Activation of Stat3 in Human Melanoma Promotes Brain Metastasis

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Abstract
Brain metastasis is a major cause of morbidity and mortality in patients with melanoma. The molecular changes that lead to brain metastasis remain poorly understood. In this study, we developed a model to study human melanoma brain metastasis and found that Stat3 activity was increased in human brain metastatic melanoma cells when compared with that in cutaneous melanoma cells. The expression of activated Stat3 is also increased in human brain metastasis specimens when compared with that in the primary melanoma specimens. Increased Stat3 activity by transfection with a constitutively activated Stat3 enhanced brain metastasis, whereas blockade of Stat3 activity by transfection with a dominant-negative Stat3 suppressed brain metastasis of human melanoma cells in animal models. Furthermore, altered Stat3 activity profoundly affected melanoma angiogenesis in vivo and melanoma cell invasion in vitro and significantly affected the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) in vivo and in vitro. Finally, Stat3 activity transcriptionally regulated the promoter activity of bFGF in addition to VEGF and MMP-2 in human melanoma cells. These results indicated that Stat3 activation plays an important role in the dysregulation of tumor invasion and metastasis of melanoma cells and contributes to brain metastasis of melanoma. Therefore, Stat3 activation might be a new potential target for therapy of human melanoma brain metastases. (Cancer Res 2006; 66(6): 3188-96)

Introduction
The major cause of death from melanoma is metastases that are resistant to conventional therapies. By the time the diagnosis of malignant melanoma is made, metastasis to regional and distant lymph nodes, liver, lung, and brain may well have occurred (1, 2). Brain metastasis occurs in ~60% to 80% of patients with metastatic melanoma and is a major cause of death in patients with melanoma (3). The distribution of metastases in the brain reflects a hematogenous route of spread from either the primary tumor or the metastases in other organs. Many patients developing brain metastases have established disease in the lungs and other sites (1, 2). When metastasis is present in the brain, the prognosis is consistently poor, with median survival duration of 4 to 6 months (3, 4). Despite our growing understanding of primary tumor progression, the biological and molecular basis of brain metastases is seriously understudied. For example, numerous genes have been shown to be involved in the pathogenesis of melanoma, but only very few have been implicated in brain metastasis of melanoma. These genes include Nm23 (5), a metastasis suppressor gene, and neurotrophin receptor and heparanase (6). A better understanding of the mechanisms of brain metastasis is important to improve current therapies and design new treatment modalities.

In general, the molecular mechanisms that control human melanoma progression and metastasis are related to alterations of various oncogenes, tumor suppressor genes, metastasis suppressor genes, and growth factors and their receptors, including Src, Ras, p16, KISS-1, Nm23, Fasl, epidermal growth factor receptor, basic fibroblast growth factor (bFGF), and interleukin (IL)-6 (7–10). These abnormalities affect the downstream signal transduction pathways involved in the control of cell growth and other malignant properties. Interestingly, one of the most recently recognized signal transduction pathways involves the Stat3 protein. In fact, Stat3 is constitutively activated in various types of human tumors, including melanoma (10–12). It has been shown that Stat3 activation plays roles in tumor growth and angiogenesis of melanoma in tumor xenograft studies (13, 14). Recent studies have also revealed that altered Stat3 activation can contribute to oncogenesis. For example, activation of Stat3 is required for cell transformation by oncogenic Src (15) and by a constitutively active form of Gα16, a heterotrimeric G-protein subunit (16). In addition, overexpression of the constitutively activated Stat3 mutant (Stat3C) into immortalized 3T3 cells and immortalized human mammary epithelial cells induces cellular transformation and tumor formation in nude mice (17, 18). The role of activated Stat3 in oncogenesis is manifested presumably through its critical role in the expression of many genes key to the regulation of multiple aspects of tumor cell survival, growth, and angiogenesis and evasion of immune surveillance (19, 20), such as cyclin D1, c-myc, vascular endothelial growth factor (VEGF), and IP-10 (17, 19–25).

Moreover, our recent study showed that activated Stat3 regulates tumor invasion of murine melanoma cells by regulating matrix metalloproteinase-2 (MMP-2) transcription (26). MMP-2, a 72-kDa type IV collagenase, is one of the key enzymes in the invasion and metastasis cascade of malignant melanoma (27). MMP-2 also contributes to tumor angiogenesis (28). A study by Dechow et al. (18) further showed that MMP-9, a 92-kDa type IV collagenase, was up-regulated in Stat3C-transfected human mammary epithelial cells.

However, primary melanomas are biologically heterogeneous and contain subpopulations of cells with different metastatic properties. Interestingly, one of the most recently recognized signal transduction pathways involves the Stat3 protein. In fact, Stat3 is constitutively activated in various types of human tumors, including melanoma (10–12). It has been shown that Stat3 activation plays roles in tumor growth and angiogenesis of melanoma in tumor xenograft studies (13, 14). Recent studies have also revealed that altered Stat3 activation can contribute to oncogenesis. For example, activation of Stat3 is required for cell transformation by oncogenic Src (15) and by a constitutively active form of Gα16, a heterotrimeric G-protein subunit (16). In addition, overexpression of the constitutively activated Stat3 mutant (Stat3C) into immortalized 3T3 cells and immortalized human mammary epithelial cells induces cellular transformation and tumor formation in nude mice (17, 18). The role of activated Stat3 in oncogenesis is manifested presumably through its critical role in the expression of many genes key to the regulation of multiple aspects of tumor cell survival, growth, and angiogenesis and evasion of immune surveillance (19, 20), such as cyclin D1, c-myc, vascular endothelial growth factor (VEGF), and IP-10 (17, 19–25). Moreover, our recent study showed that activated Stat3 regulates tumor invasion of murine melanoma cells by regulating matrix metalloproteinase-2 (MMP-2) transcription (26). MMP-2, a 72-kDa type IV collagenase, is one of the key enzymes in the invasion and metastasis cascade of malignant melanoma (27). MMP-2 also contributes to tumor angiogenesis (28). A study by Dechow et al. (18) further showed that MMP-9, a 92-kDa type IV collagenase, was up-regulated in Stat3C-transfected human mammary epithelial cells.

However, primary melanomas are biologically heterogeneous and contain subpopulations of cells with different metastatic
ability. Although it has been shown that primary melanomas have constitutive Stat3 activation, it is unclear whether human metastatic melanomas have increased Stat3 activation and whether Stat3 activation plays an important role in melanoma metastasis. The present study reported our established brain metastasis model that brain metastatic cells showed elevated Stat3 activity and the role and regulation of Stat3 activation in melanoma brain metastasis. Significantly, Stat3 activity is increased in human brain metastatic melanoma tissues when compared with that in primary melanoma tissues. Increased Stat3 activation by transfection with Stat3C mutant enhanced brain metastasis, whereas blockade of Stat3 activation by transfection with the dominant-negative Stat3 (Stat3DN) plasmid suppressed brain metastasis of human melanoma cells in animal models. Stat3C-transfected A375P melanoma cells also had increased ability to metastasize to lung. Thus, these cells mimic the behavior of human melanoma, as many patients with brain metastasis have metastases in the lungs (2). Moreover, we found for the first time that Stat3 activation regulates the expression of bFGF. Finally, we found evidence that elevated Stat3 activity drives overexpression of the metastatic molecules bFGF, MMP-2, and VEGF and promotes brain metastasis by increasing tumor invasion and angiogenesis.

**Materials and Methods**

**Animals.** Pathogen-free female athymic BALB/c nude mice were purchased from the Animal Production Area, National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

**Cell lines and culture conditions.** The human melanoma cell line A375P was established in culture from a lymph node metastasis of a patient with melanoma. The human melanoma cell lines TXM-13 and TXM-18, which are highly metastatic to the brain (29), were isolated from surgical specimens of brain metastases. The cutaneous melanoma cell lines SB-2 (30), SK-Mel-28 and SK-Mel-31 (American Type Culture Collection, Manassas, VA), and Mel888 and Mel501 (provided by Dr. Ruth Halaban, Yale University School of Medicine, New Haven, CT) were also used. All of the cell lines were maintained in Eagle's MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution (Flow Laboratories, Rockville, MD).

**Human tissue specimens and patient information.** We used primary human melanoma and brain metastasis specimens preserved at The University of Texas M. D. Anderson Cancer Center. Primary and metastasis melanoma in these patients was diagnosed and treated at The University of Texas M. D. Anderson Cancer Center. None of them underwent preoperative chemotherapy and/or radiation therapy. Brain metastases and primary melanoma cases were frequency matched by age ($\pm$ 5 years), sex, and ethnicity. The use of the archival tissue blocks was approved for this research project by the institutional review board.

**Stable transfection.** A375Br and TXM-18 cells were transfected with the Stat3DN plasmid (31, 32), and A375P cells were transfected with Stat3C plasmid (17) by using LipofectAMINE (Life Technologies, Inc., Rockville, MD). The three cell lines were also transfected with control vectors. A375Br and A375P cells were then selected with a standard medium containing 800 $\mu$g/mL G418, whereas TXM-18 cells were selected with 300 $\mu$g/mL G418 for 14 days. To avoid clonal selection and variation, three independent transfections of Stat3DN or Stat3C expression vectors were carried out for each cell line, and G418-resistant colonies were pooled to establish stable Stat3DN and Stat3C transfectants. Thus, in each experiment, only mass transfection cultures, not single cell clones, were used, each of which represented a pooled culture of >100 individual clones.

**Electrophoretic mobility shift assay.** Nuclear protein extracts were prepared as described previously (26, 30). Nuclear protein extracts (10 $\mu$g each) were incubated with the radiolabeled Stat3 consensus-binding probe 5'-GATCCCTTGCAGAATCCTAGATC-3' (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 minutes at room temperature. For supershift analyses, the cell extracts were preincubated with specific antibodies against Stat3 (Zymed Laboratories, Inc., San Francisco, CA). Protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel. The gels were then dried and autoradiographed.

**Promoter reporters and dual luciferase assays.** Plasmids containing firefly luciferase reporters driven by the full-length MMP-2 promoter (pMLuc; ref. 26), full-length bFGF promoter (pRL-bFGF; ref. 33), and full-length VEGF promoter (pGL2-VEGF; ref. 34) were used. Melanoma cells were transfected with the indicated reporter plasmids by using Lipofect-AMINE. Transfection efficiency was normalized by cotransfection with a β-actin RL or pGL2-β-actin reporter (30, 33). Both firefly luciferase and Renilla luciferase activities were quantified by using the dual luciferase assay system (Promega, Madison, WI).

**Gelatin zymography.** MMP-2 activity was measured by a standard assay using serum-free supernatants collected from cell cultures (24 hours) and normalized by the cell number (35). The samples were separated on a 7.5% SDS-PAGE gel containing 1 mg/mL gelatin. The gel was washed in wash buffer and then incubated for 24 hours at 37°C in the incubation buffer. Finally, the gel was stained with a 0.1% Coomassie blue R-250.

**In vivo model of brain metastasis.** In vivo model of brain metastasis was described previously (29). Tumor cells (3 $\times$ 10$^3$ in 0.1 mL HBSS) were slowly injected into the internal carotid artery of nude mice. The animals were killed when they became moribund or when clinical symptoms, such as immobility, weight loss, or a hunched position, developed. The animals without symptoms were sacrificed 90 or 120 days after tumor inoculation. The survival time of each mouse was recorded. Each mouse's brain was removed and cut into 2- to 3-mm sections. The presence of brain metastases was confirmed by histology.

**Northern blot analysis.** Cellular mRNA was then extracted by using a FastTrack mRNA isolation kit (Invitrogen, San Diego, CA); mRNA (2 μg) was separated electrophoretically on a 1% denaturing formaldehyde agarose gel, transferred to a nylon membrane, and hybrid with [32P]dCTP-labeled MMP-2, VEGF, and bFGF cDNA probes (26, 33, 34). Equal loading of mRNA samples was monitored by hybridizing with a β-actin cDNA probe.

**Western blot analysis.** Whole-cell lysates were prepared from cancer cell lines. Standard Western blotting was done with anti-MMP-2, anti-VEGF, or anti-bFGF antibodies (Santa Cruz Biotechnology). Equal protein sample loading was monitored by using an anti-β-actin antibody. The probe proteins were detected by using the Amersham (Piscataway, NJ) enhanced chemiluminescence system.

**Matrigel invasion assay.** An invasion assay using BioCoat Matrigel Invasion Chambers (Becton Dickinson, Franklin Lakes, NJ) was done as described previously (35). Conditioned medium from 1 $\times$ 10$^6$ IFI-251MG astrocytoma cells cultured for 48 hours in MEM containing 1% fetal bovine serum was placed in the lower chamber as a chemoattractant. Melanoma cells (2.5 $\times$ 10$^3$ in 300 μL of a serum-free medium were placed in the upper chamber and incubated at 37°C for 22 hours. The cells that penetrated through Matrigel-coated filter cells were counted at a magnification of ×<400 in 10 randomly selected fields, and the mean number of cells per field was recorded. In some experiment, tumor cells were pretreated with 5.0 $\mu$mol/L MMP-2 inhibitor I (OA-Hy, Calbiochem, San Diego, CA; ref. 36).

**Immunohistochemistry.** Sections (5 μm thick) of formalin-fixed, paraffin-embedded primary melanoma specimens, brain metastatic melanoma specimens, and experimental brain metastasis specimens were stained with anti-phosphorylated Stat3 (Tyrosine (Tyr)533) antibodies (Cell Signaling Technology, Danvers, MA; ref. 37). The level of expression of VEGF, bFGF, and MMP-2 in the experimental brain metastasis samples was determined by standard immunohistochemical staining with an anti-VEGF antibody (1:100 dilution; Santa Cruz Biotechnology; ref. 34), anti-bFGF antibody (1:200 dilution; Santa Cruz Biotechnology; ref. 33), and anti-MMP-2 antibody (1:500 dilution; Oncogene Research Products, Cambridge, MA; ref. 33). The tumor microvessel density (MVD) in the experimental brain
metastasis samples was determined by staining with anti-CD34 antibodies (1:100 dilution; Santa Cruz Biotechnology; ref. 37). Negative controls were tissue sections immunostained with nonspecific IgG. The sections were visualized by using a diaminobenzidine substrate kit. The slides were examined under a bright-field microscope.

**Statistical analysis.** The significance of the data from patient specimens was determined by χ² test. The significance of the in vitro data was determined by Student’s t test (two-tailed), whereas the significance of the in vivo data was determined by using the two-tailed Mann-Whitney test. Animal survival was compared by Kaplan-Meier survival analysis.

**Results**

**In vivo selection of highly brain metastatic melanoma cells.**

Progress in understanding the biology of melanoma brain metastasis has been limited largely by a lack of suitable cell lines and experimental models. Thus, in the present study, we first developed a reliable experimental model to study the pathogenesis of melanoma brain metastases using *in vivo* selection of a metastatic variant subline from an original human tumor cell line. The selection of a brain metastatic variant represents the emergence of a preexisting subpopulation of tumor cells and provides a useful model for studying brain metastasis (38). We injected A375P cells into the intracarotid artery of 10 nude mice (3 × 10⁵ per mouse). At 90 days after the tumor cell injection, none of the mice exhibited any systemic symptoms, and histologic examination failed to detect metastasis in the mice. However, we were able to recover human melanoma cells from tissue cultures of the brain of these mice, suggesting that those cells survived and were probably under dormancy. We then injected the recovered melanoma cells into the intracarotid artery of nude mice. Brain metastases were present in 2 of the 10 inoculated mice within 90 days. We harvested brain metastases, expanded them in culture, and reinoculated melanoma cells into the nude mice. We repeated this cycle twice to yield the cell line A375Br. After intracarotid artery inoculation, A375Br cells produced brain metastasis in 100% of the mice and died within 60 days (46-58 days), whereas A375P cells did not produce metastasis in any of them and all survived over 90 days (Fig. 1A). Therefore, we established a highly brain metastatic melanoma cell line (A375Br) from the original A375P cells.

**Direct correlation of Stat3 activity with the brain metastatic potential of melanoma cells.** We examined Stat3 activity in...
A375P and A375Br cells using electrophoretic mobility shift assay (EMSA). As shown in Fig. 1B1, we detected significantly higher Stat3 activity in the brain metastatic A375Br cells than that in the A375P cells. The Stat3 DNA-binding activity was specifically competed by an unlabeled Stat3 consensus probe but not by a mutant Stat3 probe. Moreover, the DNA-protein complex was supershifted by Stat3-specific antibodies (Fig. 1B1) but not by Stat1 or Stat5 antibodies (data not shown), indicating the specificity of the Stat3 complexes.

To be more clinically relevant, we then analyzed Stat3 activity in a panel of human melanoma cell lines. As shown in Fig. 1B2, we detected significantly higher Stat3 activity in the TXM-13 and TXM-18 brain metastatic cell lines than in most of the cutaneous melanoma cell lines, including SB-2, SK-Mel-28, SK-Mel-31, Mel888, and Mel501. These results indicated that Stat3 was constitutively activated at higher levels in highly brain metastatic melanoma cell lines.

**High activation of Stat3 in human brain metastasis specimens.** Next, we sought to determine whether activation of Stat3 was increased in melanoma brain metastasis specimens. We used 48 melanoma brain metastasis specimens and 51 primary melanoma specimens. We analyzed these samples using immunohistochemistry for nuclear staining with an antibody specific for phosphorylated Stat3, the activated form of Stat3. We scored tumors as strong positive for phosphorylated Stat3 if they displayed strong staining (i.e., at least 50% of the tumor cells displayed nuclear staining with the antibody). We scored tumors as negative for phosphorylated Stat3 if <5% of the tumor cells displayed nuclear staining with the antibody. We found that 15.7% (8 of 51) of the primary melanomas were strong positive, 56.8% (29 of 51) were weak to negative, and 27.5% (14 of 51) exhibited moderate positive for phosphorylated Stat3. In contrast, 52.1% (25 of 48) of the brain metastases were strong positive, 18.8% (9 of 48) were weak to negative, and 29.1% (14 of 48) exhibited moderate positive for phosphorylated Stat3. When the data of strong positive staining was analyzed using $\chi^2$ test, significantly higher levels of phosphorylated Stat3 were evident in melanoma brain metastases than that in primary melanoma specimens ($P = 0.001$; Fig. 1C).

Representative sections of strong staining (+++), moderate staining (++), and weak to no staining (0/+) were presented (Fig. 1D).

**Increased brain metastasis resulting from enforced up-regulation of constitutive Stat3 activity.** To directly test the hypothesis that Stat3C has a causal role in brain metastasis of melanoma cells, we sought to determine whether persistent Stat3 activation could increase brain metastasis. We stably transfected A375P cells with Stat3C. This expression vector is a constitutively activated mutant form of Stat3 that dimerizes spontaneously and is capable of binding DNA and activating transcription (17). We established three stable Stat3C-transfected A375P cell lines (A375P-Stat3C-a, -b, and -c) by performing three sets of transfection of A375P cells. The Stat3 DNA-binding activity was specifically competed by an unlabeled Stat3 consensus probe but not by Stat1 or Stat5 probes (Fig. 1B). Moreover, the DNA-protein complex was supershifted by Stat3-specific antibodies (Fig. 1B). These results indicated that Stat3C-transfected A375P cells were more malignant than the A375P cells, although Stat3 activity may not necessarily represent a mechanism for organ-specific metastasis.

**Suppression of brain metastasis by blockade of Stat3 activity.** To further determine the effect of altered Stat3 activity on brain metastasis, we transfected A375Br and TXM-18 cells with Stat3DN. This expression vector contains a phenylalanine substitution of the tyrosine residue position at 705, which results in a reduction in tyrosine phosphorylation of wild-type Stat3 and inhibition of endogenous Stat3 activation (31, 32). We established three Stat3DN-transfected A375Br cell lines (A375Br-Stat3DN-a, -b, and -c) and three Stat3DN-transfected TXM-18 cell lines (TXM-18-Stat3DN-a, -b, and -c) by performing three sets of transfection of A375Br and TXM-18. We established stably transfected cell lines only from the mixed population to avoid single-clone selection. EMSA analysis showed decreased Stat3-binding activity in the Stat3DN-transfected A375Br and TXM-18 cells compared with that of control cells (Supplementary Table S1). Thus, these data indicated that Stat3DN-transfected A375Br cells are more malignant than the A375P cells, although Stat3 activation may not necessarily represent a mechanism for organ-specific metastasis.

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in the parental and control vector-transfected cells (Fig. 2B and C). To evaluate the effect of Stat3 activation on brain metastasis, we used the cells described above in an internal carotid artery injection model. A375Br and A375Br-Neo cells produced brain metastatic lesions in all of the injected mice (Table 1). The mice became moribund ~50 days after the injection. In contrast, the Stat3DN-transfected A375Br cells produced very few or no brain metastatic lesions. Likewise, injection of TXM-18 and TXM-18-Neo cells resulted in a high incidence of brain metastatic lesions in the nude mice, whereas that of Stat3DN-transfected TXM-18 cells resulted in a very low incidence of or no brain metastatic lesions. These results showed that blockade of Stat3 activation suppressed brain metastasis of human melanoma cells. Further, the tumor growth of Stat3DN-transfected melanoma cells was similar to that of control cells in the subcutis of nude mice (Supplementary Fig. S1B and C), whereas the experimental lung metastatic ability of Stat3DN-transfected cells was also decreased compared with that of control cells to different extent among different cell lines (Supplementary Table S1). Thus, these data indicated that Stat3DN-transfected cells are less malignant than the control cells, although Stat3 activation may not necessarily represent a mechanism for organ-specific metastasis.

Effect of altered Stat3 activity on angiogenesis of brain metastases. To study the mechanisms responsible for the regulation of brain metastasis by Stat3 activation, we next tested whether altered Stat3 affects cell growth in vitro. Tumor cells (1 × 10⁵) were seeded in six-well plates and cultured for 1 to 4 days. In vitro growth of the tumor cells was determined by direct counting of viable cells from the plates. We found no discernible differences in the in vitro growth rate among A375Br, A375Br-Neo, and Stat3DN-transfected A375Br cells (data not shown). In addition, the in vitro growth of A375P, A375P-Neo, and Stat3C-transfected A375P cells was similar (data not shown).

Metastasis has been shown to depend on the development of an adequate blood supply through angiogenesis (39). Therefore, we sought to determine whether altered Stat3 activity affects angiogenesis in brain metastatic tumors. We assessed vascularization in brain metastases by staining brain metastasis tissue sections with an anti-CD34 antibody. As shown in Supplementary Fig. S2, brain metastases produced by A375Br-Neo cells were highly vascularized, whereas brain metastases produced by Stat3DN-transfected cells had a significantly decreased MVD. Moreover, brain metastases produced by A375Br-Neo cells contained a larger proportion of blood vessels with large lumens than did brain metastases produced by A375Br-Stat3DN-a cells. We obtained similar results with TXM-18-Neo control and Stat3DN-transfected cells (data not shown). In contrast, brain metastases produced by Stat3C-transfected A375P cells were highly vascularized. Because A375P cells did not produce experimental metastases in the brain, we could not perform immunohistochemistry.

Effect of altered Stat3 activity on the expression of multiple proangiogenic molecules in melanoma cells. Induction of angiogenesis is mediated by multiple molecules that are released by both tumor and host cells (39). To provide direct evidence of the contribution of Stat3 activation to the regulation of proangiogenic
molecules as well as angiogenesis, we studied the effect of altered Stat3 activity on the expression of proangiogenic molecules, including bFGF, VEGF, and MMP-2, both in vivo and in vitro. We first evaluated the protein expression of bFGF, VEGF, and MMP-2 in the tumors by using immunohistochemistry. Consistent with the MVD, staining of bFGF, VEGF, and MMP-2 was observed in brain metastatic lesions but was significantly decreased in lesions formed by Stat3DN-transfected cells. The level of staining of bFGF, VEGF, and MMP-2 in metastatic lesions formed by Stat3C-transfected A375P cells was similar to that in A375Br lesions (Supplementary Fig. S2). These results showed that altered Stat3 activity affected the expression of multiple angiogenic molecules bFGF, VEGF, and MMP-2.

We also determined the level of in vitro expression of the bFGF, VEGF, and MMP-2 genes at both mRNA and protein levels. We found a significant decrease in bFGF, VEGF, and MMP-2 mRNA and protein expression in Stat3DN-transfected A375Br cells and TXM-18 cells when compared with control cells (Fig. 3A and B). We were unable to determine whether Stat3 activation affects MMP-9 expression in A375Br and TXM-18 cells, because 92-kb MMP-9 collagenase activity was undetectable in both A375Br and TXM-18 cells. Furthermore, the promoter activities of bFGF, VEGF, and MMP-2 were significantly inhibited in Stat3DN-transfected A375Br cells and TXM-18 cells when compared with control cells (Fig. 3C and D).

Conversely, we studied the effect of altered Stat3 activity on the expression of bFGF, VEGF, and MMP-2 in Stat3C-transfected A375P cells. bFGF, VEGF, and MMP-2 mRNA and protein expression was significantly increased in Stat3C-transfected A375P cells when compared with control A375P and Neo cells (Fig. 3A and B). In addition, the promoter activities of bFGF, VEGF, and MMP-2 were significantly activated in Stat3C-transfected A375P cells (Fig. 3C). Collectively, these data showed for the first time that Stat3 activity regulates the transcription of bFGF in addition to VEGF and MMP-2 in human melanoma cells.

**Effect of altered Stat3 activity on melanoma cell invasion.** An important function of MMP-2 in tumor invasion is its ability to degrade the extracellular matrix and basement membranes. Inhibition of MMP-2 activity in A375Br cells by a potent MMP-2-specific inhibitor OA-Hy (36) significantly reduced the invasiveness of the cells (Fig. 5A). Therefore, we next analyzed whether the expression of MMP-2 by Stat3DN-transfected A375Br or Stat3C-transfected A375P cells correlated with their ability to invade through the basement membrane. As shown in Fig. 5B to D, the Stat3DN-transfected A375Br and TXM-18 cells exhibited significantly decreased invasion through a Matrigel-coated filter compared with control cells, whereas the Stat3C-transfected A375P cells exhibited significantly increased invasion. These results suggested that altered Stat3 activity directly affect the invasiveness of melanoma cells.
Discussion

In the present study, we found that Stat3 activity is increased in human brain metastatic melanoma cells when compared with that in cutaneous melanoma cells. Likewise, activated Stat3 expression is increased in brain metastases when compared with that in the primary melanoma. In an animal model, increased Stat3 activation by Stat3C transfection enhanced the brain metastasis, whereas blockade of Stat3 activation by Stat3DN transfection suppressed brain metastasis of human melanoma cells. Furthermore, altered Stat3 activity significantly affected in vitro and in vivo expression of the major proangiogenic molecules bFGF, VEGF, and MMP-2 and affected melanoma angiogenesis and invasion. Therefore, for the first time, to our knowledge, we provide clinical, experimental, and mechanistic evidence that Stat3C plays an important role in brain metastasis of human melanoma.

The process of metastasis is complex, which includes steps of shedding of cells from a primary tumor into the circulation, survival of the cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor. In the present study, we used an intracarotid injection model to study brain metastasis (29). In this model, injected tumor cells must undergo invasion into and proliferation within the brain parenchyma and induce angiogenesis leading to overt tumor formation. These processes are regulated by multiple factors. For example, increased production of degradative enzymes by tumor cells facilitates extravasation and invasion (27, 40). Interestingly, in our study, a potent MMP-2-specific inhibitor significantly reduced the invasiveness of A375Br cells, suggesting a role for MMP-2 in brain metastasis. We then determined the role of Stat3 in MMP-2 expression and tumor invasiveness in our experiments, showing that MMP-2 expression was significantly decreased in Stat3DN-transfected brain metastatic melanoma cells, whereas it was significantly increased in Stat3C-transfected parental melanoma cells. Consistently, alteration of Stat3 activity (activation and blockade) in melanoma cells significantly affected their tumor invasiveness in vitro and brain metastasis in vivo. These results suggest a critical involvement of Stat3 activation in the molecular control of extravasation and brain metastasis of melanoma, which is due at least in part to MMP-2 expression.

De novo angiogenesis is required at metastatic sites for continued tumor growth (39). In fact, the progression of neoplasms from benign to malignant state is often associated with a switch to an "angiogenic phenotype," representing an increase in proangiogenic molecules produced by the tumor cells and organ-specific environments (39). Metastatic melanoma cells secrete a variety of proangiogenic molecules, including bFGF, VEGF, IL-8, and MMP-2 (7–9, 41–43). However, the underlying molecular mechanism of imbalanced production of these molecules remains unclear. In the present study, we found that blockade of Stat3 activity in brain metastatic melanoma cells suppressed angiogenesis in brain metastases in nude mice. The inhibition of brain metastasis by blockade of Stat3 activity directly correlated with decreased blood

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**Figure 4.** Expression of the bFGF, VEGF, and MMP-2 genes in A375P, A375P-Neo, and Stat3C-transfected A375P cells. Northern blot analysis (A), Western blot analysis and gelatinase zymography (B), and bFGF, VEGF, and MMP-2 promoter activity assays (C) were done as described in Fig. 3. Fold stimulation of promoter activities was calculated relative to A375P cells. Bars, 95% confidence intervals of duplicates within the assay. *, *P* < 0.001, compared with A375P. Representative experiment of two with similar results.
vessel formation. Moreover, up-regulation of constitutive Stat3 activity increased bFGF, VEGF, and MMP-2 gene expression, whereas blockade of Stat3 activity decreased the expression of these genes, suggesting that the expression of bFGF, VEGF, and MMP-2 is critically regulated by Stat3 activation. Therefore, the mechanisms for inhibition of angiogenesis may be attributed at least in part to the down-regulation of these factors. However, it is interesting to note that altered Stat3 activity did not significantly change the s.c. growth of the melanoma cells, suggesting different molecular bases for tumor angiogenesis and growth at different tissue sites.

bFGF is one of the most important autocrine growth factors and is also a potent angiogenic factor, which plays an important role in the transformation and progression of melanoma (8). It has been shown that Stat3 can be activated by bFGF (44). Thus, the finding that Stat3 activation increases bFGF production in our study places Stat3 at the center of bFGF autocrine action. To further substantiate the notion that Stat3 regulates bFGF expression, we further analyzed the regulation of bFGF promoter by Stat3. We found significantly decreased and increased bFGF promoter activity in the cells transfected with Stat3DN and Stat3C, respectively, suggesting that the regulation of bFGF expression by Stat3 probably occurred at the transcriptional level. Little is known about transcriptional regulation of bFGF, although a few previous studies have indicated that p53 and Egr-1 regulate the bFGF promoter (45). We have analyzed the bFGF promoter for potential Stat3-binding sites with the Stat-binding consensus sequences TT(N4)AA and TT(N5)AA (24). We identified three putative Stat3-binding sites in the 1.8-kb bFGF promoter region at positions –253 to –246, –388 to –380, and –510 to –502 upstream of the transcription initiation site.7 Detailed studies designed to define the Stat3-binding site or sites in the promoter of the bFGF gene are under way.

Additionally, activated Stat3 has been shown to protect tumor cells from apoptosis and promote cell proliferation by regulating genes encoding antiapoptotic and proliferation-associated proteins, such as Bcl-xl, Mcl-1, Bcl-2, Fas, cyclin D1, and c-Myc (17, 19–23, 46–49). Alteration of Stat3 activity may affect cancer metastasis via regulation of cell survival and proliferation. However, we found that the growth of Stat3C-transfected A375P cells in vitro was not significantly different from that of A375P and control vector-transfected cells, whereas altered Stat3 activity significantly altered metastasis formation in melanoma brain metastasis models. These findings suggest that an additional mechanism for the effect of altered Stat3 activity on metastasis involves the regulation of the expression of multiple genes key to tumor cell invasion and angiogenesis. Therefore, Stat3 may affect cancer metastasis via multiple mechanisms.

Despite improvements in the early detection and treatment of primary melanoma, brain metastasis is still a disease with a rising incidence and devastating consequences. Currently, predicting which patients are likely to have brain metastasis from a primary melanoma is difficult. In the present study, we found that Stat3 activity is higher in brain metastases than in primary melanomas, suggesting a close association between Stat3 expression and brain metastasis. However, more studies are clearly needed to address whether the level of activated Stat3 protein in primary melanomas predicts brain metastasis. Clinical observations have suggested that brain metastases produced by many solid tumors occur late in the disease, and it has been proposed that some of brain metastases are produced by cells populating lymph node or visceral metastases (i.e., metastasis of metastases). Therefore, an elevated Stat3 activity in lymph node or visceral metastases is likely. In fact, activation of Stat3 increased lung metastasis in our animal models, which was consistent with clinical observations showing that many patients with brain metastasis have metastases in the lungs (2). It is also possible that the factors that affect metastases in different organs

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7 Unpublished data.
might vary. For example, Rho C overexpression is crucial for lung metastasis (50). Nonetheless, our present findings strongly suggest that Stat3 play an important role in brain metastasis and could be a potential therapeutic target for brain metastasis of melanoma. Given the critical role of Stat3 activity in regulation of multiple metastasis factors, targeting Stat3 activation may prove to be a more effective approach to controlling the brain metastatic phenotype than merely targeting individual molecules, such as bFGF, MMP-2, and VEGF.

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References


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