Pergamon

European Journal of Cancer, Vol. 35, No. 3, pp. 502–506, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00374-8

Original Paper

Thrombospondin-2 (TSP2) Expression is Inversely Correlated with Vascularity in Glioma

M. Kazuno,[1] T. Tokunaga,[2] Y. Oshika,[2] Y. Tanaka,[1] R. Tsugane,[1] H. Kijima,[2] H. Yamazaki,[2] Y. Ueyama[2] and M. Nakamura[2]

[1]Department of Neurosurgery; and [2]Department of Pathology, Tokai University, School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193 Japan

Thrombospondins (TSPs) are angiostatic factors in various cancers. However, the significance of TSPs has not been well characterised in glioma. We examined *TSP1*, *TSP2* and vascular endothelial growth factor (*VEGF*) gene expression by reverse transcription–polymerase chain reaction (RT–PCR) in 37 gliomas. Thirty of the 37 glioma specimens showed *VEGF* gene expression. Eighteen of the 37 gliomas expressed the *TSP1* gene. Seven gliomas lacked *TSP2* gene expression, while the other 30 expressed *TSP2*. The lack of *TSP2* gene expression was significantly associated with higher histological grade (Fisher's test, $P = 0.0019$) and increased vessel counts and density (Student's *t*-test, $P < 0.0001$), while there were no correlations between *TSP1* and *VEGF* gene expression and clinicopathological features. These results indicate that the lack of *TSP2* gene expression is a potent factor for enhancement of angiogenesis in glioma.

Key words: thrombospondin, angiogenesis, glioma
Eur J Cancer, Vol. 35, No. 3, pp. 502–506, 1999

INTRODUCTION

GLIOBLASTOMA MULTIFORME (GBM) is characterised by rapid growth, intense angiogenesis, vascular malformations and poor survival rate. The progressive growth of glioblastoma is thought to be dependent on angiogenesis [1]. Angiogenesis is regulated by the local balance between various molecules that induce and suppress neovascularisation. In the microenvironment around glioma, this balance shifts from neutral to angiogenic conditions. Certain angiogenic factors might be secreted from gliomas, and induce a vigorous angiogenic response overcoming local inhibitors.

Thrombospondin (TSP) is a high molecular weight multifunctional glycoprotein that is an important component of the extracellular matrix. This molecule was first described as a product of platelets, released from the alpha granules in response to activation of thrombin [2, 3]. The inhibitory properties of TSP on angiogenesis have been well characterised [4–6]. Genetically, five subtypes of TSP, i.e. TSP1, TSP2, TSP3, TSP4 and cartilage oligomeric matrix protein, have been identified [7–9]. Of the five structurally different TSPs, TSP1 and TSP2 show similar molecular architecture and inhibitory properties against angiogenesis [10–12]. TSP1 has been implicated in tumour progression in various cancer cell lines, whilst a human breast carcinoma cell line transfected with *TSP1*-cDNA showed reduced tumour growth, metastatic potential and angiogenesis [4]. Recently, TSP1 overexpression has been reported to inhibit angiogenesis in xenografts of human glioblastoma cell lines [13]. We previously reported that *TSP2* expression was correlated with decreased vascularity in lung and colon cancer specimens [14, 15]. However, the cell biological behaviour and expression pattern of *TSP*s have not been well characterised in primary glioma.

In this study, we evaluated the expression of the angiostatic factors *TSP1*, *TSP2* and angiogenic factor vascular endotheliel growth factor (*VEGF*) genes in 37 glioma specimens. The significance of the local balance of these factors with regard to vascularisation in glioma is also discussed.

MATERIALS AND METHODS

Tissue samples

Tissue samples were obtained from 37 glioma patients (10 females and 27 males, mean age 44 years) who underwent surgical resection between October 1990 and December 1996, at Tokai University Hospital. All gliomas were examined

Correspondence to M. Nakamura.
Received 3 Jul. 1998; revised 4 Sep. 1998; accepted 10 Sep. 1998.

under the microscope by two pathologists and classified according to WHO criteria [16]. There was one pilocytic astrocytoma, four fibrillary astrocytomas, one protoplasmic astrocytoma, one mixed oligoastrocytoma, 13 anaplastic astrocytomas and 17 GBMs. Surgical specimens were rapidly frozen and stored at −80°C until analyses. Total cellular RNA was prepared from frozen specimens. These tumour specimens were also fixed with 10% formalin for immunohistochemical examination.

TSP1, TSP2 *and* VEGF *gene expression*

We evaluated *TSP1* and *TSP2* gene expression by reverse transcription–polymerase chain reaction (RT–PCR) using the following sets of specific primers: *TSP1* sense primer, 5-ACCGCATTCCAGAGTCTGGC-3 (Th1-S); *TSP1* antisense primer, 5-ATGGGGACGTCCAACTCAGC-3 (Th1-A); *TSP2* sense primer, 5-CTGTGTCAACACTCAGCCTGGC-3 (Th2-S); *TSP2* antisense primer, 5-TCCTTCTCATCGGTCACACCG-3 (Th2-A). Reverse transcription was performed at 42°C for 60 min (1 μg total cellular RNA; 100 pM random primers, 40 U reverse transcriptase, GIBCO-BRL, Maryland, U.S.A.). DNA fragments were amplified by 30 rounds of PCR (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min; Gene Amp PCR System 9600, Perkin Elmer, California, U.S.A.) and *Taq* DNA polymerase (1.3 U, Toyobo, Osaka, Japan). RT–PCR amplified a 492 bp fragment of *TSP1* cDNA and a 433 bp fragment of *TSP2* cDNA. Blots (Zeta-Probe, BIO-RAD, California, U.S.A.) were hybridised with photochemically labelled *TSP1*-/or *TSP2*-specific cDNA probes (ECL; enhanced chemiluminescence, Amersham, Buckinghamshire, U.K.), and exposed to Kodak AR film. The quality of the sample RNA was also estimated by RT–PCR for β2-microglobulin.

We evaluated the expression of *VEGF* mRNA by RT–PCR according to the previously described procedure [17]. We also confirmed the pattern of *VEGF* mRNA isoforms.

Human glioblastoma xenografts

Three xenografts were established from the 17 GBMs and maintained by serial subcutaneous transplantation in severe combined immune deficiency (SCID) mice (Clea Japan, Tokyo, Japan; 10–20 passages). We obtained xenografts from mice sacrificed under deep anaesthesia. No significant morphological differences were observed between xenografts and primary tumours.

Vascularisation in glioma

We evaluated vascularisation in glioma according to the previously described procedure [14, 15]. Formalin-fixed, paraffin-embedded sections of the glioma tissue were examined immunohistochemically with mouse antihuman CD34 monoclonal antibody (NCL-end, Novo Castra, Newcastle upon Tyne, U.K.). After blockage of endogenous peroxidase activity (methylalcohol, 3% H_2O_2) and non-specific binding (10% normal goat serum), the specimens were incubated with anti-CD34 antibody (1:20) at room temperature for 60 min. Sections were serially incubated with biotin-labelled antimouse IgG (Nichirei, Tokyo, Japan) and horseradish peroxidase-conjugated streptavidin (Nichirei).

Immunoreaction products were visualised with 3,3′-diaminobenzidine and H_2O_2. Light microscopy was used to identify two regions within or immediately adjacent to the cancer containing the highest number of vessels. The microvessel counts and densities were evaluated at ×200 magnification (×20 objective and ×10 ocular, 0.739 mm^2/field) using a computerised image analyser (Interactive Build Analysis System, Carl Zeiss, Jena, Germany).

Statistical analysis

Differences in survival between subgroups of patients were compared with the log-rank test and survival curves were plotted according to the method of Kaplan and Meier. The χ^2 test or Fisher's exact test was applied for comparisons between group frequencies. Differences in mean vessel counts and density among the groups were analysed by Student's *t*-test.

RESULTS

TSP1, TSP2 *and* VEGF *gene expression*

Eighteen of the 37 glioma specimens examined expressed *TSP1*, and all of 18 specimens were also positive for *TSP2* gene expression (Figure 1a and b). *TSP2* gene expression was detected in 30 of the 37 specimens. Seven glioma specimens lacking *TSP2* gene expression were also negative for *TSP1* gene expression.

Thirty of the 37 glioma specimens showed *VEGF* gene expression (81%). The isoform patterns were classified into two groups: VEGF121, and VEGF121 + VEGF165 + VEGF189. Five of the 37 glioma specimens expressed VEGF121 alone (14%), and 25 gliomas were positive for VEGF121, VEGF165 and VEGF189 (68%). None of the gliomas examined showed VEGF206 expression.

Correlation between VEGF *and* TSP *gene expression and WHO grade*

Expression of the *TSP2* gene was significantly correlated with lower WHO grade (grade 1–3; 20/30) (Fisher's test, $P = 0.0019$) as compared with gliomas lacking *TSP2* (grade 1–3; 0/7). However, *TSP1* gene expression was not

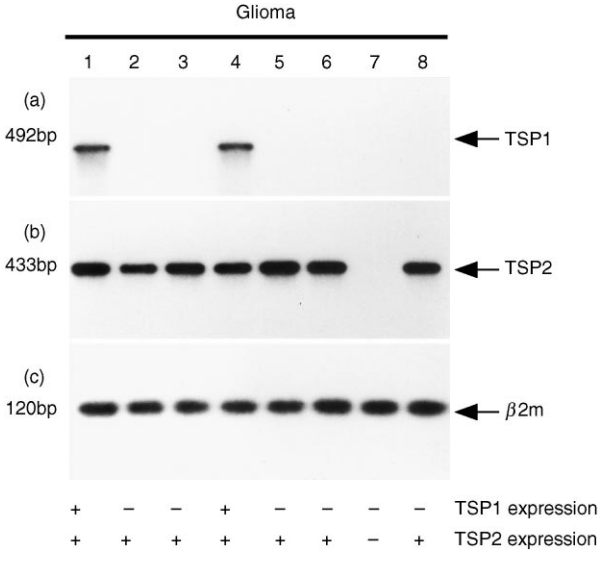

Figure 1. Examples of *TSP1* and *TSP2* gene expression in glioma: (a) *TSP1* expression was detected by reverse transcription–polymerase chain reaction (RT–PCR) with primers Th1-S and Th1-A, yielding a 492 bp specific fragment. (b) *TSP2* expression was detected by RT–PCR with primers Th2-S and Th2-A, yielding a 433 bp specific fragment. (c) β2 microglobulin (β2m) gene expression was evaluated to qualify RNA samples.

apparently correlated with any WHO grade (Table 1). *VEGF* gene expression including isoform pattern did not show any correlations with WHO grade.

Expression of VEGF and TSP in xenografts

One of the three xenograft specimens expressed *TSP1*. All the specimens (3/3) were positive for *TSP2* and *VEGF* gene expression. The isoform pattern was VEGF121 + - VEGF165 + VEGF189 for all the specimens. The expression pattern of *VEGF* and *TSP*s in xenograft tumours was identical to those of primary specimens.

Vascularisation and VEGF *and* TSP *gene expression*

The mean vessel count in gliomas expressing *TSP2* transcripts was $15.9 \pm 5.9/\times 200$ field (range 8–32); that in gliomas lacking *TSP2* expression was $65.3 \pm 25.1/\times 200$ field (range 29–118). The mean vessel density in gliomas expressing *TSP2* transcripts was $4.5 \pm 1.1\%/\times 200$ field (range 0.3–4.7), whilst that in those without *TSP2* expression was $9.0 \pm 1.4\%/\times 200$ field (range 2.7–22.6). The differences were significant between these two groups for vessel counts ($P < 0.0001$, Student's *t*-test) and vessel densities ($P < 0.0001$, Student's *t*-test) (Figure 2, Table 2). There were no correlations between *TSP1* expression and any histological feature of glioma (Table 1). Neither *VEGF* gene expression nor isoform pattern showed any correlation with the degree of vascularisation.

Correlation between clinical characteristics and VEGF *and* TSP *gene expression*

According to the pre-operational estimation, 20 patients demonstrated increased vascularity around the tumours by brain angiography and 8 patients revealed recurrence during the follow-up period. We used 'vessel increase' to indicate radiological features.

Table 1. *Univariate analysis of the associations between* TSP *gene expression and tumour characteristics*

	TSP1			*TSP2*		
	+	–	*P* value	+	–	*P* value
Sex			0.17			1
Male	14	13		22	5	
Female	4	6		8	2	
Age			0.64			1
< 60	12	14		21	5	
≥ 60	6	5		9	2	
Grade			0.51			0.0019*
1–3	11	9		20	0	
4	7	10		10	7	
Vessel increase			0.75			0.10
Yes	9	11		14	6	
No	9	8		16	1	
Recurrence			0.23			0.16
Yes	2	6		5	3	
No	16	13		25	4	

**TSP2* expression was significantly correlated with histological grade in glioma ($P = 0.0019$, Fisher's exact test). 'Vessel increase' was used to indicate an increased number of vessels around the tumour, by brain angiography, and recurrence was used to indicate the number of patients who recurred during the follow-up period.

2 of 8 (25%) recurrent patients showed *TSP1* expression, whereas 13 of the 29 recurrence free patients (45%) did not express this gene ($P > 0.05$, Fisher's test, Table 1). 5 of 8 (63%) recurrent patients showed *TSP2* expression, whereas 4

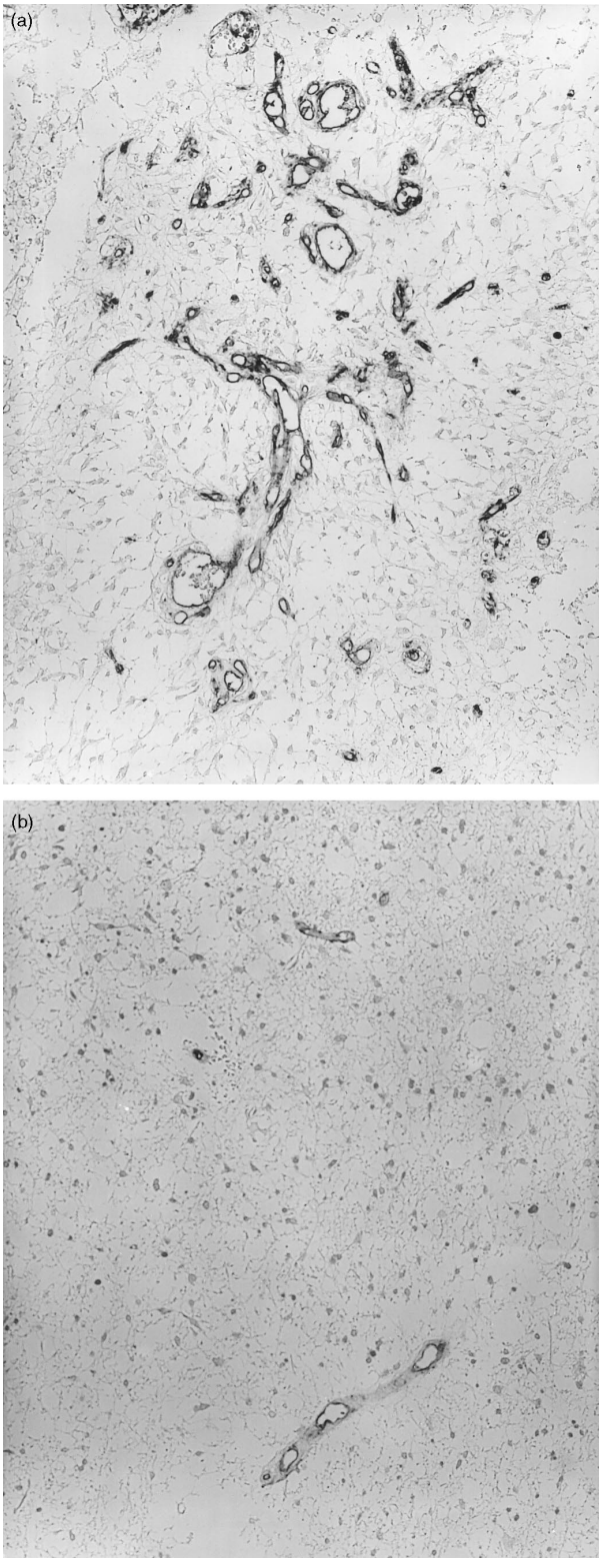

Figure 2. Vascularisation in gliomas was shown by CD34 immunostaining. (a) Glioblastoma multiforme (TSP2–) showed increased vascular density. (b) Low grade astrocytoma (TSP2+) showed lower vascular density (x 131).

Table 2. Associations between TSP2 *gene expression and tumour vascularity*

	TSP2 +	*TSP2* −	*P* value
Vessel counts	15.9 ± 5.9	65.3 ± 25.1	< 0.0001*
Vessel density	4.5 ± 1.1	9 ± 1.4	< 0.0001*

TSP2 expression was inversely correlated with vessel counts and density ($P < 0.0001$, Student's *t*-test).

of the 29 recurrence free patients (14%) did not express this gene ($P > 0.05$, Fisher's test, Table 1).

9 of 20 (45%) patients with 'vessel increase' showed *TSP1* expression, while 8 of 17 (47%) patients without 'vessel increase' did not express this gene ($P > 0.05$, χ^2 test, Table 1). 14 of 20 (70%) patients with 'vessel increase' showed *TSP2* expression, while 1 of 17 (6%) patients without 'vessel increase' did not express this gene ($P > 0.05$, Fisher's test, Table 1). There was also no correlation found between *VEGF* expression and clinical characteristics.

DISCUSSION

The prognostic significance of neovascularisation in solid neoplasms has been demonstrated in several kinds of tumours including malignant melanoma, breast, prostate, bladder, stomach and non-small cell lung cancer [18–23]. The relevance of angiogenesis and vascularity to the progression and malignancy of glioma is a major focus of current research. These phenomena are thought to be regulated and controlled by the local balance between inducing and suppressing factors produced in the glioma. TSPs are well known to be negative angiogenic or angiostatic factors, while the roles of *TSP1* and *TSP2* have not been well characterised in glioma *in vivo*.

In this study, we demonstrated a significant correlation between lack of *TSP2* transcripts and increased vascularisation in glioma. *TSP1* gene expression did not show any significant correlation with histological grade or vascularity of glioma. These results are similar to those of our previous studies in that *TSP1* gene expression showed no significant correlations with vascularity in colon cancer [15] or non-small cell lung cancer [14], while stromal angiogenesis was correlated with *TSP2* gene expression in these cancers. These results suggest that *TSP2* gene expression might be an important factor for neovascularisation in human glioma.

We also confirmed the preserved expression of the *TSP2* gene in human glioblastoma xenografts, in which stromal elements were replaced by the host SCID murine tissue (data not shown). Certain glioblastoma cells expressed the *TSP2* gene, while it is still unclear whether the neoplastic cells or stromal cells predominantly express *TSP2*. Further analyses are required of the localisation of cells producing TSP2 in glioma. We are now preparing an antihuman TSP2 antibody to estimate the localisation of TSP2 protein in glioblastoma.

VEGF has been suggested to be a potent factor positively regulating the angiogenesis in glioma [24–26]. *VEGF* gene expression of astrocytic tumours was upregulated with increasing grade of malignancy [27]. We analysed gene expression and isoform pattern of *VEGF* mRNA by RT–PCR. *VEGF* was expressed in 81% (30/37) of the glioma specimens examined and the isoform patterns of *VEGF* were varied (VEGF121, 14%, 5/37; VEGF121 + VEGF165 + -VEGF189, 68%, 25/37). No significant correlations were apparent between the expression and isoform pattern of *VEGF* and the histological grade or vascularity in glioma. We previously reported that cell-associated VEGF189 isoform was correlated with malignant progression of colon cancer [17] and non-small cell lung cancer [28]. However, we did not find such correlations in glioma. Stromal vascularisation which may support tumorigenicity can arise in a stepwise manner in response to both a decrease in the secretion of inhibitors and the sequential upregulation of the secretion of inducers of angiogenesis [29, 30]. However, no close correlation was apparent between *TSP2* and *VEGF* expression in glioma (data not shown). These results suggest that *TSP1* and *TSP2* gene expression are independent of *VEGF* expression in human glioma.

We demonstrated that lack of *TSP2* transcripts (19%, 7/37) was significantly correlated with histological grade IV of glioma. Higher grades of glioma are known to be the most vascularised tumours among human malignancies. This suggests that the angiogenic properties of glioma cells tend to be more aggressive in accordance with progression of grade via reductions in levels of angiostatic factor TSP2. This result is consistent with previous studies indicating that *TSP2* gene expression is significantly correlated with progression of non-small cell lung cancer [14] and colon cancer [15]. The prognostic relevance of *TSP2* gene expression is not yet clear, and prospective analyses in patients with glioma are required.

This study demonstrated that the major angiostatic factor in glioma was TSP2. The identification of TSP2 as an intrinsic angioinhibitory product of glioma may be a useful marker for prognostic evaluation. Angioinhibitory molecules mimicking TSP2 function will be potential candidates for therapeutic use in glioma.

1. Brem S. The role of vascular proliferation in the growth of brain tumors. *Clin Neurosurg* 1976, **23**, 440–453.
2. Baenziger NL, Brodie GN, Majerus PW. Isolation and properties of a thrombin sensitive protein of human platelets. *J Biol Chem* 1972, **247**, 2723–2731.
3. Lawler J. The structural and functional properties of thrombospondin. *Blood* 1986, **67**, 112–123.
4. Weinstat-Saalow DL, Zabrenetzky VS, VanHoutte K, Frazier WA, Roberts DD, Steeg PS. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumour growth, metastatic potential, and angiogenesis. *Cancer Res* 1994, **54**, 6504–6511.
5. Volpert OV, Tolsma SS, Pellerin S, *et al*. Inhibition of angiogenesis by thrombospondin-2. *Biochem Biophys Res Commun* 1995, **217**(1), 326–332.
6. Zabrenetzky V, Harris CC, Steeg PS, Roberts DD. Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung, and breast carcinoma cell lines. *Int J Cancer* 1994, **59**, 191–195.
7. Vos HL, Devarayalu S, de Vries Y, Bornstein P. Thrombospondin 3 (Thbs3), a new member of the thrombospondin family. *J Biol Chem* 1992, **267**, 12192–12196.
8. Lawler J, Duquette M, Whittaker CA, Adams JC, McHenry K, DeSimone DW. Identification and characterization of thrombospondin-4, a new member of the thrombospondin gene family. *J Cell Biol* 1993, **120**, 1059–1067.
9. Oldberg A, Antonsson P, Lindblom K, Heinegard D. COMP (cartilage oligomeric matrix protein) is structurally related to the thrombospondins. *J Biol Chem* 1992, **267**, 22346–22350.
10. Bornstein P, O'Rourke K, Wikstrom K, *et al*. A second, expressed thrombospondin gene (Thbs2) exists in the mouse genome. *J Biol Chem* 1991, **266**, 12821–12824.
11. Bornstein P. Thrombospondins: structure and regulation of expression. *FASEB J* 1992, **6**, 3290–3299.

12. Laherty CD, O'Rourke K, Wolf FW, Katz R, Seldin MF, Dixit VM. Characterization of mouse thrombospondin 2 sequence and expression during cell growth and development. *J Biol Chem* 1992, **267**, 3274–3281.
13. Hsu SC, Volpert OV, Steck PA, *et al.* Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. *Cancer Res* 1996, **56**, 5684–5691.
14. Oshika Y, Masuda K, Tokunaga T, *et al.* Thrombospondin 2 gene expression is correlated with decreased vascularity in non-small cell lung cancer. *Clin Cancer Res* 1998, **4**, 1785–1788.
15. Tokunaga T, Nakamura M, Oshika Y, *et al.* Thrombospondin-2 expression is correlated with inhibition of angiogenesis and metastasis of colon cancer. *Br J Cancer* (in press).
16. Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumours. *Brain Pathol* 1993, **3**, 255–268.
17. Tokunaga T, Oshika Y, Abe Y, *et al.* Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 1998, **77**, 998–1002.
18. Grossfeld GD, Ginsberg DA, Stein JP, *et al.* Thrombospondin-1 expression in bladder cancer: Association with P53 alterations, tumour angiogenesis, and tumour progression. *J Natl Cancer Inst* 1997, **89**, 219–227.
19. Graham C, Rivers J, Kerbel R, Stankiewicz K, White W. Extent of vascularisation as a prognostic indicator in thin (< 0.76 mm) malignant melanomas. *Am J Pathol* 1994, **145**, 510–514.
20. Weidner N, Semple JP, Welch WR, Folkman J. Tumour angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991, **324**, 1–8.
21. Weidner N, Carro PR, Flax J, Blumenfeld W, Folkman J. Tumour angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993, **143**, 401–409.
22. Maeda K, Chung YS, Takatsuka S, *et al.* Tumour angiogenesis as a predictor of recurrence in gastric carcinoma. *J Clin Oncol* 1995, **13**, 477–481.
23. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992, **340**, 145–146.
24. Kim KJ, Li B, Winer J, *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 1993, **362**, 841–844.
25. Saleh M, Stacker SA, Wilks AF. Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial sequence. *Cancer Res* 1996, **56**, 393–401.
26. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature* 1994, **367**, 576–579.
27. Chan AS, Leung SY, Wong MP, *et al.* Expression of vascular endothelial growth factor and its receptors in the anaplastic progression of astrocytoma, oligodendroglioma, and ependymoma. *Am J Surg Pathol* 1998, **22**, 816–826.
28. Oshika Y, Nakamura M, Tokunaga T, *et al.* Expression of cell-associated isoform of vascular endothelial growth factor 189 and its prognostic relevance in non-small cell lung cancer. *Int J Oncol* 1998, **12**, 541–544.
29. Volpert OV, Tolsma SS, Pellerin S, *et al.* Inhibition of angiogenesis by thrombospondin-2. *Biochem Biophys Res Commun* 1995, **217**, 326–332.
30. Volpert OV, Dameron KM, Bouck N. Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* 1997, **14**, 1495–1502.

Acknowledgements—The authors thank Mr. Yuichi Tada, Masashi Tomisawa, Johbu Itoh and Ms Kyoko Murata for their technical assistance. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture (MN, 09670205; HK, 9670239; NT, 07457588; 08877040; YU, 09680835) and Tokai University School of Medicine Research Aid (HK, YO and TT).