Macrophage Infiltration and Heme Oxygenase-1 Expression Correlate with Angiogenesis in Human Gliomas[1]

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ABSTRACT

Macrophages are key participants in angiogenesis. In this study on human brain tumors, we first investigated whether macrophage infiltration is associated with angiogenesis and malignant histological appearance. Immunostaining of macrophages and small vessels in resected glioma specimens indicated that numbers of infiltrating macrophages and small vessel density were higher in glioblastomas than in astrocytomas or anaplastic astrocytomas. Macrophage infiltration was closely correlated with vascular density in human gliomas. Heme oxygenase-1 (HO-1), which is the rate-limiting enzyme in heme catabolism, was also associated with activated macrophages. Expression of mRNA encoding HO-1 was correlated with macrophage infiltration and vascular density in human glioma samples. Infiltrating macrophages were positively stained with anti-HO-1 antibody by immunohistochemical analysis, and *in situ* hybridization for HO-1 indicated that HO-1 was expressed in infiltrating macrophages in gliomas. *HO-1* gene may be a useful marker for macrophage infiltration as well as neovascularization in human gliomas.

INTRODUCTION

Angiogenesis is necessary for the continued growth of solid tumors, and acceleration of tumor growth accompanies neovascularization (1, 2). Development of blood vessels within tumor tissue is closely correlated with invasion and metastasis as well as growth, as has been demonstrated in malignant melanoma and cancers in breast, lung, prostate, and other organs (1, 3). Endogenous angiogenic factors as well as angiogenesis inhibitors released by tumor cells and other type cells are thought to regulate tumor angiogenesis (4). Such angiogenic factors include VEGF,[3] bFGF, IL-8, platelet-derived growth factor, epidermal growth factor/transforming growth factor-α, and TNF-α, whereas angiogenesis inhibitors include thrombospondins, platelet factor 4, IFN-α, angiostatin, and endostatin (4).

In addition to endogenous angiogenic factors, the stroma of the neoplasm is essential for tumor growth, invasion, and neovascularization. The stroma intermingles with and surrounds neoplastic cellular elements in almost all solid tumor cells and includes interstitial connective tissues, basal lamina, and constituents such as type IV collagen, laminin, fibronectin, and proteoglycans (5). Blood vessels and inflammatory cells such as lymphocytes, neutrophils, macrophages, and natural killer cells are also observed frequently in the stroma. Interaction of stroma with malignant cells is considered to be critical for the development of neovascularity in tumors (6). Among stromal cells, macrophages carry out various biological functions, including participation in tumor angiogenesis (7, 8). Macrophages are important among the key angiogenic effector cells that produce a number of growth stimulators and inhibitors, proteolytic enzymes, and cytokines capable of modulating new vessel formation (9). Polverini (9) has demonstrated that conditioned medium derived from tumor-associated macrophages can induce angiogenesis. Implantation of syngeneic fibrosarcomas reportedly induces much less neovascularity in mice depleted of monocytes than in undepleted mice, and degree of vascularization correlates with macrophage infiltration in transplanted tumor cell lines (8). Macrophages activated by IFN-γ enhance angiogenesis in cultures of endothelial cells from rat aorta (10). Moreover, inhibition of macrophage infiltration by IL-10 is correlated with reduced tumor growth (11). Leek *et al.* (7) have further demonstrated that focally increased numbers of macrophages are closely related to vascularization and prognosis in patients with breast cancers. These studies suggest a close association of the monocyte/macrophage with angiogenesis in human tumors.

Human gliomas often show hypervascularity (12). Gliomas produce high levels of bFGF, which appears to mediate paracrine control of angiogenesis (13–15). Although bFGF has no conventional signal sequence, it acts as an angiogenic factor in the chorioallantoic membrane and cornea bioassays as well as in

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; TNF-α, tumor necrosis factor α; HO-1, heme oxygenase-1; DIG, digoxigenin; GAPDH, glyceraldehyde phosphate dehydrogenase.

in vitro angiogenesis models. (16–19). Glioma cell lines also produce high levels of a monocyte-macrophage-derived cytokine, IL-8, which stimulates chemotaxis of human vascular endothelial cells and angiogenesis in the cornea (20–22) and also induces formation of tube-like structures by human microvascular endothelial cells (23, 24). VEGF, which, unlike bFGF, has a signal sequence, is also produced abundantly in gliomas and glioma cell lines. VEGF shows potent angiogenic activity both *in vitro* and *in vivo* (12, 24–28). Glioblastoma growth is inhibited by a dominant-negative mutation for the protein of flk-1, a VEGF receptor, in an animal model (29), suggesting that VEGF-induced angiogenic signaling is critical in glioma growth. Gliomas are thus believed to produce several potent angiogenic factors such as VEGF, IL-8, and bFGF. Although such angiogenic factors are produced by human gliomas, how their expression is regulated by stromal or environmental effector cells is largely unknown. Glioma cells are highly susceptible to environmental stresses such as hypoxia and cytokines, resulting in induction of both VEGF production and neovascularization (26, 28, 30).

Here, we asked whether the macrophage component of the stroma participates in the neovascularization of human gliomas. Macrophage infiltration in surgically resected gliomas was determined by immunostaining with an antibody against the macrophage-specific marker, CD68. Microvessels were determined using an antibody against von Willebrand factor. Moreover, expression of *HO-1* gene is often enhanced in macrophage-like cells activated by lipopolysaccharide or phorbol myristate acetate (31–34) as well as brain tumors (35): HO-1, a heme-catabolizing and free radical-scavenging enzyme (36, 37), cleaves heme to release carbon monoxide, iron, and biliverdin (38, 39). We also determined whether expression of HO-1 in human glioma samples was correlated with macrophage infiltration or vascular density and whether activated macrophages were specifically stained using immunohistochemistry and *in situ* hybridization for HO-1.

MATERIALS AND METHODS

Samples. Resected specimens from 38 patients (ages 2–73 years) with primary gliomas who underwent operations in the Department of Neurosurgery at Kyushu University Hospital from 1993 to 1997 were evaluated for this study. Histological confirmation of the diagnosis was obtained in all cases. According to the revised WHO classification, tumors included 3 pilocytic astrocytomas, 6 fibrillary astrocytomas, 1 oligoastrocytoma, 1 oligodendroglioma, 8 anaplastic astrocytomas, 1 anaplastic oligodendroglioma, and 18 glioblastomas. All surgical specimens were snap-frozen immediately after removal and stored at −80°C.

Immunohistochemistry. Resected specimens of gliomas were fixed in 10% formalin solution, routinely processed, and embedded in paraffin. Six-μm-thick sections were stained immunohistochemically using an avidin-biotinylated peroxidase complex method with a mouse monoclonal antibody against the macrophage marker CD68, KP-1 (DAKO Glostrup, Denmark), a rabbit polyclonal antibody that reacts with Factor VIII (DAKO), and a rabbit polyclonal antibody against rat HO-1, SPA-895 (StressGen, Victoria, British Columbia, Canada), which exhibits cross-reactivity with human HO-1. The sections were counterstained lightly with hematoxylin.

Quantification of Macrophages and Microvessels. In each case, macrophage infiltration and microvascular density were assessed microscopically in the three hottest areas following a brief scan of the entire section at low power, and the numbers of macrophages and blood vessels per microscopic field (×400 magnification) were recorded.

***In Situ* Hybridization.** For *in situ* hybridization, DIG-labeled sense and antisense RNA probes were synthesized with T7 RNA polymerase from 360-bp template cDNA using DIG-RNA Labeling Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Prior to hybridization, 6-μm-thick sections were treated with proteinase K (1 μg/ml) at 37 °C for 5 min and postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. Thereafter, the sections were treated with 0.2 N HCl to inactivate internal alkaline phosphatase and acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine (pH 8.0) for 10 min. The pretreated sections were dehydrated, air-dried, and hybridized overnight with DIG-labeled RNA probe in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 10 mM DTT, 0.25% SDS, and 250 μg/ml *Escherichia coli* tRNA) at 50°C. After hybridization, each section was washed with 5× SSC (1× SSC = 0.15 M NaCl-0.015 M sodium citrate) briefly and then with 50% formamide-2× SSC for 30 min at 50°C. The sections were then washed with 2× SSC for 10 min, followed by 0.2× SSC for 20 min twice at 50°C. Detection of hybridization was performed immunohistochemically with alkaline phosphatase-conjugated Fab fragment of anti-DIG antibody using DIG-Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Northern Blot Analysis. Northern blot analysis was performed as described previously (23, 28, 40). The tumor tissue specimens were homogenized and suspended in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 M β-mercaptoethanol. Total RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, transferred onto a nylon membrane (Hybond N+; Amersham), and UV cross-linked at 0.25 J/cm^2 with Fluo-Link (Viler Lourmat, Marne-La-Vallee, France). The membrane was hybridized to ^{32}P-labeled DNA probes in Hybrisol (Oncor, Gaithersburg, MD) at 42°C for 24 h and washed once in 2× SSC with 0.1% SDS and finally in 0.2× SSC with 0.1% SDS at 42°C. mRNA levels were quantified by densitometry with a Fujix BAS 2000 bioimage analyzer. The expression indices of HO-1 and IL-8 mRNA were presented, normalized by the GAPDH mRNA level in each case.

Materials. IL-8 cDNA and [α-^{32}P]dCTP have been described previously (23, 40). HO-1 cDNA was purchased from the Swiss Institute for Experimental Cancer Research.

Statistics. Data were analyzed using Pearson's correlation coefficient. Student's *t* test was used to evaluate differences for statistical significance, represented by $P < 0.05$.

RESULTS

Macrophage Infiltration and Angiogenesis in Human Gliomas. We first examined the number of infiltrating macrophages and vascular density in gliomas from 38 patients, includ-

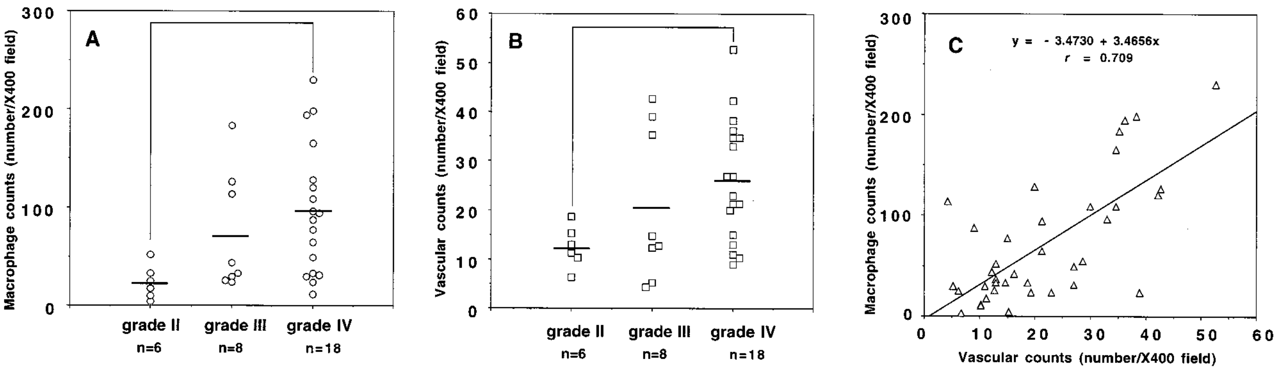

Fig. 1 Correlation between glioma grade and macrophage infiltration (*A*) or vascular density (*B*). Macrophage infiltration and microvascular density were assessed microscopically in the three hottest areas, and the numbers of macrophages and blood vessels per microscopic field (×400 magnification) were recorded. Mean macrophage counts were 23.6 ± 17.3 in grade II, 72.2 ± 60.6 in grade III, and 96.7 ± 65.5 in grade IV tumors. Mean small vessel counts were 12.5 ± 4.3 in grade II, 20.8 ± 15.6 in grade III, and 26.1 ± 12.3 in grade IV tumors. Both macrophage counts and vascular counts in grade IV were significantly higher ($P < 0.05$) than those in grade II tumors. *C*, correlation between macrophage infiltration and vascular density for 38 glioma samples.

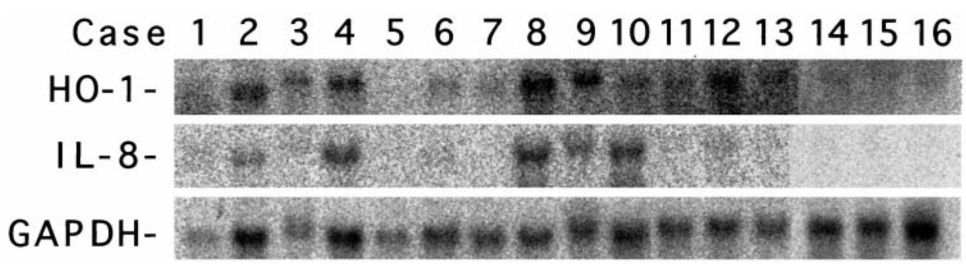

Fig. 2 Northern analysis of HO-1 and IL-8 in 16 glioma samples. Total RNA (20 μg) was electrophoresed on a 1% agarose-formaldehyde gel, which was then transferred to a nylon membrane and hybridized with radiolabeled cDNA probes. A GAPDH cDNA probe was used, as a control. (Results in each case are shown in the Table 1.)

ing three histological categories: fibrillary astrocytomas (grade II; $n = 6$), anaplastic astrocytomas (grade III; $n = 8$), and glioblastomas (grade IV; $n = 18$). We excluded pilocytic astrocytomas (grade I) from this part of study because these are a unique subtype.

Fig. 4, *A* and *B*, shows immunostaining for a grade IV tumor. We examined macrophage counts and small vessel counts in grade II, III, and IV tumors. Mean macrophage counts were 23.6 ± 17.3 in grade II, 72.2 ± 60.6 in grade III, and 96.7 ± 65.5 in grade IV tumors; counts in grade IV were significantly higher than those in grade II tumors ($P < 0.05$). The number of macrophages in grade III was higher than in grade II tumors (Fig. 1*A*), suggesting that the number of infiltrating macrophages is correlated with histological malignancy. Vascular density also showed an association with histological grade. Mean small vessel counts were 12.5 ± 4.3 in grade II, 20.8 ± 15.6 in grade III, and 26.1 ± 12.3 in grade IV tumors. Vascular density in grade IV was significantly higher ($P < 0.05$) than in grade II tumors. Vascular density in grade III was higher than in grade II (Fig. 1*B*). We also observed a significant correlation ($r = 0.709$) between high vascular grade and increased macrophage number (Fig. 1*C*).

HO-1 and IL-8 Expression. Expression of IL-8, which is also derived from monocytes and macrophages, was increased in various glioma cell lines. Northern analysis for *IL-8* gene expression in 16 glioma samples demonstrated IL-8 mRNA expression at various levels (Fig. 2), which was normalized to GAPDH mRNA levels. IL-8 mRNA levels were higher in grade IV than in grades III and II gliomas (Table 1). We found correlation coefficients between IL-8 mRNA expression and macrophage counts ($r = 0.634$). Weak staining was immunohistochemically observed in infiltrating macrophages when anti-IL-8 antibody was used (data not shown).

Expression of HO-1 was enhanced in macrophage-like cells activated by lipopolysaccharide or phorbol myristate acetate, which may make it a useful marker (31–34). We next determined HO-1 mRNA levels in human gliomas ($n = 16$) by Northern analysis (Fig. 2). HO-1 mRNA level in each glioma sample was normalized to mRNA levels for the *GAPDH* gene (Table 1). We examined whether expression of HO-1 mRNA was correlated with macrophage infiltration and vascular density in 16 glioma samples. On the basis of the data in the Table 1, we found correlation coefficients between HO-1 mRNA expression and both macrophage counts ($r = 0.769$, Fig. 3*A*) and vascularity ($r = 0.823$, Fig. 3*B*).

Immunostaining with anti-CD68 antibody showed appearance of many infiltrating macrophages in grade IV tumor (Fig. 4*A*). Immunostaining with anti-Factor VIII antibody showed appearance of many microvessels in the same grade VI tumor (Fig. 4*B*).

To determine whether HO-1 was specifically expressed in infiltrating macrophages, several grade IV samples were immu-

Table 1 HO-1 and IL-8 mRNA levels, macrophage counts, and vascular counts in gliomas

Case no.	Expression index[a] HO-1	IL-8	Macrophage counts[b]	Vascular counts[c]	Histology
1	0.736	0.23	120.0	42.3	Glioblastoma
2	0.396	0.17	128.3	20.0	Glioblastoma
3	0.450	0.16	23.7	23.0	Glioblastoma
4	0.396	0.39	94.0	21.3	Glioblastoma
5	0.127	0.07	29.3	5.3	Anaplastic astrocytoma
6	0.475	0.06	113.3	4.3	Anaplastic astrocytoma
7	0.540	0.01	23.3	39.0	Anaplastic astrocytoma
8	0.982	0.83	229.3	52.7	Glioblastoma
9	0.510	0.40	33.0	14.7	Anaplastic astrocytoma
10	0.255	0.45	87.3	9.0	Glioblastoma
11	0.261	0.08	54.3	28.7	Anaplastic oligoastrocytoma
12	0.766	0.13	183.3	35.3	Anaplastic astrocytoma
13	0.320	0.07	23.7	19.3	Pilocytic astrocytoma
14	0.285	0.01	52.0	13.0	Astrocytoma
15	0.300	0.01	3.7	15.3	Astrocytoma
16	0.221	0.01	2.7	6.7	Pilocytic astrocytoma

[a] Normalized to the GAPDH mRNA level.
[b] Macrophage counts are the numbers of macrophages (see Fig. 4*A*) observed in a ×400 field using light microscopy.
[c] Vascular counts are the numbers of vessels (see Fig. 4*B*) observed in a ×400 field using light microscopy.

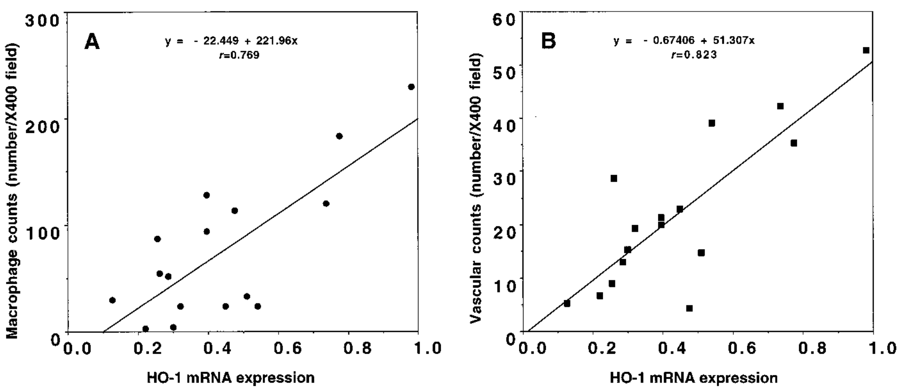

Fig. 3 Correlation between expression of HO-1 mRNA and macrophage infiltration (*A*) or vascular density (*B*). HO-1 expression indices, macrophage infiltration, and vascular density are based on the data in the Table 1. Expression of HO-1 mRNA was closely correlated with both macrophage infiltration and vascular density in human gliomas.

nohistochemically analyzed with anti-HO-1 antibody. As seen in Fig. 4, *C* and *D*, positive staining for HO-1 was specifically observed in infiltrating macrophages, but only weak staining (if there was any staining at all) was present in tumor cells. Other several samples also showed staining patterns that were almost similar to those in Fig. 4, *C* and *D* (data not shown). And cells expressing mRNA of HO-1 were examined by *in situ* hybridization analysis. Infiltrating macrophages are strongly stained in the sections hybridized with antisense probe, whereas other cells, including tumor cells and vascular endothelial cells, were not or were only weakly stained (Fig. 4*E*). Virtually no positive signal from any cells in the sections hybridized with the sense probe was observed (Fig. 4*F*).

DISCUSSION

By producing a number of growth stimulators and inhibitors, cytokines, and proteolytic enzymes, macrophages play a key role in the angiogenesis cascade (7–9). Macrophages in the stromal compartment of tumors, often called tumor-associated macrophages, are closely correlated with neovascularization and prognosis in patients with breast cancer (7). In this study, we examined whether macrophage infiltration was associated with neovascularity in resected gliomas. The number of infiltrating macrophages in human gliomas was closely correlated with vascular density (Fig. 1*C*). Tumor vascularization is often a limiting factor in metastasis and other clinically malignant behavior in various tumor types (1, 3). We also observed an apparent increase in vascular density in grade IV gliomas (glioblastomas), compared to grade II or III gliomas, and macrophage infiltration was observed more frequently in grade IV glioblastomas than in grade II or III gliomas (Fig. 1, *A* and *B*). Macrophage infiltration could be closely associated with neovascularization and also malignancy in human gliomas, suggesting tumor-associated macrophages as a possible prognostic factor.

One could argue how macrophages could induce angiogenesis in gliomas. Macrophages represent a terminally differentiated cell type within the mononuclear phagocyte system, and they produce a number of growth stimulators and inhibitors, including VEGF, bFGF, epidermal growth factor/transforming

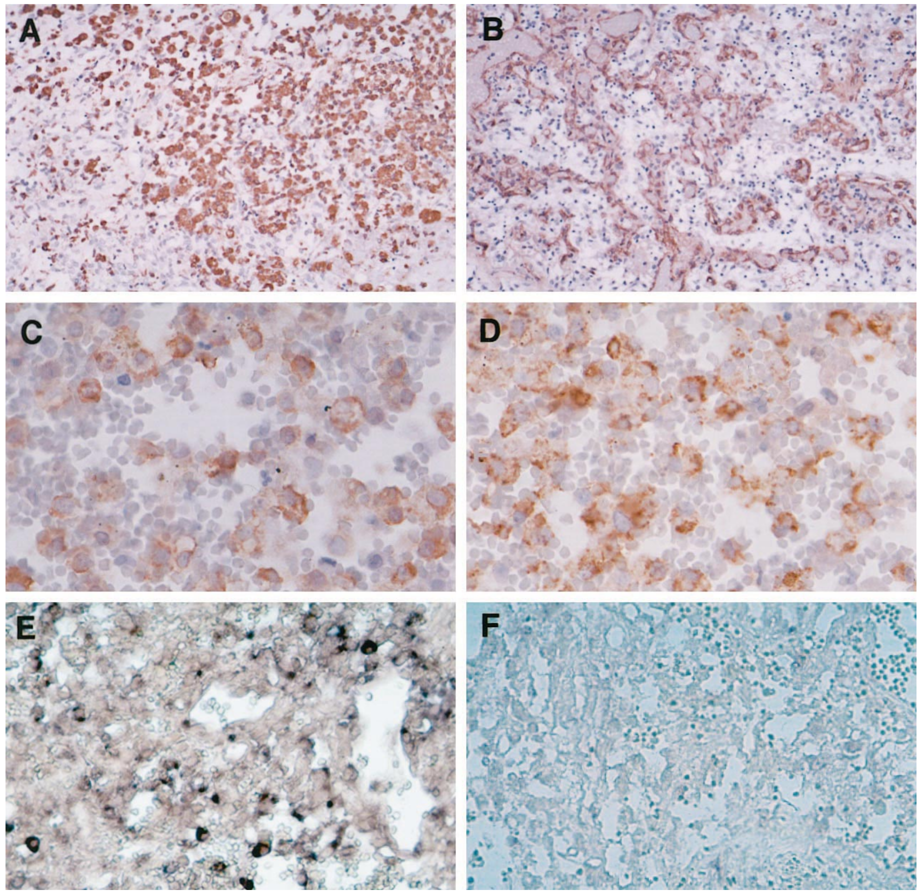

Fig. 4 *A* and *B*, immunohistochemical staining for CD68 (*A*) and Factor VIII (*B*) in grade IV glioma. Paraffin sections were stained immunohistochemically with anti-CD68 or Factor VIII antibody using an avidin-biotinylated peroxidase complex method. Prominent macrophage infiltration is evident in *A*. Prominent tumor vascularization is observed in *B*. Magnification, ×200. *C* and *D*, immunohistochemical staining for CD68 (*C*) and HO-1 (*D*) in grade IV glioma. Positive staining of HO-1 was observed in infiltrating macrophages, but weak or negative in tumor cells. *C* and *D* represent a series of specimens. Magnification, ×1000. *E* and *F*, *in situ* hybridization of grade IV glioma using the DIG-labeled antisense (*E*) and sense (*F*) HO-1 probe. This case is the same as that in *C* and *D*. Large and round cells, which are supposed to be macrophages, mainly demonstrate HO-1 mRNA with cytoplasmic staining pattern in *E*. When the sections were treated with RNase in the process of washing, the result was the same though the entire signal reduced. Negative controls included hybridization with the sense RNA probe and use of neither antisense RNA probe nor anti-DIG antibody. None of the controls showed positive signals. Magnification, ×400.

growth factor-α, platelet-derived endothelial cell growth factor, insulin-like growth factor-I, and IL-8 (7–9). Macrophages also modulate events in the extracellular matrix, either by direct secretion of degradative enzymes, including matrix metalloproteinases, urokinase-type plasminogen activator, tissue-type plasminogen activator, and plasminogen activator inhibitor-1, or by production of extracellular matrix-modulating cytokines. Moreover, activated macrophages produce TNF-α and IL-1. TNF-α up-regulates expression of the angiogenic factors VEGF and bFGF in vascular endothelial cells and in human glioma cells (27, 40, 41). Expression of IL-8 is also up-regulated in both vascular endothelial cells and glioma cells by TNF-α (20, 40). We observed that production of both VEGF and IL-8 was remarkably increased by TNF-α or IL-1 treatment in several glioma cell lines, but production of bFGF was not significantly increased by TNF-α or IL-1 treatment.[4] TNF-α and IL-1, derived from activated macrophages, would stimulate glioma cells to produce VEGF and IL-8. Macrophages, thus, not only produce angiogenic stimulators by themselves but also promote expression of VEGF and IL-8 in glioma cells through TNF-α and IL-1. TNF-α also stimulates vascular endothelial cells to produce VEGF, IL-8, and bFGF (40). Interaction of such angiogenic factors with vascular endothelial cells could result in development of neovasculature in human gliomas. Further study is needed to validate the diagrammed sequences for the *in vivo* angiogenesis network in human gliomas.

One major question is how tumors attract macrophages (7–9). Various factors that are produced by tumor cells are chemotactic toward macrophages, including monocyte chemotactic protein-1, macrophage colony-stimulating factor, IL-8, and others (7). In this study, IL-8 mRNA levels were found to be much higher in all six glioblastomas and two anaplastic astrocytomas than in eight other gliomas (Table 1). Levels of IL-8 mRNA were moderately correlated with macrophage counts (Table 1), suggesting that IL-8 might be mainly produced by activated macrophages or that IL-8 production by gliomas might play an important role in attracting and activating macrophages. Although IL-8 appears to be produced by macrophages as far as we examined several grade IV samples, further study with more samples must be performed for precise evaluation. It remains unclear which chemotactic factor is mainly involved in attracting and activating macrophages in gliomas.

HO-1 mRNA levels in gliomas were also closely associated with vascular density (Table 1 and Figs. 2 and 3). Induction of HO-1 is considered as a defense mechanism against free radicals (36, 37), and expression of the *HO-1* gene is activated in response to various environmental insults, including hypoxia,

[4] A. Nishie, Y. Ohmoto, M. Ono, and M. Kuwano, unpublished data.

heat shock, heavy metal toxicity, and UV light irradiation (31, 42). The *HO-1* gene is expressed selectively in brain tumors (35) and also in reactive astrocytes and macrophage-like cells (31). Moreover, Kurata *et al.* (32, 33) have reported that expression of both inducible nitric oxide synthase and *HO-1* genes is increased simultaneously on activation of macrophages. Muraoka *et al.* (34) have reported that increased HO activity reflects the functional state of activated macrophages. Consistent with these reports, expression of HO-1 mRNA was apparently induced in human macrophage-like U937 cells activated by phorbol myristate acetate.[5] Both immunostaining analysis and *in situ* hybridization assay apparently demonstrate that HO-1 is specifically expressed in macrophages in brain tumors. *HO-1* gene expression appears to be related to the number of activated macrophages. A correlation of HO-1 expression with vascular density again supports an association of macrophages with neovascularization. *HO-1* gene expression could be a useful marker for macrophage infiltration as well as neovascularization in human gliomas.

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REFERENCES

1. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, *79:* 185–188, 1994.

2. Folkman, J., Watson, K., Ingber, D., and Hanahan, D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature (Lond.), *339:* 58–61, 1989.

3. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1–8, 1991.

4. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.

5. Dvorak, H. F. Tumors. Wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med., *17:* 122–137, 1996.

6. Nagy, J. A., Brown, L. F., Senger, D. R., Lanir, N., Van De Water, L., Dvorak, A. M., and Dvorak, H. F. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochim. Biophys. Acta, *948:* 305–326, 1988.

7. Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast cancer. Cancer Res., *56:* 4625–4629, 1996.

8. Sunderkötter C., Steinbrank, K., Goebeler, M., Bhardwaj, R., and Sorg, C. Macrophages and angiogenesis. J. Leukocyte Biol., *55:* 410–422, 1994.

9. Polverini, P. J. Role of the macrophage in angiogenesis-dependent diseases. EXS, *79:* 11–28, 1997.

10. Kobayashi, S., Nagaura, T., Kimura, I., and Kimura, M. Interferon-γ-activated macrophages enhance angiogenesis from endothelial cells of rat aorta. Immunopharmacology, *27:* 23–30, 1994.

11. Richter, G., Kruger, K. S., Hein, G., Huls, C., Schmitt, E., Diamantstein, T., and Blankenstein, T. Interleukin 10 transfected into Chinese hamster ovary cells prevents tumor growth and macrophage infiltration. Cancer Res., *53:* 4134–4137, 1993.

12. Plate, K. H., and Mennel, H. D. Vascular morphology and angiogenesis in glial tumors. Exp. Toxicol. Pathol., *47:* 89–94, 1995.

13. Stefanik, D. K., Rizkalla, L. R., Soi, A., Goldblatt, S. A., and Rizkalla, W. M. Acidic and basic fibroblast growth factors are present in glioblastoma multiforme. Cancer Res., *51:* 5760–5765, 1991.

14. Takahashi, J. A., Mori, H., Fukumoto, M., Igarashi, K., Jame, M., Oda, Y., Kikuchi, H., and Hatanaka, M. Gene expression of fibroblast growth factors in human gliomas and meningiomas: demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. Proc. Natl. Acad. Sci. USA, *87:* 5710–5714, 1990.

15. Zagzag D., Miller, D. C., Sato, Y., Rifkin, D. B., and Burstein, D. E. Immunohistochemical localization of basic fibroblast growth factor in astrocytomas. Cancer Res., *50:* 7393–7398, 1990.

16. Lobb, R. R., Alderman, E. M., and Fett, J. W. Induction of angiogenesis by bovine brain derived class 1 heparin-binding growth factor. Biochemistry, *24:* 4969–4973, 1985.

17. Montesano, R., Pepper, M. S., and Mohlesteinlein, U. Increased proteolytic activity is responsible for the aberrant morphogenetic behaviour of endothelial cells expressing the middle T-oncogene. Cell, *62:* 435–445, 1990.

18. Rifkin, D. B., and Moscatelli, D. Recent developments in the cell biology of basic fibroblast growth factor. J. Cell. Biol., *109:* 1–6, 1989.

19. Abe, T., Ono, M., Kohno, K., Mori, T., Hori, S., and Kuwano, M. Induction of vascular endothelial tubular morphogenesis by human glioma cells. A model system for tumor angiogenesis. J. Clin. Invest., *92:* 54–61, 1993.

20. Kasahara, T., Mukaida, N., Yamashita, K., Yagisawa, H., Akahoshi T., and Matsushima, K. IL-1 and TNF-α induction of IL-8 and monocyte chemotactic and activating factor (MCAF) mRNA expression in a human astrocytoma cell line. Immunology, *74:* 60–67, 1991.

21. Van Meir, E., Ceska, M., Effenberger, F., Walz, A., Grouzmann, E., Desbaillets, I., Frei, K., Fontana, A., and de Tribolet, N. Interleukin-8 is produced in neoplastic and infectious disease of the human central nervous system. Cancer Res., *52:* 4297–4305, 1992.

22. Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., and Strieter, R. M. Macrophage inflammatory protein-1 α. A novel chemotactic cytokine for macrophages in rheumatoid arthritis. J. Clin. Invest., *93:* 921–928, 1994.

23. Shono, T., Ono. M., Izumi, H., Jimi, S., Matushima, K., Okamoto, T., Kohno, K., and Kuwano, M. Involvement of the transcription factor NFκB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. Mol. Cell. Biol., *16:* 4231–4239, 1996.

24. Wakabayashi, Y., Shono, T., Isono, M., Hori, S., Matsushima, K., Ono, M., and Kuwano, M. Dual pathways to tubular morphogenesis of vascular endothelial cells by human glioma cells: vascular endothelial growth factor/basic fibroblast growth factor and interleukin-8. Jpn. J. Cancer Res., *86:* 1189–1197, 1995.

25. Berkman, R. A., Merril, M. J., Reinhold, W. C., Monacci, W. T., Saxena, A., Clark, W. C., Robertson, J. T, Ali, I. U., and Oldfield, E. H. Expression of the vascular permeability factor/vascular endothelial growth factor gene in the central nervous system neoplasm. J. Clin. Invest., *91:* 153–159, 1993.

26. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature (Lond.), *362:* 841–844, 1993.

27. Ryuto, M., Ono, M., Izumi, H., Yoshida, S., Weich, H. A., Kohno, K., and Kuwano, M. Induction of vascular endothelial growth factor by tumor necrosis factor α in human glioma cells. J. Biol. Chem., *271:* 28220–28228, 1996.

28. Samoto, K., Ikezaki, K., Ono, M., Shono, T., Kohno, K., Kuwano, M., and Fukui, M. Expression of vascular endothelial growth factor and

[5] A. Nishie, unpublished data.

its possible relation with neovascularization in human brain tumors. Cancer Res., *55:* 1189–1193, 1995.

29. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A. Glioblastoma growth inhibited *in vivo* by a dominant negative Flk-1 mutant. Nature (Lond.), *367:* 576–579, 1994.

30. Shuweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia initiated angiogenesis. Nature (Lond.), *359:* 843–845, 1992.

31. Dwyer, B. E., Nishimura, R. N., Lu, S. L., and Alcaraz, A. Transient induction of heme oxygenase after cortical stab wound injury. Mol. Brain Res., *38:* 251–259, 1996.

32. Kurata, S., Matsumoto, M., and Nakajima, H. Transcriptional control of the heme oxygenase gene in mouse M1 cells during their TPA-induced differentiation into macrophages. J. Cell. Biol., *62:* 314–324, 1996.

33. Kurata, S., Matsumoto, M., Tsuji, Y., and Nakajima, H. Lipopolysaccharide activates transcription of the heme oxygenase gene in mouse M1 cells through oxidative activation of nuclear factor κB. Eur. J. Biochem., *239:* 566–571, 1996.

34. Muraoka, V., and Shibahara, S. Identification of a *cis*-regulatory element and putative *trans*-acting factors responsible for 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated induction of heme oxygenase expression in myelomonocytic cell lines. Mol. Cell. Biol., *13:* 7881–7891, 1993.

35. Hara, E., Takahashi, K., Tominaga, T., Kumabe, T., Kayama, T., Suzuki, H., Fujita, H., Yoshimoto, T., Shirato, K., and Shibahara, S. Expression of heme oxygenase and inducible nitric oxide synthase mRNA in human brain tumors. Biochem. Biophys. Res. Commun., *224:* 153–158, 1996.

36. Stocker, R., Yamamoto, Y., Mcdonagh, A. F., Glazer, A. N., and Ames, B. N. Bilirubin is an antioxidant of possible physiological importance. Science (Washington DC), *235:* 1043–1046, 1987.

37. Stocker, R., Glazer, A. N., and Ames, B. N. Antioxidant activity of albumin-bound bilirubin. Proc. Natl. Acad. Sci. USA, *84:* 5918–5922, 1987.

38. Tenhunen, R., Marver, H. S., and Schmid, R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc. Natl. Acad. Sci. USA, *61:* 748–755, 1968.

39. Tenhunen, R., Marver, H. S., and Schmid, R. Microsomal heme oxygenase. Characterization of the enzyme. J. Biol. Chem., *244:* 6388–6369, 1969.

40. Yoshida, S., Ono., M., Shono, T., Izumi, H., Ishibashi, T., Suzuki, H., and Kuwano, M. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor α-dependent angiogenesis. Mol. Cell. Biol., *17:* 4015–4023, 1997.

41. Okamura, K., Morimoto, A., Hamanaka, R., Ono, M., Kohno, K., Uchida, Y., and Kuwano, M. A model system for tumor angiogenesis: involvement of transforming growth factor-α in tube formation of human microvascular endothelial cells induced by esophageal cancer cells. Biochem. Biophys. Res. Commun., *186:* 1471–1479, 1992.

42. Lee, P. J., Jiang, B., Chin, B. Y., Iyer, N. V., Alam, J., Semenza, G. L., and Choi, A. M. K. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J. Biol. Chem., *272:* 5375–5381, 1997.

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