Identification and Localization of the Cytokine SDF1 and Its Receptor, CXC Chemokine Receptor 4, to Regions of Necrosis and Angiogenesis in Human Glioblastoma

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ABSTRACT

Glioblastoma multiforme (GBM) tumors display extensive histomorphological heterogeneity, with great variability in the extent of invasiveness, angiogenesis, and necrosis. The identification of genes associated with these phenotypes should further the molecular characterization, permitting better definition of glioma subsets that may ultimately lead to better treatment strategies. Therefore, we performed a differential mRNA display analysis comparing six GBM-derived primary cell cultures from patients having tumors with varied histomorphological features. We identified stromal cell-derived factor 1 (*SDF1*) as a gene with varied expression. SDF1 (cytokine) and CXC chemokine receptor 4 (CXCR4) interactions are implicated in modulating cell migration. They are also implicated in modulating the immune response in AIDS patients by macrophage-mediated T-cell apoptosis. GBM patients also fail to mount an immune response, although their tumors are seemingly exposed to immune cells in regions of angiogenesis, where the blood-brain barrier is absent, or in areas of necrosis. To determine whether the expression and localization of SDF1 and CXCR4 are consistent with such a role in these brain tumors, immunohistochemical analyses of these proteins were performed on normal brain and astrocytomas (grades II-IV). In normal brain tissue, low levels of SDF1 (0.5+) were observed in astrocytic processes, in neurons, and in the occasional phagocytic cells around vessels. CXCR4 expression was negative in brain tissue but was observed in phagocytic cells within the vessel lumen. In tumors, SDF1 and CXCR4 expression was colocalized when both were expressed, and SDF1 and CXCR4 expression increased with increasing tumor grade (from 0.5+ to 6+). Additionally, CXCR4 was expressed in neovessel endothelial cells. The proteins were expressed in regions of angiogenesis and degenerative, necrotic, and microcystic changes. Those tumors displaying greater amounts of these features had greater staining intensity of the proteins. The expression of SDF1 and CXCR4 did not colocalize with the proliferation marker MIB-1. Thus, our data suggest that SDF1 and CXCR4 expressions: (*a*) increase with increasing grade; (*b*) colocalize to regions within these tumors where their interaction may contribute to angiogenesis and/or modulation of the immune response; and (*c*) may serve to characterize subsets of GBMs.

INTRODUCTION

Astrocytic neoplasms are graded as astrocytoma (grade II), anaplastic astrocytoma (grade III), and GBM[2] (grade IV). The GBMs are particularly aggressive, and patients with this diagnosis seldom survive longer than 2 years. However, even within this tumor grade, there is a range of patient survival that is currently not predictable and may be a consequence of several factors. GBMs display extensive morphological heterogeneity that may reflect their origin from different populations of astrocytes and possibly from oligodendrocytic and ependymal cell lineages. Furthermore, they demonstrate variability in invasiveness, angiogenesis, and the extent of necrosis. The identification of genes associated with these phenotypes will further the molecular analysis of GBMs and may lead to a better definition of glioma subsets (1) that may result in better treatment strategies. To identify genes that are differentially expressed between such GBMs, we performed a differential mRNA display analysis comparing six primary cell cultures derived from GBMs that demonstrated a range of histomorphological features. We identified *SDF1* as a gene with varied expression. Consequently, we characterized the immunohistochemical expression and localization of SDF1 and its receptor, CXCR4, between GBMs and lower grade astrocytomas.

SDF1 is a highly conserved gene localized to chromosome 10q11.1 (2). The gene encodes two isoforms, SDF1-α and SDF1-β, that arise from alternative splicing. These isoforms differ only in that SDF1-β contains four additional 3′ amino acids (2). In all tissues examined to date, SDF1-α mRNA predominates (2). The SDF1 cytokines belong to the intercrine CXC chemokine subfamily, so designated because they have four conserved cysteines that form two essential disulfide bonds,

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[2] The abbreviations used are: GBM, glioblastoma multiforme; SDF1, stromal cell-derived factor 1; CXCR4, CXC chemokine receptor 4; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; TNF-R, TNF receptor.

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with the first two cysteines separated by one amino acid (3). SDF1 was originally described as pre-B cell growth-stimulating factor and implicated in lymphocyte maturation (4). Human SDF1 is chemotactic for T lymphocytes, monocytes, and neutrophils (5). However, its expression is not restricted to the leukocyte lineage, with high levels of message found in the human pancreas, spleen, ovary, and small intestine, and lower levels associated with the brain, colon, and placenta (2). Evidence implicating SDF1 in a broader role in development was obtained from mice lacking the SDF1 gene. These mice die perinatally and not only have severely impaired lymphopoiesis and abnormally low numbers of B-cell and myeloid bone-marrow precursors, as expected, but also display a defective ventricular septum of the heart (6) and defects in the central nervous system (7), suggesting that SDF1 may play a role in diverse cellular aspects during morphogenesis and development.

The SDF1s exert their influence by interaction with the seven-transmembrane G protein-coupled receptor CXCR4 localized to chromosome 2q2 (8). CXCR4 was first identified as HM89, a novel cDNA that was amplified using degenerate primers made against leukocyte chemotactic factor receptors (9), and was subsequently cloned by other groups and named LESTR (10). When LESTR was found to be the cofactor necessary to fuse HIV-1 and $CD4^+$ cells, it was given the name fusin (11). Once the ligand for the receptor was identified as SDF1, a CXC chemokine family member, it was renamed CXCR4. The expression of CXCR4 in T lymphocytes, monocytes, and neutrophils (3) mediates the chemotactic response to SDF1 by these cells. However, as observed with SDF1, expression is not restricted to leukocytes. Expression is reported in human neurons (12), cultured rodent neurons, glial cells (13, 14), microglial cells (14), and endothelial cells (15, 16). Recently, CXCR4 expression was observed to be up-regulated in glioblastoma, the inhibition of which impeded cell proliferation *in vitro* (17, 18). CXCR4 also plays a role in AIDS. CXCR4 is the cofactor necessary for the $CD4^+$-mediated infection of T cells by the HIV virus (19). This entry can be inhibited specifically by SDF1 (20, 21). SDF1/CXCR4 interactions are also implicated in the modulation of the immune response by inducing macrophage-mediated apoptosis of $CD8^+$ T cells (22, 23). As observed in SDF1-deficient mice, those mice lacking CXCR4 also exhibit hematopoietic, cardiac, and cerebellar defects (7, 24).

Thus, this chemokine-receptor interaction has been implicated in the modulation of cell migration in morphogenesis and development *in vivo*, in cell proliferation *in vitro*, in HIV infection, and in modulation of the immune response. Although the signaling pathways that affect these events are not well understood, SDF1 appears to activate distinct signaling pathways that may mediate each of these responses (25–27).

In this study, we identified SDF1 as a gene that is differentially expressed among GBMs with varying histomorphological features. We further characterized both SDF1 and CXCR4 expression immunohistochemically in normal brain and astrocytomas of all grades. Our data indicate that SDF1 and CXCR4 expressions increase with tumor grade. Their colocalization to predictable areas within the tumor suggests that their interactions may contribute to angiogenesis and/or modulation of the immune response.

MATERIALS AND METHODS

Primary Cell Cultures. Primary GBM cell cultures were derived from freshly resected tumor tissues. Tumors were minced, and the tissue was dissociated in HBSS with 0.2 mg/ml collagenase and 0.5 mg/ml pronase at 37°C for 30 min. Cells were grown and passaged in DMEM plus 10% FCS. Cells from each passage were frozen and stored in liquid nitrogen. Passage 3 cells were removed from liquid nitrogen and harvested at passages 6–7 for differential display and RT-PCR analysis.

Primary Cell Culture Selection. Six primary GBM cell cultures were selected by assessment of the histopathology of the corresponding tumors according to the WHO classification system (28). Although all tumors met the requirements necessary for the diagnosis of GBM, the tumors selected ranged in presentation from morphologically more homogeneous to morphologically more heterogeneous, with extensive angiogenesis and necrosis. We used primary cultures instead of the bulk frozen tumor tissue to eliminate contamination from normal tissue. The primary cell cultures derived from the selected tumors were used for the differential display analysis.

Differential Display and Gene Identification. Total RNA was extracted from the six GBM primary cell cultures by the guanidinium thiocyanate extraction procedure for Northern blot analysis as described previously (29) and for differential display. Total RNA (6 μg) was treated with 1 unit of DNase I in the presence of 1–3 units of RNase inhibitor at 37°C for 30 min in DNase buffer [0.1 M sodium acetate (pH 5.0) and 5 mM $MgSO_4$]. Purified total RNA (approximately 0.2 μg) was reverse-transcribed by Superscript II (Life Technologies, Inc.) using T12 MN primers (Genhunter). First-strand cDNA was treated for 15 min with RNase H before PCR amplification with arbitrary 10-mers in the presence of α-dATP. PCR parameters were 40 cycles of 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s. PCR amplification of each sample was performed in triplicate. PCR products were fractionated on a 7.6% denaturing polyacrylamide gel. Gels were exposed to X-ray film overnight. Candidate fragments were isolated from the gel and PCR-amplified as described above. Amplified fragments were labeled with ^{32}P using a random priming kit (Life Technologies, Inc.) and hybridized to Northern blots of total RNA from the primary cell cultures. Differential display fragments that exhibited differential expression between the more aggressive *versus* less aggressive primary cell cultures were cloned into pGEM-T vector (Promega). Cloned fragments were reprobed to ensure that the pattern of expression and the size of the transcript were the same as those observed with the initial probing. Clones were then sequenced using Sequenase version 2.0 kit (United States Biochemical) according to the manufacturer's instructions. Sequences were then submitted to GenBank for gene identification.

Quantitative PCR. RT-PCR was performed on the same RNA samples used to perform the differential display. First-strand cDNA synthesis was generated using Superscript II (Life Technologies, Inc.) and T12 MN primers (Genhunter). First-strand cDNA was treated with RNase H for 15 min before PCR. SDF1-specific primers were synthesized (Genosys) as follows: (*a*) 5′ primer, ATGAACGCCAAGGTCGTGGTC; and (*b*) 3′ primer, GGTCTGTTGTGCTTACTTGTTT. The 305-bp frag-

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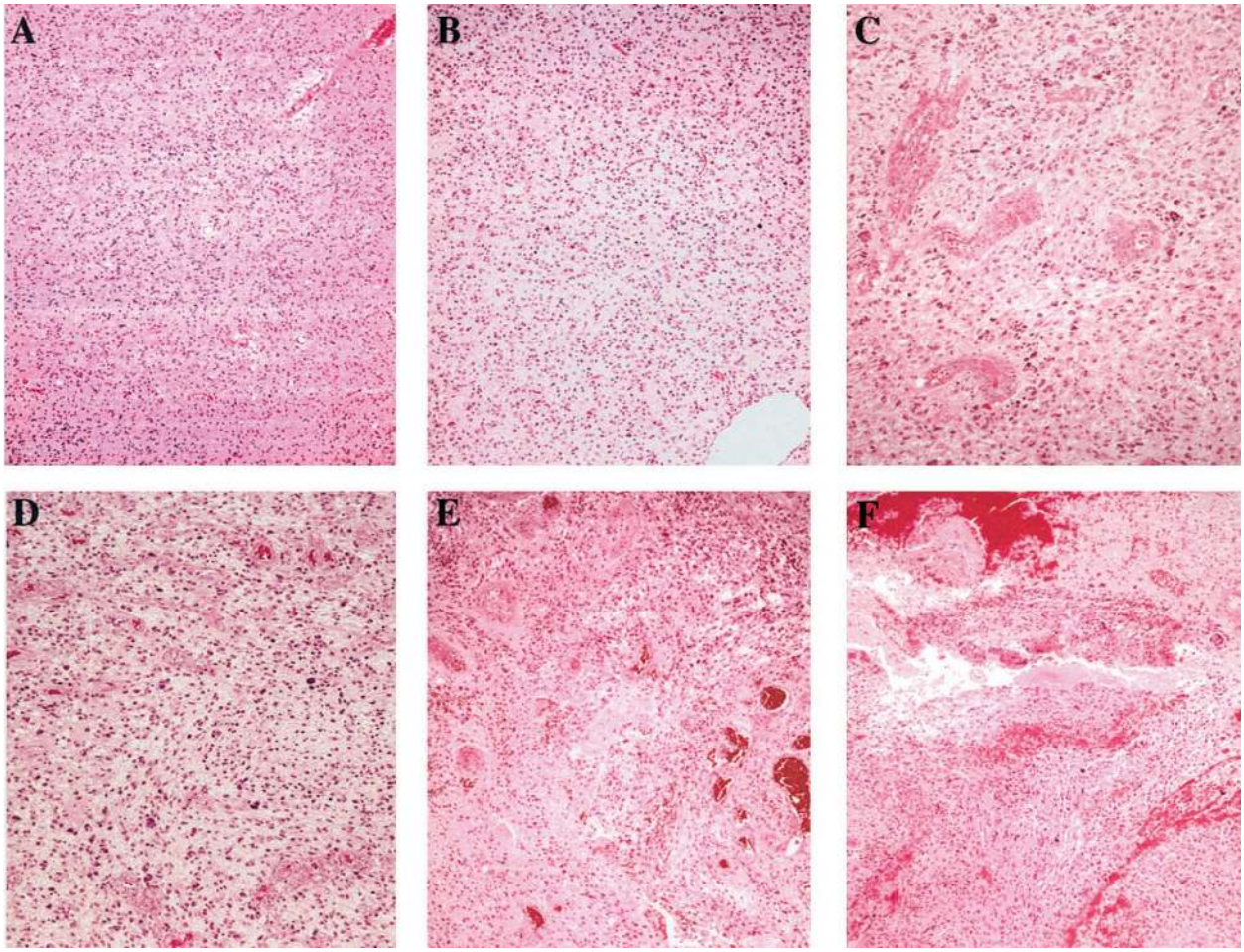

Fig. 1 GBM histomorphology. Representative H&E-stained, paraffin-embedded tumor sections (magnification, ×20) of the six GBM tumors used to derive the primary cell cultures used for differential mRNA display. The tumors were ranked according to their histomorphological features from the least heterogeneous (*A*) to the most heterogeneous (*F*).

ment generated by these primers was cloned into pGEM-T (Promega) and sequenced. Primers specific for GAPDH (Clontech) were used as an internal control. PCR conditions were as follows: denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. Primers for GAPDH were added to each of the SDF1 PCR samples after cycle 7 of the reaction. The DNA was visualized on an agarose gel with ethidium bromide staining. Images of four replicate gels were obtained. The images were digitally captured using an image analysis system (UVP, Inc., San Gabriel, CA) and densitometrically quantitated using the NIH image analysis software program as described previously (29).

Immunohistochemistry. Formalin-fixed, paraffin-embedded 5-μm tissue sections of normal brain specimens (tumor-adjacent brain tissues taken at the time of surgery) and tumors were used for immunohistochemical analyses, as reported previously (29, 30), with minor changes. Sections were subjected to routine deparaffinization and rehydration, and the subsequent steps were performed at room temperature unless otherwise specified. Sections were incubated for 10 min in 3% hydrogen peroxide in distilled water and washed in PBS three times for 5 min. Slides were then immersed in 10 mM sodium citrate buffer (pH 6.0), boiled for 10 min on a hot plate, and then allowed to cool for 20 min. Sections were rinsed in PBS solution and incubated with 10% normal horse serum in PBS for 30 min. The sections were then incubated overnight at 4°C with a 1:1000 dilution (0.2 μg/ml) of primary anti-SDF1 antibody or a 1:400 dilution (0.5 μg/ml) of primary anti-CXCR4 antibody (Santa Cruz Biotechnology) in PBS. After three washes in PBS buffer, the sections were incubated for 30 min with biotinylated secondary antibody (1:200 dilution in PBS), and washed and incubated for 45 min with the avidin-biotin complex according to manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Finally, the sections were washed and reacted with diaminobenzidine in 0.1 M Tris buffer (pH 7.6) with 0.03% hydrogen peroxide, rinsed in tap water, counterstained, and mounted. Controls were performed by omitting the primary SDF1 and CXCR4 antibodies or by substituting the primary antibodies with goat IgG isotype. Slides were blindly reviewed and scored by a neuropathologist. Staining intensity was graded as negative (−), weak (0.5+ and +), moderate (++ and +++), or strong (++++, +++++, and ++++++).

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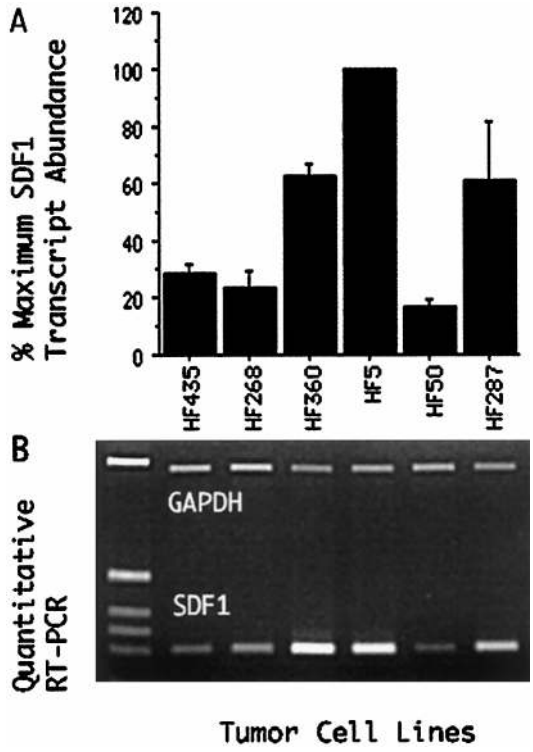

Fig. 2 Quantitation of SDF1 in the GBM primary cell cultures. The same samples of total RNA used for the differential display of the six GBM primary cell cultures were quantitated for SDF1 transcript abundance. SDF1-α was PCR-amplified using primers specific for the open reading frame of the gene. *A*, densitometric quantitation of the SDF1-α in the GBM primary cell cultures. Levels of mRNA were calculated as a ratio of relative expression of the gene to the internal control GAPDH and as a percentage of the maximum signal within a blot. *Error bars* represent the SD of four replicates. *B*, ethidium bromide-stained agarose gel of the RT-PCR coamplified *SDF-1* and *GAPDH* products. The *bottom signals* are the amplified SDF1-α PCR products. The *top signals* are the amplified GAPDH internal control PCR products.

MIB-1 (Immunotech, Marseilles, France) immunohistochemistry was performed with a 1:600 dilution as described above. Western blot analysis of tumor cell lysates using both anti-SDF1 and anti-CXCR4 antibodies was performed as reported previously (29) and demonstrated specificity for the respective proteins (data not shown).

RESULTS

Tumor Cell Culture Selection Based on Tumor Histomorphological Assessment. All tumors were originally graded as GBMs by the WHO classification system (28). Six tumors were selected because they represented a range of morphological differences in the extent of angiogenesis and necrosis (Fig. 1). They were ordered from the histomorphologically most homogeneous to the most heterogeneous, based on the extent of angiogenesis and necrosis, as follows: (*a*) HF360; (*b*) HF268; (*c*) HF435; (*d*) HF50; (*e*) HF5; and (*f*) HF287. The corresponding primary cell cultures were used in the differential display analysis to identify genes differentially expressed between these tumors.

Differential Display and SDF1 RT-PCR Quantitation. Total RNA was isolated from the six GBM primary cell cultures for the differential mRNA display analysis. From this analysis, 21 DNA fragments were identified that exhibited differential expression between the GBMs. After confirmation of differential expression by Northern blot analysis (data not shown), one differentially displayed fragment (LC5-8) was cloned and sequenced. The sequence was submitted to GenBank and found to be 100% homologous to the 3′ end of the previously known cytokine SDF1. Through the literature, the receptor was found to be CXCR4.

SDF1 was then quantitated in the GBM primary cell cultures by RT-PCR using SDF1 sequence-specific primers and GAPDH internal control primers. The primary cell cultures were ranked (Fig. 2) according to their levels of SDF1, from the lowest to the highest, as follows: (*a*) HF50; (*b*) HF268 = HF435; (*c*) HF287 = HF360; and (*d*) HF5, with an approximate 5-fold difference between the lowest and highest levels (Fig. 2). These data indicated that the differential display had indeed identified a gene that was differentially expressed between the primary cell cultures. However, because the primary cell cultures represented only a subpopulation of the tumor cells that grow out in culture, we next examined the *in vivo* immunohistochemical localization of SDF1 and its receptor, CXCR4, in the tumor sections.

Immunohistochemical Expression Profiles of SDF1 and CXCR4 in the Six GBMs. We performed immunohistochemistry for SDF1 and CXCR4 on paraffin-embedded GBM sections to determine which cells expressed these proteins. The lowest levels of SDF1 (1–2+) and CXCR4 (0.5+) were observed in tumors HF360 and HF268 (Fig. 3; Table 1). These cellular tumors were the most histomorphologically homogeneous of the six GBMs, displaying frequent mitoses and vascular proliferation, but no necrosis. A striking increase in both SDF1 (3+ and 5+) and CXCR4 (2+) was observed in tumors HF435 and HF50 (Fig. 3; Table 1). These tumors were histomorphologically intermediate GBMs containing both regions of angiogenesis and regions of necrosis. Further increases in SDF1 were not observed in HF5 and HF287; however, CXCR4 (4+ and 5+) was more highly expressed. These tumors were the most histomorphologically heterogeneous, with large regions of both angiogenesis and necrosis. SDF1 and CXCR4 expression patterns were colocalized regionally within the tumors. These proteins were expressed in areas of angiogenesis (Fig. 3, *HF287*), in tumor tissue located between regions of angiogenesis where morphology suggested that the tumors were displaying signs of degeneration (Fig. 3, *HF435* and *HF5*), and in cells adjacent to regions of necrosis (Fig. 3, *HF50*). Both SDF1 and CXCR4 were observed within neutrophils and/or monocytes (data not shown). Additionally, CXCR4 was often observed in endothelial cells, whereas SDF1 was only occasionally seen in endothelial cells (Table 1). These tumors were then ranked according to their overall SDF1 and CXCR4 levels from lowest to highest as follows: (*a*) HF360; (*b*) HF268; (*c*) HF435; (*d*) HF50; (*e*) HF5; and (*f*) HF287 (Fig. 3).

Immunohistochemical Expression Profiles in Normal Brain and Astrocytomas. We performed immunohistochemistry to determine the expression levels and localization of SDF1 and CXCR4 in normal brain and astrocytomas (grades II-IV). In

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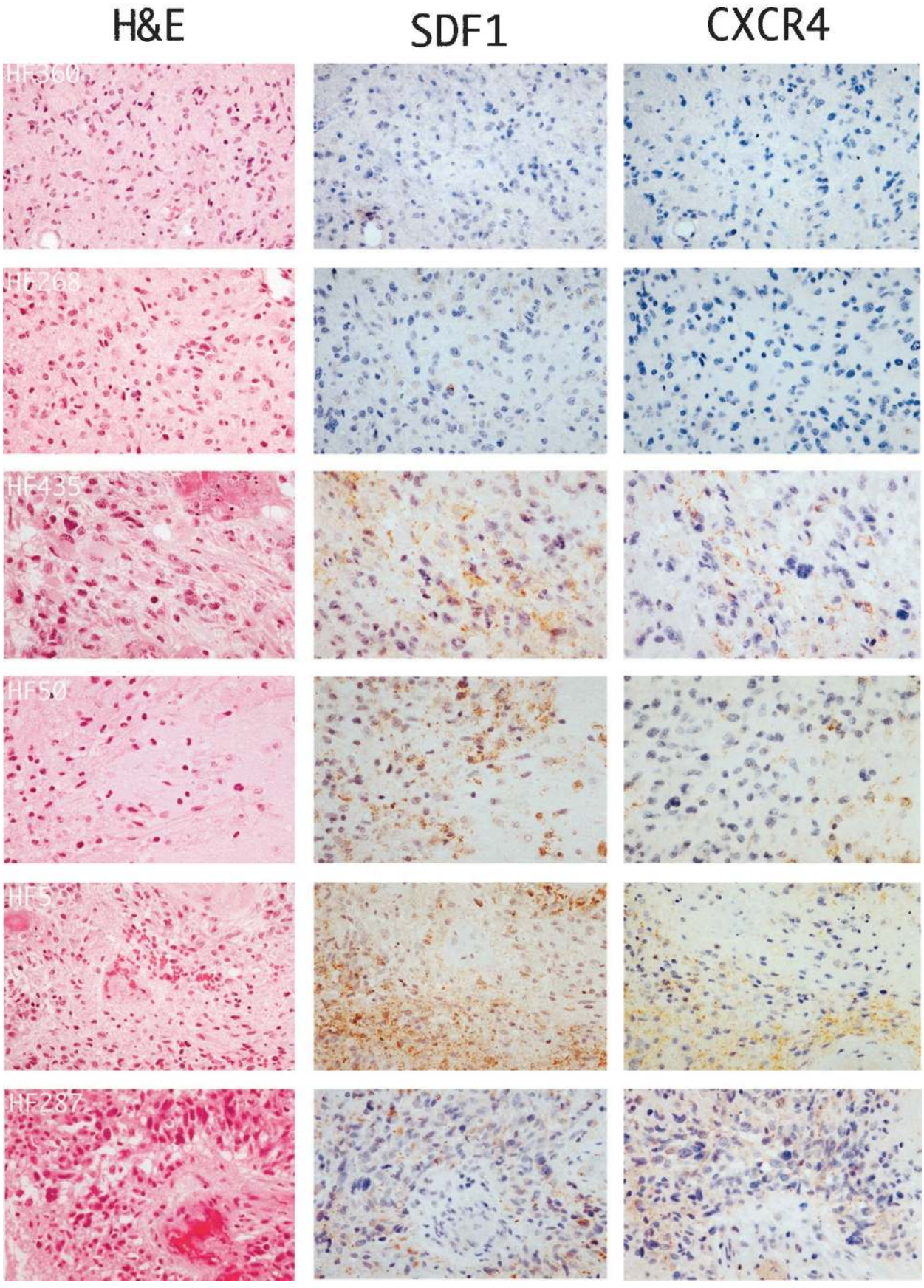

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Table 1 SDF1 and CXCR4 immunohistochemical quantitation and localization in normal brain, astrocytoma, anaplastic astrocytomas and GBMs

The bold tumor numbers are the original six GBMs used for differential display. In normal brain tissue, SDF1 overall staining was low (0.5+). Positive reactivity was noted in astrocytic processes, in neurons, and in phagocytic cells in vessels. The staining appeared lysosomal, and the staining appeared polar in neurons. Positive reactivity was observed in reactive astrocytes of one specimen, HF191. CXCR4 overall staining was negative. Occasionally, a finely granular and diffuse signal was observed. Positive reactivity was observed in phagocytic cells within the vessels. In tumors, SDF1 overall staining ranged from 0.5+ to 6+, increasing with grade. In general, when positive reactivity was observed in a tumor, it was associated in regions between angiogenic vessels, in cells undergoing early degeneration, and/or regions of necrosis. Occasional reactivity was observed in endothelial cells. When present, phagocytic cells, which were identified phenotypically, were also positive. CXCR4 overall staining ranged from 0.5+ to 6+, increasing with grade. In general, when positive reactivity was observed, it was colocalized to the same regions expressing SDF1, including regions of angiogenesis, degeneration (tissue with swelling, hyperchromasia, and pyknosis), microcystic changes (well-preserved tumor tissue with fibrillary, vacuolar background), necrosis, and phagocytic cells. In addition, reactivity was often observed in endothelial cells.

Tissues		SDF1						CXCR4						Angiogenic/ neovessels	Necrosis
A. Adjacent normal brain		OS[a]	AP	N	RA	P	EC	OS	AP	N	RA	P	EC		
HF442		0.5+	+	+	−	+	−	−	−	−	−	+	−	No	No
HF457		0.5+	+	+	−	+	−	−	−	−	−	+	−	No	No
HF252		0.5+	+	+	−	+	−	−	−	−	−	+	−	No	No
HF191		0.5+	+	+	+++	+	−	−	−	−	−	+	−	No	No
HF491		0.5+	+	+		+	−	−	−	−	−	+	−	No	No
B. Tumor		OS	A	D	Ne	P	EC	OS	A	D	Ne	P	EC	Angiogenic/ neovessels	Necrosis
HF250	A	0.5+	−	−	−	+	−	0.5+	−	−	−	+	−	No	No
HF108	AA	0.5+RA	−	−	−		−	0.5+	−	−	−		+	No	No
HF152	AA	0.5+	−	−	−	−	−	0.5+	−	−	−		−	No	No
HF34	AA	+	−	−	−	+	−	+	−	−	−		−	Yes (V)	No
HF189	AA	+	−	−	−		+	+	−	−	−		+	No	No (microcystic)
HF26	AA	+++	−	+	−	+	−	++	−	+	−	+	+	No	No (microcystic)
HF166	AA	+++	−	+	−		−	++++	−	+	−	+	+	No	No (degenerative)
HF73	GBM	0.5+	−	−	+	+	−	0.5+	−	−	+	+	+	No	Small fragment
HF360	GBM	+	+	−	−	+	−	0.5+	+	−	−	+	−	No	No
HF268	GBM	++	+	−	−	+	−	0.5+	+	−	−		+	No	No
HF435	GBM	+++	−	+	+	+	−	++	−	+	+	+	−	Yes (A)	Yes
HF138	GBM	++++	+	+	−	+	−	+++	+	+	−	+	−	Yes (V)	No
HF130	GBM	++++		+	−		−	+++++	+	+	−	+	+	Yes (V)	No (degenerative)
HF50	GBM	+++++	+	+	+	+	−	++	+	+	+	+	−	Yes (A)	Yes
HF287	GBM	+++++	+		+	+	−	++++	+	+	+	+	+	Yes (A)	Yes*
HF5	GBM	+++++	+	+	+	+	−	+++++	+	+	+	+	−	Yes (A)	Yes*
HF140	GBM	++++++	+	+	+	+	−	++++	+	+	+	+	+	Yes* (A)	Yes*
HF142	GBM	+++	+	+	+	+	+	++++++	+	+	+	+	++	Yes* (A)	Yes*
HF330	GBM	++++	−	+	+	+	−	+++++	−	+	+	+	−	No	Yes
HF350	GBM	+++++	+	+	+	+	−	++++++	+	+	+	+	+	Yes (A)	Yes*

[a] OS, overall staining; AP, astrocytic processes; N, neurons; RA, reactive astrocytes; P, phagocytic cells, EC, endothelial cells; D, degeneration; Ne, necrosis; A, pleomorphic angiogenic vessels; V, nonpleomorphic vessels; +, positive reactivity; −, negative reactivity; *, tumors with the most angiogenesis or necrosis observed in the data set.

normal brain tissue, low levels of SDF1 (0.5+) were observed in astrocytic processes and in neurons (Fig. 4; Table 1). SDF1 was also observed in the occasional phagocytic cells around vessels. Reactive astrocytes in the molecular layer of one sample were positive (Table 1, HF191). CXCR4 expression was negative in normal brain tissue (Fig. 4; Table 1) but was also present in phagocytic cells within the vascular lumen (Table 1). Thus, the level of expression for both SDF1 and CXCR4 was low in normal brain tissue compared to the GBMs and was in agreement with the reported literature. To examine the expression patterns in astrocytomas, we examined the expression of SDF1 and CXCR4 in one astrocytoma, five anaplastic astrocytomas, and six additional GBMs (Table 1). Overall, the lower grade tumors expressed lower levels of both SDF1 and CXCR4 than did the GBMs. However, three anaplastic tumors had intermediate levels of expression of SDF1 (2–

Fig. 3 Immunohistochemical quantitation of SDF1 and CXCR4 proteins in GBMs. SDF1 and CXCR4 were immunohistochemically localized in the six GBMs. The H&E staining, and SDF1 and CXCR4 localizations were performed as described in "Materials and Methods" and are illustrated for each tumor. SDF1 and CXCR4 were regionally colocalized when both were expressed. Low SDF1 immunoreactivity was noted in HF360 and HF268. Increasing reactivity was noted for tumors HF435, HF50, HF5, and HF287. CXCR4 immunoreactivity was virtually negative in HF360 and HF268. Increasing reactivity was also observed for tumors HF435, HF50, HF5, and HF287. A positive correlation was noted between increased SDF1 and CXCR4 expression and increased tumor heterogeneity. Magnifications, ×40.

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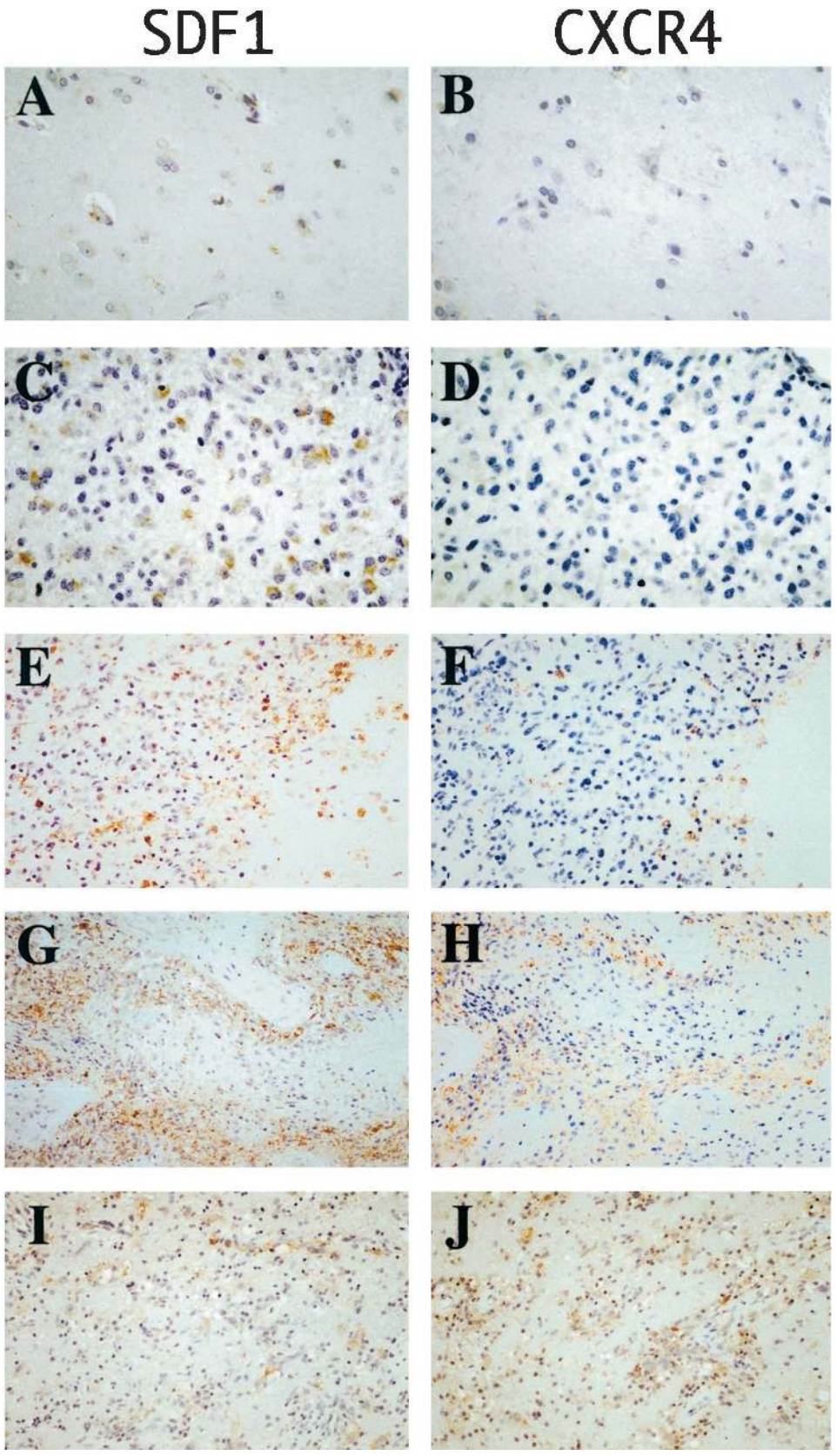

Fig. 4 Immunohistochemical localization of SDF1 and CXCR4 in normal brain tissue and tumors. SDF1 and CXCR4 were immunohistochemically localized in normal brain tissue, low-grade tumors, and additional GBMs (Table 1). In normal brain tissue (*A* and *B*), SDF1 was expressed at low levels in astrocytic processes and neurons. CXCR4 was not expressed in normal brain tissue. A finely granular reactivity was occasionally noted. Phagocytic cells in all tissues were also positive when present. In the tumors, SDF1 reactivity ranged from low (0.5+ to +), to intermediate (2+ to 3+; HF268; C), and high (4+ to 6+; HF140; *E, G,* and *I*). CXCR4 reactivity ranged from low (0.5+; HF268; *D*), to intermediate (2+ to 3+) and high (4+ to 6+; HF140; *F, H,* and *J*). Reactivity for both SDF1 and CXCR4 was colocalized to regions of necrosis (*E* and *F*), regions of degeneration in highly angiogenic areas (*G* and *H*), and angiogenic vessels (*I* and J). Magnifications, ×40 (*A–D*) and ×20 (*E–J*).

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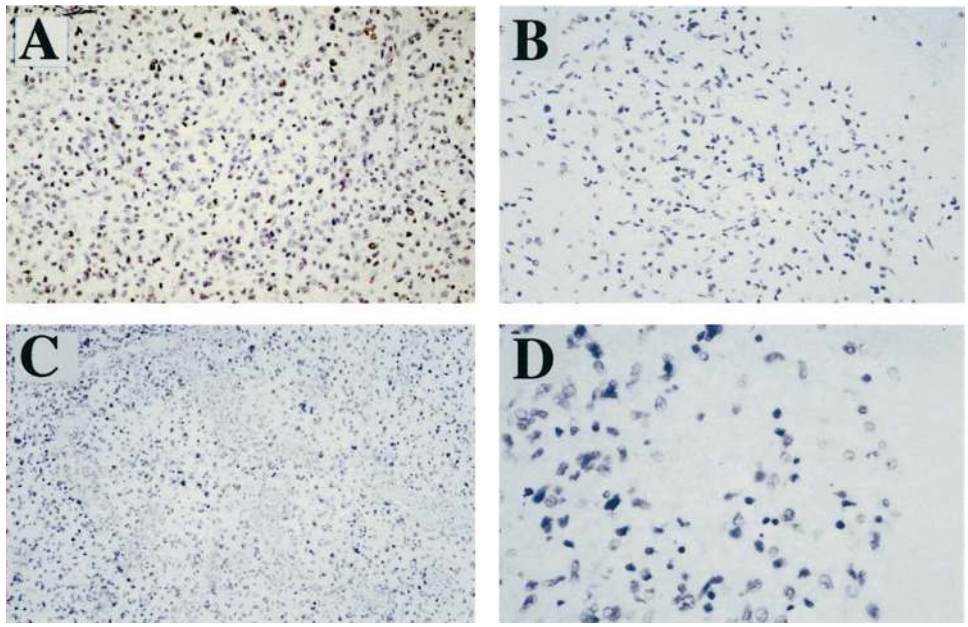

Fig. 5 Immunohistochemical localization of MIB-1 in tumors. MIB-1 reactivity was noted in the regions of low SDF1/CXCR4 expression (HF268; *A*). MIB-1 positive cells were abundant in highly cellular, proliferated regions (*A*). However, MIB-1 was not expressed in the regions of necrosis (*B* and *D*) or angiogenesis (*C*) associated with high SDF1/CXCR4 expression. Magnifications, ×20 (*A–C*) and ×40 (*D*).

3+) and CXCR4 (2–4+). These tumors were described as having microcystic or degenerative regions (Table 1). Thus, SDF1 and CXCR4 expression was colocalized, increased with increasing tumor grade, and was associated with regions of necrosis/microcystic change and angiogenesis. Those tumors displaying greater amounts of these features expressed greater intensity of the proteins. Not all GBMs with elevated SDF1 and CXCR4 had both angiogenesis and necrosis (Table 1), suggesting that the proteins may be associated with both phenotypes. Overall, the expression of both proteins was low in normal brain tissue (Fig. 4, *A* and *B*), slightly elevated in low-grade tumors or in GBMs without extensive regions of angiogenesis and necrosis (Fig. 4, *C* and *D*), elevated in GBMs in regions of necrosis (Fig. 4, E and F) and in regions of degeneration (Fig. 4, *G* and *H*), and occasionally increased in neovessels (Fig. 4, *I* and *J*).

Expression Relative to MIB-1 Expression. Because CXCR4 has been implicated in the regulation of proliferation in GBM *in vitro* (17), we performed MIB-1 immunohistochemistry on adjacent sections to determine whether SDF1 and CXCR4 colocalized regionally to those areas high in proliferation. We observed an inverse relationship. Regions high in MIB-1 (Fig. 5*A*, HF268) expression were low in SDF1 and CXCR4 (Fig. 4, *C* and *D*, HF268). In contrast, regions of obvious necrosis (Fig. 5, *B* and *D*, HF140) and degeneration (Fig. 5*C*, HF140) were low in MIB-1 but expressed SDF1 and CXCR4 (Fig. 4, *G* and *H*).

Thus, the differential display analysis between GBMs of varying histomorphological features successfully identified differentially expressed genes associated with histomorphologically defined regions.

DISCUSSION

GBM refers to a morphologically heterogeneous group of malignant tumors derived from astroglial and possibly oligodendroglial and ependymal cell lineages. Although patients generally survive only 1–2 years, there is a range in response and in length of survival within this group of tumor patients. Molecular genetic studies have demonstrated that GBMs are genetically heterogeneous and that genetic markers can be used to subtype the progression pathways leading to GBM (1, 31). However, molecular defects have not previously been associated with a specific histomorphological malignant phenotype. In an attempt to identify genes that specifically correlate with one of these phenotypes, we performed differential mRNA display analyses on primary cell cultures derived from six GBMs that demonstrated a wide range of histomorphological features with regard to the extent of necrosis and angiogenesis.

We identified *SDF1* as a gene that is differentially expressed between the GBMs. SDF1 is a chemokine that binds to its seven-transmembrane G-coupled receptor, CXCR4, to modulate several biological functions through signal transduction pathways, including cell proliferation, cell migration, and transcriptional activation (25–27). Therefore, we further immunohistochemically characterized both SDF1 and CXCR4 protein expression and localization in formalin-fixed, paraffin-embedded tissue sections of normal brain tissue and astrocytic tumors of all grades. SDF1 expression is low in normal brain tissue (2). Both SDF1 expression and CXCR4 expression have been observed in the brain of developing embryos, particularly in neurons in the fetal cerebellum (7). *In vitro*, both SDF1 and CXCR4 have been detected in astrocytes, neurons, and microglial cells (13). Immunohistochemically, we observed low levels of SDF1 [in agreement with overall levels in the brain (2)] and negligible levels of CXCR4, probably because these specimens were not taken from the cerebellum. The lower grade tumors (astrocytoma and anaplastic astrocytoma) demonstrated intermediate levels of SDF1 and CXCR4 expression. The highest levels of expression of both proteins were observed in the GBMs, and the expression was associated with histomorphologically defined

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regions within the GBMs. Specifically, we observed high levels of SDF1 expression in regions adjacent to tumor necrosis, in regions in-between angiogenic vessels where the tumor appeared to be undergoing degeneration, and occasionally in endothelial cells of neovessels. CXCR4 was colocalized to the same histomorphological regions as SDF1 and was often observed in the endothelial cells of neovessels. Immunohistochemical localization of MIB-1 (a proliferation marker) on adjacent sections indicated that regions high in SDF1 and CXCR4 expression were not associated with MIB-1 staining. This is not surprising, because necrotic areas are poor in MIB-1. However, the converse was also observed: regions high in MIB-1 staining were low in SDF-1 and CXCR4. These observations suggest that SDF1 and CXCR4 interactions may play a role in tumor angiogenesis and/or immune surveillance but probably do not play a role in proliferation.

We observed SDF1 expression in neovessel endothelial cells in an occasional tumor. However, expression was high in tumor cells adjacent to the neovessels. In contrast, we observed CXCR4 expression in the endothelial cells of neovessels in many tumors. These data suggest that the cytokine released from tumor cells may play a role in promoting angiogenesis by inducing the migration of endothelial cells. This hypothesis is supported by reports that CXCR4 expression has been observed in other types of endothelial cells including human umbilical vascular endothelial cells, bovine aortic endothelial cells, and bovine pulmonary artery endothelial cells (15, 16) and that SDF1 was found to be a potent inducer of endothelial cell chemotaxis (16). During normal development, CXCR4 is highly expressed in the endothelium of developing blood vessels, including the brain (32). Furthermore, mice lacking either SDF1 or CXCR4 have defective vascular development, particularly in the gastrointestinal tract, suggesting that SDF1 and CXCR4 represent an important signaling system for organ vascularization (32).

The greatest reactivity of SDF1 and CXCR4 was noted both in the regions of tumor situated between areas of angiogenesis and in the tumor immediately adjacent to areas of necrosis. Histomorphologically, these tumor regions between the neovessels appeared to be undergoing degeneration and probably progressing to necrosis, where the tumor was presumably outgrowing its blood supply. Expression of SDF1 in such regions could serve two purposes. One would be to promote new angiogenesis to supply nutrients to further tumor growth, as indicated previously. However, the observation that the highest expression was observed in these seemingly degenerative regions, in areas of necrosis, and around neovessels suggests another function. In all of these cases, expression is elevated in regions where the integrity of the tumor is compromised by destroyed tissue or leaky vessels, where the tumor is vulnerable to immune attack. Recently, SDF1 has been shown to modulate the immune response by its interactions with CXCR4 present on both macrophages and $CD8^+$ T cells (22, 23). SDF1 attracts macrophages, binds to CXCR4, and induces macrophages to secrete surface TNF. SDF1 also attracts $CD8^+$ T cells, binds to the CXCR4 receptor, and induces T cells to express the TNF-R. Macrophages and T cells bind to each other through the TNF/TNF-R interaction, inducing a death signal in the T cells. Thus, it is postulated that SDF1 and CXCR4 interactions act to suppress the immune response.

Surprisingly, GBMs fail to mount a successful immune response, although the tumors are seemingly exposed to the immune cells in regions of angiogenesis, where blood-brain barrier is absent, or in areas of tumor necrosis or degeneration. Our findings suggest that in the regions where an immune access would normally be expected, high SDF1 expression may protect the tumor by inducing macrophages and probably microglial cells to attack T cells via the TNF/FNF-R interaction. In support of this hypothesis, SDF1 has been recognized as a strong chemoattractant for microglial cells (14), and TNF expression increases with tumor grade [17% of astrocytomas and oligoastrocytomas and 80% of anaplastic and GBM tumors express the factor (33)].

CXCR4 has also been implicated in the regulation of glioma proliferation when transfection of an antisense to CXCR4 inhibited cell proliferation of glioma cells *in vitro* (17). However, in our immunohistochemical analysis of MIB-1 labeling of cycling cells, SDF1/CXCR4 and MIB-1 were generally mutually exclusive, as determined by immunohistochemical staining of adjacent sections. This discrepancy may reflect methodological differences because cells in culture are not subject to the same environment that they are within the tumor, and the assessment of a candidate gene *in vitro* may not always reflect its major function *in vivo.* For example, we found a fairly good correlation between increased SDF1 expression as assessed by RT-PCR and immunohistochemistry for four of the six tumors. However, levels observed for HF50 and HF360 did not agree with the immunohistochemistry, suggesting that the cell culture results did not always reflect the *in vivo* expression patterns.

In conclusion, differential display combined with immunohistochemical analysis was successful in the identification of genes differentially expressed between heterogeneous GBMs. The localization of these genes to distinct histomorphological regions within the tumor suggests clues as to their possible functions, *i.e.,* promoting angiogenesis and/or inhibiting T-cell-mediated immune response.

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