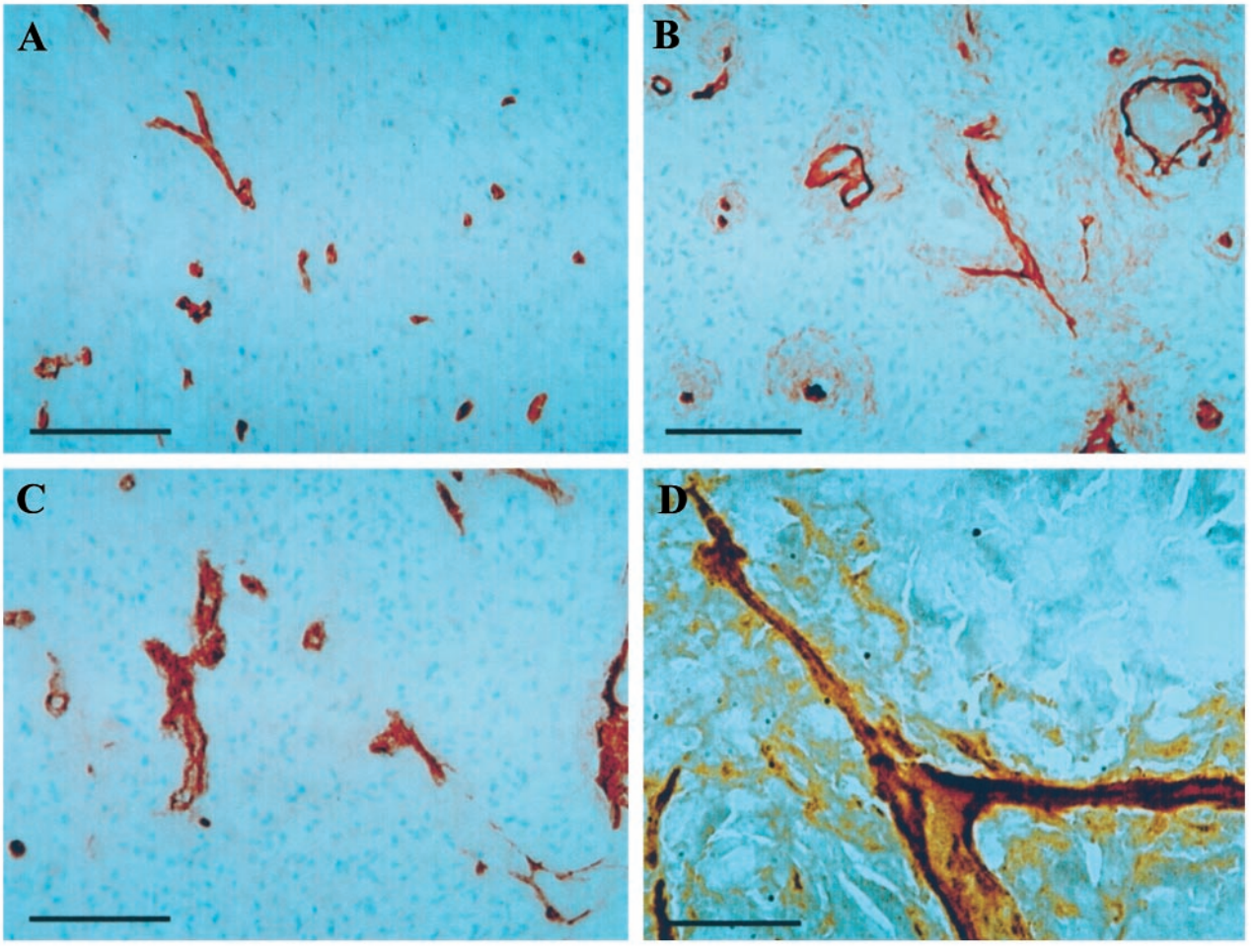

Fig. 2 Immunohistochemical detection of endostatin in tissue sections from gliomas. Immunostaining for endostatin in tissue sections of astrocytoma (grade 2, *A*) and glioblastoma (grade 4, *B* and *D*). Immunostaining for factor VIII-related antigens in a section of glioblastoma (*C*). Although positive immunoreactivity for endostatin is confined to the tumor blood vessels in a section from an astrocytoma, it can be seen distributed widely around tumor blood vessels with hyperplasia in a section from a glioblastoma. The immunoreactivity for endostatin is seen even around tumor cells near the perivascular zone and seems to be localized extracellularly at a higher magnification (*D*). The immunoreactivity for factor VIII-related antigens is confined to the tumor blood vessels. *Bars: A–C,* 100 μm; *D,* 20 μm.

elevated levels of tissue endostatin and malignancy grades in gliomas.

Increase in the Tumor Vascular Density and the Levels of VEGF in Malignant Gliomas. In addition to an increase in the number of tumor blood vessels, glioblastomas characteristically show endothelial cell hyperplasia (3, 4). To correlate the concentrations of endostatin protein in glioma tissues with tumor angiogenesis, we examined tumor blood vessel density in glioma tissues (Fig. 3). The number and the area of tumor capillary blood vessels in glioma tissues increased with increasing tumor grade. The areas of tumor capillary blood vessels/field in grade 4 gliomas were significantly greater than those in grade 2 and grade 3 gliomas ($P < 0.001$ *versus* grade 2 and $P < 0.05$ *versus* grade 3; Fig. 3*A*). The correlation between endostatin concentrations and the number of tumor capillary vessels/field was weakly positive in glioma tissues ($P < 0.05$; correlation coefficient, $r = 0.30$). However, a strong positive correlation was found when endostatin concentrations were compared with the areas of tumor capillary vessels/field (Fig. 3*B*; $P < 0.001$, $r = 0.70$). The result was consistent with the presence of immunoreactivity for endostatin around tumor cells near the hyperplastic tumor blood vessels in malignant gliomas.

We then examined the concentrations of angiogenic growth factors, VEGF and basic FGF, in the tumor tissue lysates by enzyme immunoassay (Fig. 4). Sundberg *et al.* (25) reported recently that VEGF is both sufficient for the generation and necessary for the maintenance of glomeruloid vascular proliferation, a defining histological feature of glioblastoma and some other cancers. The present study showed that VEGF concentrations in glioblastoma tissues were significantly greater than in lower-grade glioma tissues ($P < 0.01$ *versus* grade 2 and $P < 0.05$ *versus* grade 3), whereas the levels of basic FGF were the same. Yamaguchi *et al.* (16) showed recently that endostatin could inhibit VEGF-induced endothelial cell migration *in vitro*.

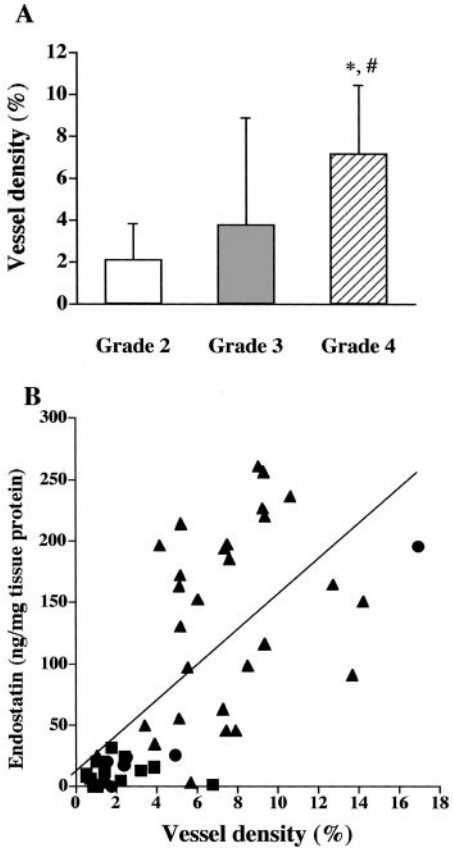

Fig. 3 *A,* tumor blood vessel densities (means) in sections from gliomas of various grades; *bars,* SD. Frozen sections were immunostained with antibodies against von Willebrand factor. The areas of tumor capillary vessels/high power field were calculated, and the vessel density was expressed as the mean percentage of vessel areas/field from three highly vascularized areas. *, $P < 0.001$ *versus* grade 2; #, $P < 0.05$ *versus* grade 3. *B,* correlation between endostatin concentration and tumor blood vessel density in human glioma tissues. ■, astrocytoma (grade 2); ●, anaplastic astrocytoma (grade 3); ▲, glioblastoma (grade 4). The regression line was obtained by the method of least squares. $y = 5.74 + 15.0x$, correlation coefficient (r) = 0.70, $P < 0.001$.

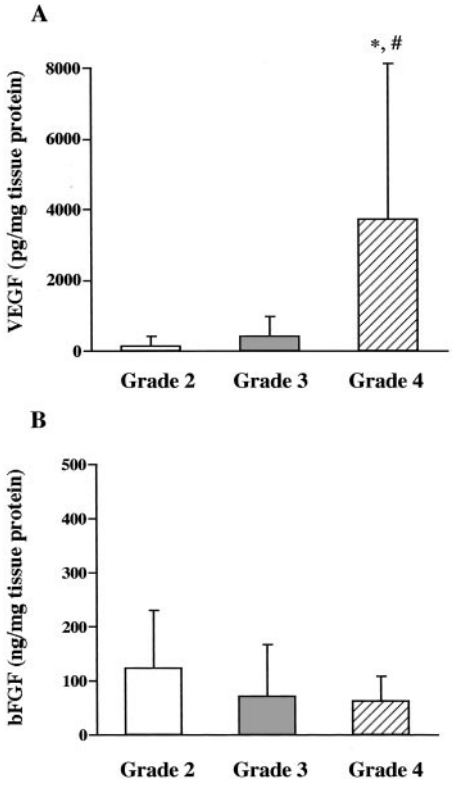

Fig. 4 Tissue concentrations of VEGF (*A*) and basic FGF (*B*) in gliomas. Concentrations of VEGF and basic FGF in the tumor tissue lysates were measured with enzyme immunoassay kits for human VEGF and basic FGF. *, $P < 0.01$ *versus* grade 2; #, $P < 0.05$ *versus* grade 3.

Our findings indicate that endostatin is processed or activated near the hyperplastic tumor microvessels in malignant gliomas. The endostatin that increased around the hyperplastic tumor microvessels in malignant gliomas potentially counteract angiogenic stimuli such as VEGF.

The Role of Endostatin in Malignant Gliomas. Recent evidence indicates that several proteinases, such as cathepsins, elastase, and MMPs, process NC1 of collagen XVIII to endostatin, and even endostatin is degraded by cathepsins (12, 13). Malignant gliomas are known to express high levels of MMP activity (26). Thereby, angiogenesis seems to be controlled in a feedback mechanism. In addition, large amounts of VEGF are secreted from malignant glioma cells and act on endothelial cells to promote angiogenesis (27). The elevated levels of endostatin might represent an attempt at a compensatory response to the angiogenic phenotype of malignant gliomas (23). Thus, an overall net balance between angiogenic stimulators and inhibitors may deviate toward angiogenesis, despite the high levels of endostatin production in malignant gliomas. Alternatively, Ratel *et al.* (14) showed recently the presence of endostatin antibodies in tissues and serum of aggressive cancers and suggested the possibility that tumors can escape the effects of antiangiogenic molecules. Such complexities in the regulation of angiogenesis may explain why some glioblastoma tissues contain low levels of endostatin protein. Further investigations that focus on the regulation of the processing or inactivation mechanisms of endostatin in malignant gliomas will facilitate the creation of effective antiangiogenic therapeutic approaches for currently incurable malignant gliomas.

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