Title

Angiogenesis and anti-angiogenic therapy for malignant gliomas

Shingo Takano・Hiroshi Kamiyama・Koji Tsuboi・Akira Matsumura

Address to which proofs are to be sent:

Department of Neurosurgery, Institute of Clinical Medicine, University of Tsukuba

1-1-1 Tennoudai, Tsukuba city, Ibaraki 305-8575, Japan

E-mail shingo4@md.tsukuba.ac.jp

Key words: Glioma, Angiogenesis, VEGF, soluble VEGF receptor, thrombospondin-1

Abstract

[PURPOSE] Angiogenesis is crucial to malignant glioma growth. Therefore, antiangiogenic therapy represents as new, promising therapeutic modality for malignant gliomas. This study designed to define the malignant glioma cases most suitable for anti-angiogenic therapy in human and to demonstrate the efficacy of anti-angiogenic therapy in the animal.

[METHODS & RESULTS] In human malignant glioma samples (24 glioblastomas, 13 anaplastic astrocytomas), the protein expression of most potent angiogenic factor, VEGF and its specific natural inhibitor, soluble Flt-1 as well as vessel architectures, including vessel density, area, and diameter were evaluated. Among these, VEGF>1000 ng/ml, VEGF/soluble Flt1 ratio >1, vessel density >30, vessel area >7% were prognostic factors for malignant gliomas. Based on these results, we demonstrated three different anti-angiogenic experiments targeted to inhibit VEGF expression in human malignant glioma (U87) mouse model. 1) Anti-VEGF neutralized antibody intraperitoneal injection, 2) interferon-beta intramusclar injection, and 3) an endogenous non-specific angiogenesis inhibitor, thrombospondin-1, transfection into glioma cells caused inhibition of VEGF secretion and/or mRNA expression and resulted in glioma growth inhibition of 70%, 84%, and 50% respectively compared to control.

[CONCLUSION] We conclude that malignant gliomas with high degree of VEGF expression and vessel area are good candidate for anti-angiogenic therapy, especially when designed to target to inhibit VEGF expression.

Introduction

Angiogenesis is crucial to malignant glioma growth. [1-3] Therefore, antiangiogenic therapy represents as promising therapeutic modality for malignant gliomas.[4] Current defined endogenous angiogenic stimulators and inhibitors in neoplasms have been well described.[5] Especially the balance between stimulators and inhibitors regulate angiogenesis of the tumors, resulting tumor growth.[6] Almost all of these factors have been investigated for gliomas and these factors should be designed for glioma anti-angiogenesis therapy. The evidence of glioma anti-angiogenesis therapy is described in many animal experiments combined with and without chemotherapy, where showed dramatic inhibition of glioma growth.[7,8] These include many anti-VEGF strategies, for example, antisence, antibody, receptor antibody etc.[9] Interestingly, four angiogenesis inhibitors, interferon-beta, marimastat, suramin, thalidomide, are now under the clinical trials. However, the results of these clinical trials have been limited. Further studies are needed to improve the efficacy of the therapy. This study designed to define the malignant glioma cases most suitable for anti-angiogenic therapy in human and to demonstrate the efficacy of anti-angiogenic therapy in the animal.

Materials and Methods

I. Angiogenic profiles of gliomas

Human malignant glioma samples (24 glioblastomas, 13 anaplastic astrocytomas) were stored at -80 degree. The protein expression of most potent angiogenic factor, VEGF and its specific natural inhibitor, soluble Flt-1 were measured with ELISA (R&D systems). Vessel architectures, including vessel density, area, and diameter were measured by CD31 stained sections using Windroof morphometry software (Sankou Shoji). By marking each vessel outline, vessel density per 0.13mm^2, vessel occupied area, vessel diameter, vessel perimeter, and vessel roundness were measured on three different areas. MIB-1(Immunotech) and p53 (DO7) positivities were measured. Based on these profiles, prognostic factors of malignant gliomas were investigated.

II. Anti-angiogenic therapy for U87 mouse glioma model

We designed three different anti-angiogenic experiments targeted to inhibit VEGF expression in human malignant glioma, U87 mouse model.[9] First, anti-VEGF neutralizing antibody experiment. Second, an endogenous non-specific angiogenesis inhibitor, thrombospondin-1, transfection into glioma cells, U87. Third, interferon-beta intraperitoneal injection. In each experiment, tumor growth, angiogenic profiles, and VEGF expression were evaluated.

Results

I. Angiogenic profiles and their relation to survival of gliomas

Our treatment strategy for malignant gliomas is total or subtotal removal, following by 40 Gy whole brain and 20 Gy local boast irradiation combined with PAV (procarbazine ACNU, Vincristine) chemotherapy and interferon-β. At the recurrence, PE (cisplatin, etoposide) chemotherapy and / or immunotherapy, including locolesional natural killer or cytotoxic T lymphocyte injection were carried out. Median survival rate of all malignant gliomas were 19.2 mo, (glioblastoma 11.2 mo, and anaplastic astrocytoma 30.9 mo).

VEGF concentration and sFlt-1 concentration more than 1000 pg/mg were prognostic factors. VEGF/ sFlt-1 ratio over than 1 was a strongest prognostic factor among each factor. Median survival time was 11.3 month for ratio over 1 and 28.7 month for ratio under 1 (Table). These results suggest the balance of stimulators and inhibitors of angiogenesis is especially important for the angiogenic evaluation of malignant gliomas. Other angiogenic parameters, vessel density more than 30, and vessel area more than 7% also predicted malignant glioma survival (Table).

II. Anti-angiogenic therapy for U87 mouse glioma model

1) Anti-VEGF neutralizing antibody experiment

Intraperitoneal injection of VEGF antibody 100 μg, twice a week for 4 weeks, inhibited glioma growth with subcutaneous model (Figure 1) and prolonged survival of intracranial glioma bearing mouse (data not shown).

2) Thrombospondin-1 transfection

Thrombospopndin-1 cDNA transfected into U87 using fugene6. Thrombospondin-1 transefectant secreted not only a large amount of thrombospondin in the conditioned medium but also a decreased amount of VEGF in the conditioned medium compared to parent and control glioma cells. The growth of the transfectant in vivo in the mouse subcutaneous model was significantly slow compared to control (Figure 2). Western blot analysis of transfectant glioma tissues shows decreased expression of VEGF as well as increased expression of thrombospondin-1 compared to control glioma tissues (data not shown).

3) Interferon-beta intraperitoneal injection

Low dose ($1x10^5$ unit every day for 15 days) and high dose ($5x10^5$ unit) interferon-beta systemic treatment significantly inhibited glioma subcutaneous growth. After discontinuation of the treatment, glioma growth re-started, suggesting the growth inhibitory effect of interferon-beta is cytostatic, not cytosidal (Figure 3A). In the glioma

tissues, interferon-beta treatment remarkably up-regulated the protein expression of IP-10, an endogenous inhibitor of angiogenesis and down-regulated the VEGF protein expression (Figure 3B).

Discussion

In this study, we demonstrated 1) angiogenic profiles of malignant gliomas which are related to prognosis and 2) three types of VEGF targeting anti-angiogenesis experimental therapy for malignant gliomas.

1) Angiogenic profile of malignant gliomas

Importance of angiogenesis of malignant gliomas has been demonstrated by many investigations. However, definite value of the degree of angiogenesis is variable between the methods which measure the angiogenesis. Therefore, which malignant gliomas are most suitable for the anti-angiogenesis therapy is not clear. Among the parameters of angiogenesis, VEGF[10] and its specific inhibitor, soluble Flt-1 are important. Soluble Flt-1 is a soluble form of VEGF receptor-1 called Flt1. soluble Flt1 is a part of intracellular domain of Flt1 and is existed in the tissue with the soluble form. Because soluble Flt-1 has VEGF binding site, it inhibits VEGF binding to the receptor.[11] In our study, VEGF>1000pg/mg, VEGF/sFlt-1 ratio>1, vessel density >30 / 0.13mm^2, vessel area >7% were prognostic factors for malignant gliomas. Especially, VEGF/

sFLT-1 ratio was the strongest prognostic factor, meaning not only the amount of stimulators but also the balance with a specific stimulator (e.g. VEGF) and its inhibitor (e.g. sFlt-1) is more reliable. Malignant gliomas which possess these angiogenic values should be suitable candidates for VEGF targeting anti-angiogenesis therapy.

2) VEGF targeting anti-angiogenesis therapy

Next, anti-VEGF antibody intraperitoneal injection, thrombospondin-1 transfection into glioma cell, and interferon-beta intraperitoneal injection caused inhibition of VEGF expression (secretion and/or mRNA level), and resulted in glioma growth inhibition compared to control

VEGF neutralizing antibody is now clinically relevant for colon cancer and renal cell carcinoma.[12] These data as well as our data promptly us to use these compounds as anti-angiogenesis molecules for malignant gliomas. Anti-angiogenic function of Thrombospondin-1 has been investigated for a long time.[13] In vitro, TSP-1 transfectant was inhibited the secretion of VEGF and in vivo, TSP-1 transfectant glioma tissues had few VEGF expression compared to control. This molecular link between TSP1 and VEGF is quite new finding and promising the possibility of anti-angiogenic gene therapy[14] utilizing TSP1 molecule. Interferon-β has been clinically used systemically and genetically.[15,16] Our experimental result clearly its anti-angiogenesis effect and its

cytostatic, not cytosidal, effect. We emphasize the target molecule of interferon-β is VEGF as well as bFGF[17] as a stimulator and IP10 as an inhibitor of angiogenesis in the view point of anti-angiogenesis.

We conclude that malignant gliomas with high degree of VEGF expression and vessel area are good candidate for anti-angiogenic therapy. Targeting to inhibit VEGF expression is a promising therapy for malignant gliomas.

Acknowledgments

We gratefully acknowledge Yoshiko Tsukada and Makiko Miyakawa for their excellent technical assistance. This study was supported by a Grant-in-Aid for Scientific Research from Japanese Ministry of education, Science, and Culture and by a grant provided by the Tsukuba Advanced Research Alliance.

References

1. Godard S, Getz G, Delorenzi M, Farmer P, Kobayashi H, Desbaillets I, Nozaki M, Diserens AC, Hamou MF, Dietrich PY, Regli L, Janzer RC, Bucher P, Stupp R, de Tribolet N, Domany E, Hegi ME (2003) Classification of human astrocytic gliomas on the basis of gene expression: a correlated group of genes with angiogenic activity emerges as a strong predictor of subtypes. Cancer Res 63: 6613-6625
2. Jensen, R.L. (1998) Growth factor-mediated angiogenesis in the malignant progression of glial tumors: a review. Surg. Neurol. 49, 189-196
3. Zagzag D, Amirnovin R, Greco MA, Yee H, Holash J, Wiegand SJ, Zabski S, Yancopoulos GD, Grumet M (2000) Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis. Lab Invest 80: 837-849
4. Lund E., Spang-Thomsen M, Skovgaard-Poulsen H, Kristjansen PEG (1998) Tumor angiogenesis - a new therapeutic target in gliomas. Acta Neurol Scand. 97: 52-62
5. Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nature Reviews Cancer 2: 727-739
6. Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the

angiogenic switch during tumorigenesis. Cell 86: 353-364

7. Kirsch M, Strasser J, Allende R, Bello L, Zhang J, Black PM (1998) Angiostatin suppresses malignant glioma growth in vivo. Cancer Res 58: 4654-4659
8. Reed TA, Sorensen DR, Mahesparan R, Enger PO, Timpl R, Olsen BR, Hjelstuen MH, Haraldseth O, Bjerkvig R (2001) Local endostatin treatment of gliomas administered by microcapsulated producer cells. Nat Biotechnol 19: 29-34
9. Takano S, Tsuboi K, Matsumura A, Nose T (2003) Anti-vascular endothelial growth factor antibody and nimustine as combined therapy: effects on tumor growth and angiogenesis in human glioblastoma xenografts. Neuro-Oncol 5: 1-7
10. Takano S, Yoshii Y, Kondo S, Maruno T, Shirai S, Nose T (1996) Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. Cancer Res 56: 2185-2190
11. Lamszus K, Ulbricht U, Matschke J, Brockmann MA, Fillbrandt R, Westphal M (2003) Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. Clin Cancer Res 9: 1399-1405
12. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab,

an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 349: 427-434

13. Bocci G, Francia G, Man S, Lawler J, Kerbel RS (2003) Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronic chemotherapy. Proc Natl Acad Sci USA 100: 12917-12922
14. Tanaka T, Cao Y, Folkman J, Fine HA (1998) Viral vector mediated “anti-angiogenic” gene therapy utilizing angiostatin cDNA. Cancer Res 58: 3362-3369
15. Yoshida, J.A., Kajita, T., Wakabayashi, T., and Sugita, K. (1994) Long-term follow-up results of 175 patients with malignant glioma: Importance of radical tumour resection and postoperative adjuvant therapy with interferon, ACNU and radiation. Acta Neurochir (Wien) 127, 55-59
16. Yoshida J, Mizuno M, Fujii M, Kajita Y, Nakahara N, Hatano M, Saito R, Nobayashi M, Wakabayashi T (2004) Human gene therapy for malignant gliomas (glioblastoma multiforme and anaplastic astrocytoma) by in vivo transduction with human interferon beta gene using cationic liposomes. Hum Gene Ther 15: 77-86
17. Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ (1995) Interferons alpha and beta down-regulate the expression of basic fibroblast growth

factor in human carcinomas. Proc Natl Acad Sci USA 92: 4562-4566

Figure legend

Figure 1　anti-VEGF antibody treatment (100 μg intraperitoneal injection, every 4 days, 8 times) inhibited U87 subcutaneous glioma growth. * $p < 0.01$ compared to control

Figure 2　The subcutaneous growth of Thrombospondin-1 transfected U87 glioma cells is significantly ($p < 0.01$) inhibited compared to ones of vector-transfected and non-transfected parent cells.

Figure 3　A. Intereferon-β treatment (low and high dose intraperitoneal injection, every day for 14 days) significantly ($p < 0.01$) inhibited U87 subcutaneous glioma growth. After discontinuation of the treatment (NO INF-β), tumor restarted to grow, meaning the cytostatic effect of the inhibition.　B. Western blotting with IP10 and VEGF of Glioma tissue treated with interferon-β. Interferon-β treatment increased expression of interferon-induced protein 10 (IP10) and decreased expression of VEGF.

Figure 1

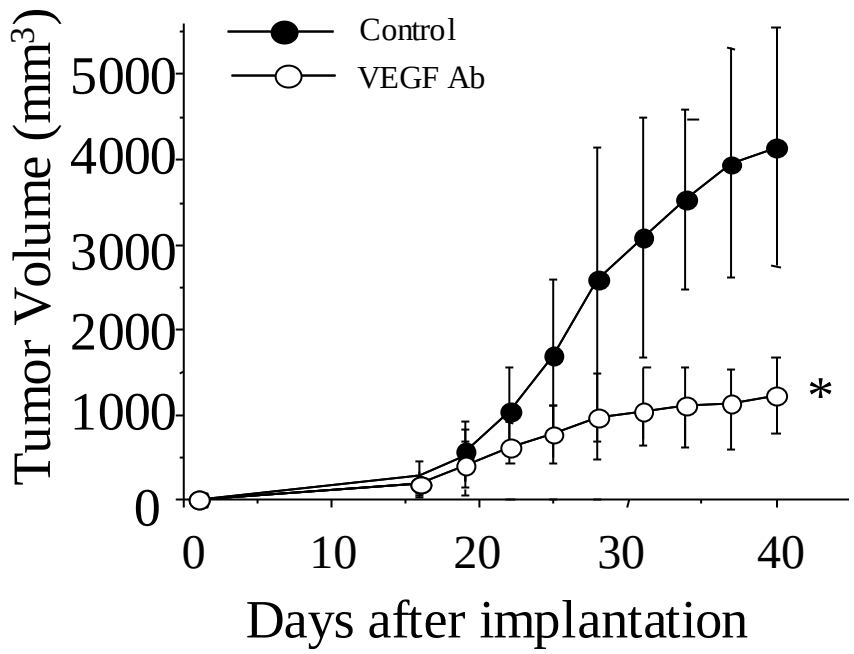

Figure 2

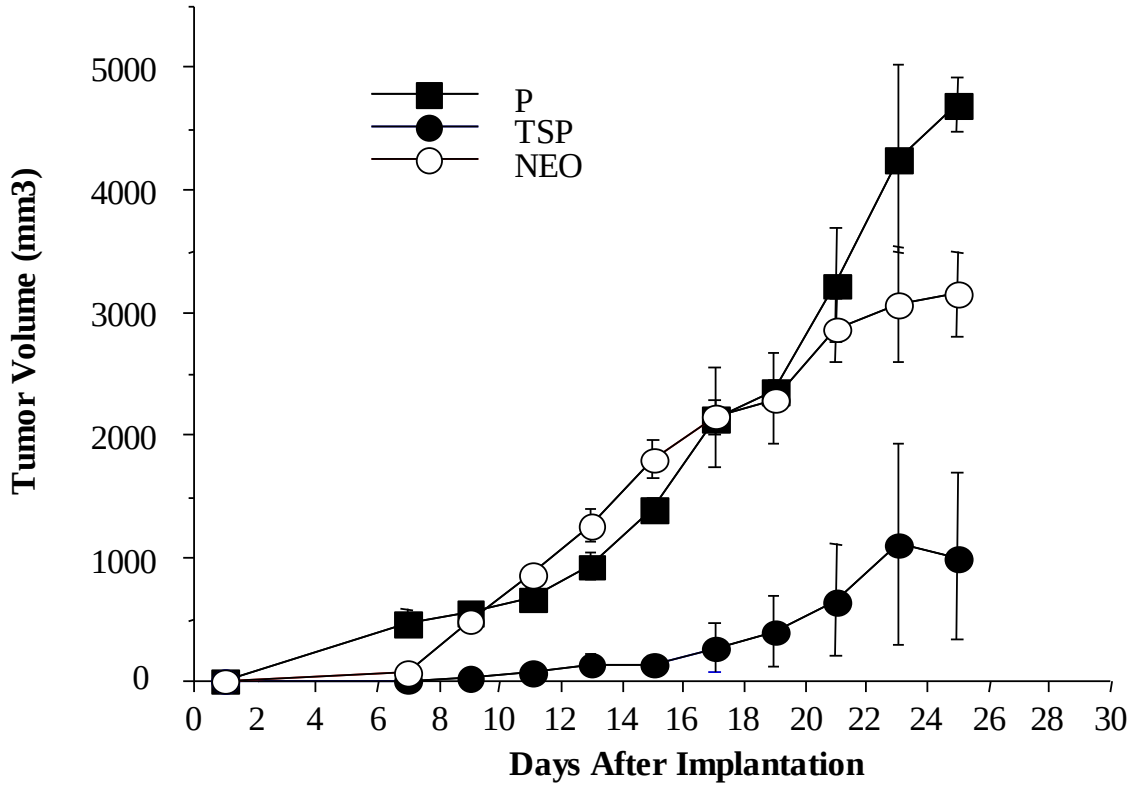

Figure 3A

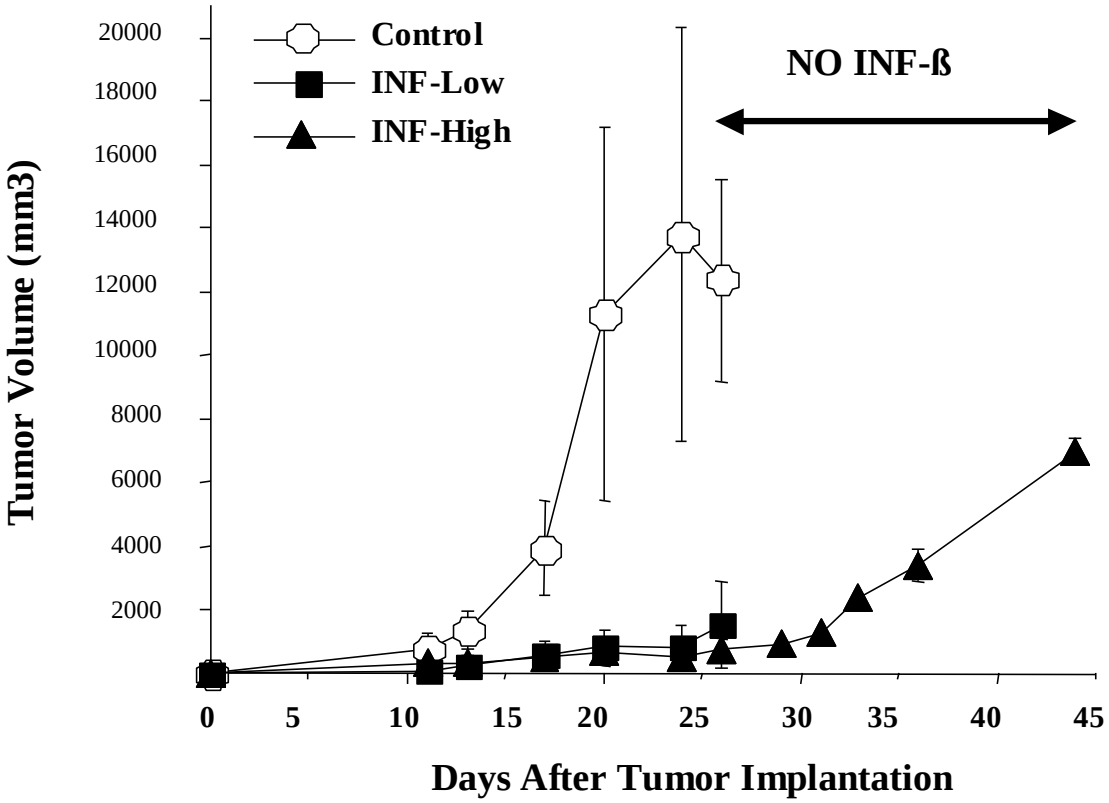

Figure 3B

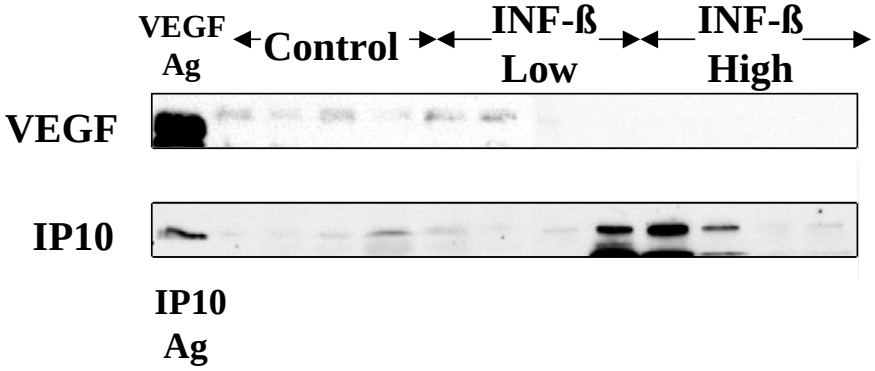

Table Angiogenic profiles relating to survival of malignant gliomas

Parameter		number	MST mo	p value univariate
VEGF (pg/mg)	>1000	20	11.8	0.0025
	<1000	17	24.8	
sFlt1 (pg/mg)	>1000	6	9.3	0.0145
	<1000	31	21	
Ratio	>1	24	11.3	<0.0001
	<1	13	28.7	
Density	>30	22	12.3	0.0177
	<30	15	24.6	
Area	>7%	14	11.6	0.0134
	<7%	23	23.1	
Pathology	GBM	24	11	<0.0001
	AA	13	28.8	
MIB-1	>20%	12	9.3	0.1742
	<20%	25	20.6	
p53	>20%	14	13.4	0.284
	<20%	23	21.1	

Figure 1

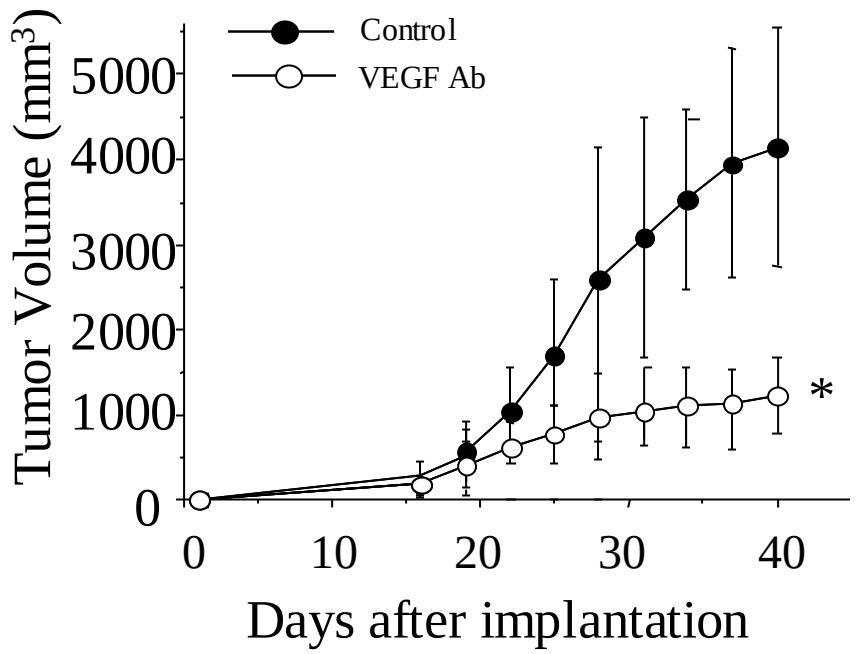

Figure 2

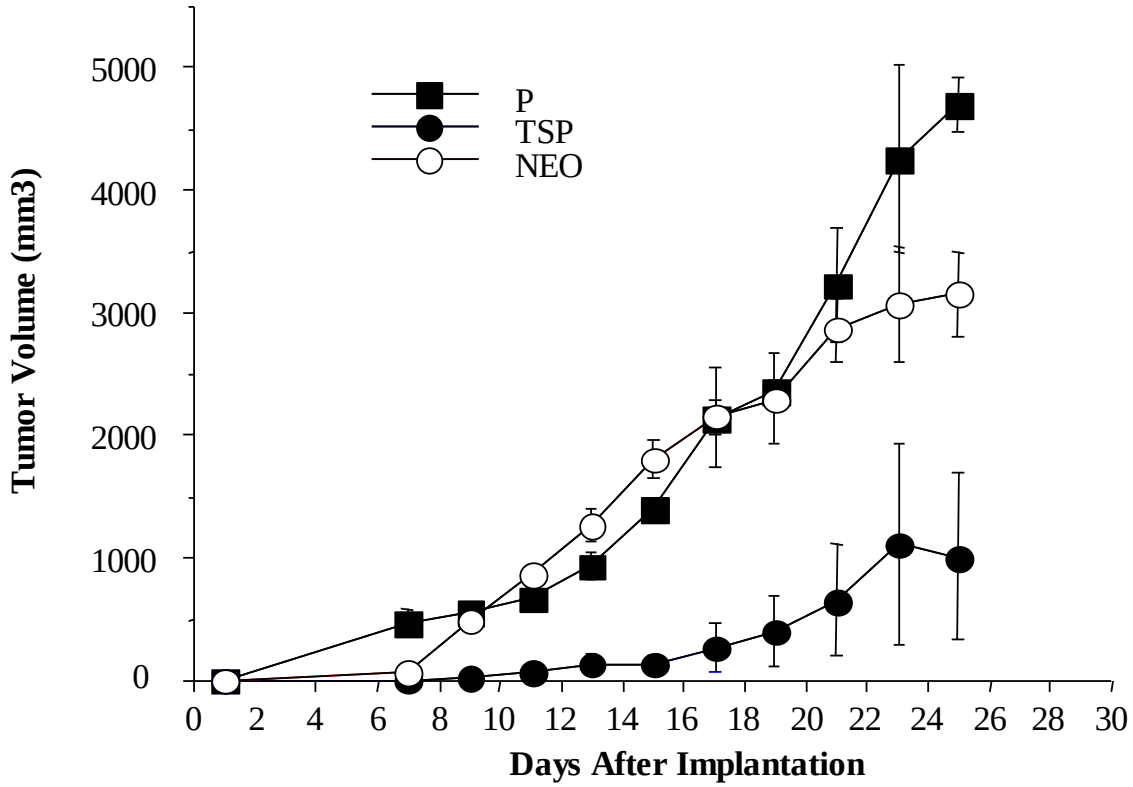

Figure 3A

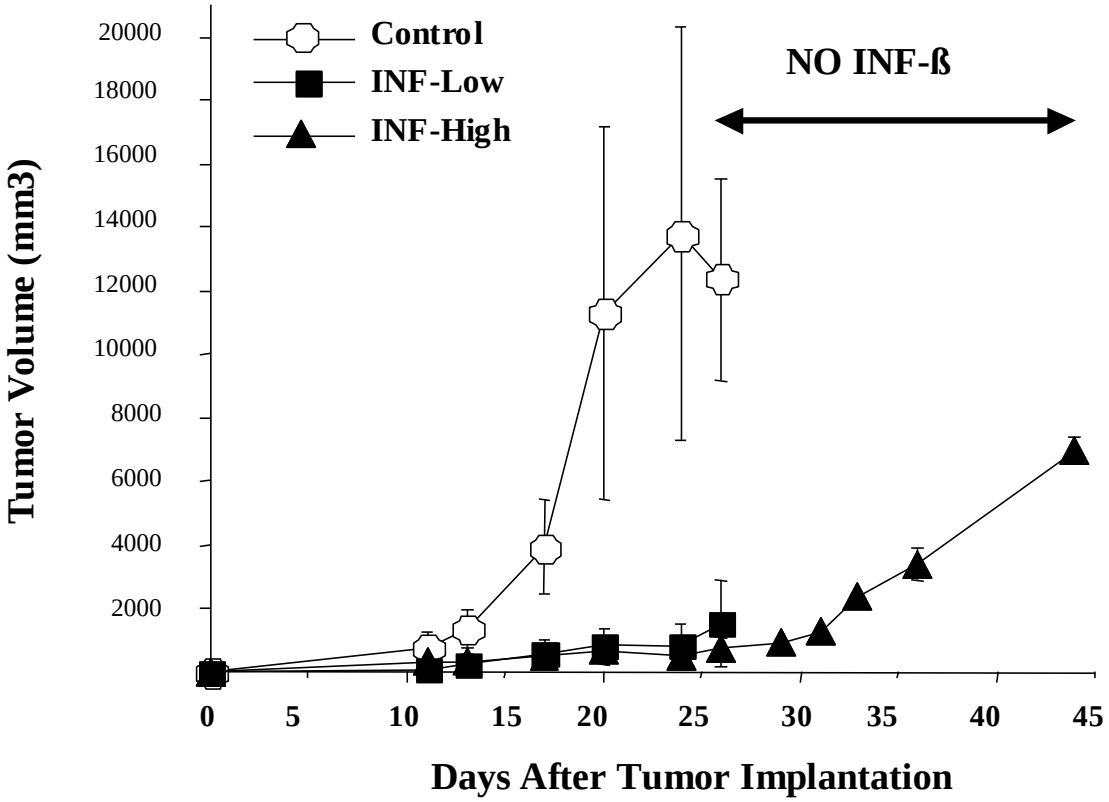

Figure 3B

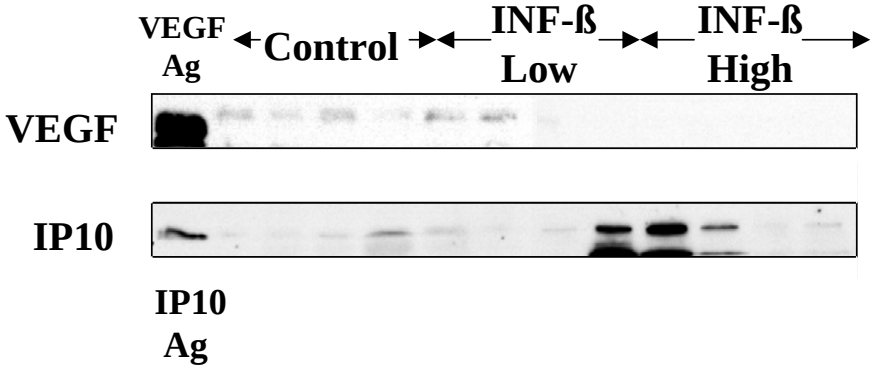

Table Angiogenic profiles relating to survival of malignant gliomas

Parameter		number	MST mo	p value univariate
VEGF (pg/mg)	>1000	20	11.8	0.0025
	<1000	17	24.8	
sFlt1 (pg/mg)	>1000	6	9.3	0.0145
	<1000	31	21	
Ratio	>1	24	11.3	<0.0001
	<1	13	28.7	
Density	>30	22	12.3	0.0177
	<30	15	24.6	
Area	>7%	14	11.6	0.0134
	<7%	23	23.1	
Pathology	GBM	24	11	<0.0001
	AA	13	28.8	
MIB-1	>20%	12	9.3	0.1742
	<20%	25	20.6	
p53	>20%	14	13.4	0.284
	<20%	23	21.1	