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

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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, 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, angiotatin, 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, fibronecin, 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 forma-

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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, 6 fibrillary astrocytomas, 1 oligoastrocytoma, 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 anhydride 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 μl NaCl-0.015 μl 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 guanidium isothiocyanate, 25 mm sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 μl 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/cm² with Fluo-Link (Viler Lourtmat, Marne-La-Vallee, France). The membrane was hybridized to 32P-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-32P]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-
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 the 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. 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.

**Case 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

**HO-1**

**IL-8**

**GAPDH**

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 \((r = 0.769, \text{Fig. 3A})\) and vascularity \((r = 0.823, \text{Fig. 3B})\).

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

To determine whether **HO-1** was specifically expressed in infiltrating macrophages, several grade IV samples were immu-
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. 4E). Virtually no positive signal from any cells in the sections hybridized with the sense probe was observed (Fig. 4F).

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. 1C). 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 growth factor-α (TGF-α), and transforming growth factor-β (TGF-β) (9, 30).

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

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* Normalized to the GAPDH mRNA level.

b Macrophage counts are the numbers of macrophages (see Fig. 4A) observed in a ×400 field using light microscopy.

Vascular counts are the numbers of vessels (see Fig. 4B) 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.
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 neovascularity 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 chemoattractant 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. 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


5 A. Nishie, unpublished data.
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