Antiangiogenic and Antimetastatic Activity of JAK Inhibitor AZD1480

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Abstract

STAT3 has important functions in both tumor cells and the tumor microenvironment to facilitate cancer progression. The STAT regulatory kinase Janus-activated kinase (JAK) has been strongly implicated in promoting oncogenesis of various solid tumors, including the use of JAK kinase inhibitors such as AZD1480. However, direct evidence that JAK drives STAT3 function and cancer pathogenesis at the level of the tumor microenvironment is yet to be established clearly. In this study, we show that AZD1480 inhibits STAT3 in tumor-associated myeloid cells, reducing their number and inhibiting tumor metastasis. Myeloid cell–mediated angiogenesis was also diminished by AZD1480, with additional direct inhibition of endothelial cell function in vitro and in vivo. AZD1480 blocked lung infiltration of myeloid cells and formation of pulmonary metastases in both mouse syngeneic experimental and spontaneous metastatic models. Furthermore, AZD1480 reduced angiogenesis and metastasis in a human xenograft tumor model. Although the effects of AZD1480 on the tumor microenvironment were important for the observed antiangiogenic activity, constitutive activation of STAT3 in tumor cells themselves could block these antiangiogenic effects, showing the complexity of the JAK/STAT signaling network in tumor progression. Together, our results indicated that AZD1480 can effectively inhibit tumor angiogenesis and metastasis mediated by STAT3 in stromal cells as well as tumor cells. Cancer Res; 71(21); 1–10. ©2011 AACR.

Introduction

Tumor development is affected by signaling within the cancer cells and their interactions with surrounding tissues composed of extracellular matrix components and stromal cells including endothelial cells and immune cells (1). The local tumor microenvironment responds to signaling through inflammatory cells, which release cytokines, chemokines, and growth factors to stimulate tumor growth via increased invasion potential of tumor cells. These signals also create immunosuppressive networks that enhance tumor survival (2). STAT3 is a point of convergence for multiple oncogenic signaling pathways. Constitutive activation of STAT3 within tumor cells as well as stromal cells promotes cancer cell proliferation, invasion, angiogenesis, and immune evasion (3). Activated STAT3 downregulates Th1 cytokines and other mediators critical for potent antitumor immune responses. STAT3-driven tumor-derived factors, including interleukin (IL)-6, IL-10, and VEGF, establish a crosstalk between tumor cells and tumor-associated immune cells to ensure persistent STAT3 activation in the tumor microenvironment, thereby creating a “feed-forward loop” (4–7). Activated STAT3 in tumor-associated immune cells leads to expression of a large number of growth factors, angiogenic factors, and other molecules crucial for invasion and metastasis (8–10).

The importance of IL-6 in cancer development and progression has been widely documented (11–13). A critical role of Janus-activated kinase (JAK) in mediating IL-6–induced STAT3 activation has also been established. Although JAK has been viewed as a critical target for treating malignancies of hematopoietic origins, recent studies show its importance in various solid tumors (14). Recently, JAK has also been shown to facilitate sphingosine-1-phosphate receptor-1 (SIPR1)-induced persistent STAT3 activation in both tumor cells and tumor stromal cells (9). We recently showed that AZD1480 is a potent, competitive small-molecule inhibitor of JAK1/2 kinase, and that it is capable of inhibiting STAT3 phosphorylation and tumor growth in a STAT3–dependent manner (14). Although tumor growth was inhibited directly in vivo in each tumor model tested, in some tumor cell lines, AZD1480 did not block tumor cell growth in vitro at levels that produced maximal
inhibition of STAT3 phosphorylation (14). This suggests the potential important effects of AZD1480 on the tumor microenvironment by inhibiting JAK/STAT signaling. AZD1480 is currently in early clinical trials for solid and hematologic malignancies (15). Our current study shows that AZD1480 inhibits tumor angiogenesis and metastasis in part by affecting the tumor microenvironment.

Materials and Methods

Reagents
AZD1480 was provided by AstraZeneca and dissolved in dimethyl sulfoxide (DMSO) for in vitro studies. For in vivo experiments, AZD1480 was suspended in water supplemented with 0.5% hypromellose and 0.1% Tween 80. All solvents are from Sigma. Mouse IL-6 was purchased from R&D Systems. Antibodies against phosphorylated STAT3 (p-STAT3; Tyr705), phosphorylated JAK2 (p-JAK2; Tyr1007/1008), JAK2, cleaved caspase-3 (Asp175; 5A1E), and matrix metalloproteinase 9 (MMP9; G657) were purchased from Cell Signaling Technology. Antibodies against STAT3 (C-20) and VEGF (A-20) were obtained from Santa Cruz Biotechnology.

Cell lines
Renca murine cell line was a gift from Dr. Alfred Chang (University of Michigan Medical Center, Ann Arbor, MI). Human renal cell carcinoma cell line 786-O was generously provided by Dr. William G. Kaelin (Harvard Medical School, Boston, MA). The 4T1 mouse mammary tumor cell line and the Calu-6 lung carcinoma cell line were from American Type Culture Collection. Mouse endothelial cell line derived from prostate and colon was kindly provided by S. Huang and J. Fidler (MD Anderson Cancer Center, Houston, TX). All the above cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) or RPMI-1640 with 10% FBS (Sigma). Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and cultured on collagen I–coated plates in their complete medium (Clonetics). 786-O constitutively active STAT3 mutant (STAT3C) and vector-expressing control cell lines were generated and maintained as described previously (16).

Animal models and drug administration
Female BALB/c and athymic nude (NCR<sup>–</sup> nu/nu) mice (7–8 weeks old) were obtained from National Cancer Institute (Bethesda, MD) and Taconic Laboratories. Animal use procedures were approved by the Institutional Animal Care and Use Committees of Beckman Research Institute at City of Hope and AstraZeneca. For subcutaneous tumor model, 2.5 × 10<sup>6</sup> Renca or 786-O cells suspended in 100 µL PBS were injected into the flank of BALB/c or nude mice, respectively. When average tumor volume reached approximately 100 to 150 mm<sup>3</sup>, AZD1480 or vehicle was administered by oral gavage either once a day at the dose of 50 mg/kg or twice daily at 30 mg/kg, as indicated. Tumor size was measured by caliper every other day. For experimental lung metastasis model, 0.1 × 10<sup>6</sup> Renca or 1 × 10<sup>6</sup> 786-O cells suspended in 500 µL PBS were injected via tail vein to BALB/c or nude mice, respectively. Three days later, mice were orally treated with AZD1480 (50 mg/kg/d) or vehicle for 21 days for Renca tumors and 60 days for 786-O tumors, respectively. For the Calu-6 model, 3 × 10<sup>5</sup> tumor cells in Matrigel were implanted subcutaneously into the flanks of nude mice, randomized into vehicle (twice daily [BID]) and drug treatment (AZD1480, 30 mg/kg BID) groups, and dosed orally daily for 19 days. For spontaneous lung metastasis model, 2 × 10<sup>5</sup> 4T1 cells suspended in 100 µL PBS were injected in the mammary gland of female BALB/c mice by gently penetrating the skin. AZD1480 (50 mg/kg/d) or vehicle was given orally for 21 days.

Flow cytometry
Cell suspensions from spleen, tumor, or lung were prepared as described previously (7) and stained with fluorochrome-conjugated CD11b and Gr1 antibodies (BD Biosciences). Data were collected by CyAn ADP Violet Cytometer (Dako Cytomation) and analyzed by FlowJo (Tree Star).

In vivo Matrigel plug assay
Growth factor–reduced Matrigel (BD Biosciences) containing Renca tumor cells and splenic CD11b<sup>+</sup>/CD11c<sup>+</sup> myeloid cells enriched from Renca tumor–bearing mice (ratio, 1:10) were implanted subcutaneously into BALB/c mice. Five days after implantation, AZD1480 (50 mg/kg/d) or vehicle was given orally for 4 days. For the Calu-6 Matrigel plug assay, 5 × 10<sup>6</sup> tumor cells in Matrigel were implanted into nude mice which were then treated twice daily, beginning on day 2, with vehicle, 30 mg/kg AZD1480, or 6 mg/kg VEGF receptor (VEGFR) inhibitor, orally for 7 days. The plugs were harvested for hemoglobin content measurement by colorimetry with Dabkin’s reagent (Sigma), and frozen sections of the Renca tumor plugs were stained for CD31.

In vitro tube formation assay
Mouse endothelial cells or HUVECs (5 × 10<sup>4</sup> cells per well in 1% FBS RPMI-1640 medium) were seeded on 48-well plates coated with 100 µL of growth factor–reduced Matrigel. Five percent of Renca tumor–conditioned medium (collected from cultured Renca tumor cells) with varying doses of AZD1480 or DMSO was added. After 16 hours, capillary-like tube formation was quantified by manually counting the cord junctions with at least 3 branches formed by endothelial cells.

Wound-healing migration assay
Mouse endothelial cells were grown on 6-well plates, “wounds” were made by scratching on the confluent cells with a pipette tip. The number of cells migrated into the wound area was counted after incubation with DMSO or AZD1480 (1 µmol/L) for 24 hours.

Cell viability assay
Renca or 786-O cells suspended in DMEM medium with 5% FBS were seeded in 96-well plates (5,000 per well) to allow adhesion and then treated with DMSO or AZD1480 for 48 hours. Cell viability was determined by MTS assay (Promega) according to instructions. Absorbance at 490 nm was measured with Mikrotek Laborsysteme. Mouse endothelial cells and splenic CD11b<sup>+</sup>/CD11c<sup>+</sup> myeloid cells enriched from tumor-bearing mice were cultured in 5% FBS RPMI-1640 medium.
HUVECs were cultured on collagen 1–coated plates in complete medium (Clonetics). All cells are treated with DMSO and AZD1480 at various doses for 24 hours. Cell viability was determined by counting cell number manually. All the experiments were repeated 3 times.

Immunofluorescence

Immunofluorescent staining of tumor or lung frozen tissue sections was described previously (10). To prepare lung sections, mouse lungs were perfused with PBS to eliminate circulatory blood. All the representative images were obtained under 200× magnification. CD31+ blood vessels or CD11b+ myeloid cells were counted in 6 random fields (200×).

Quantitative real-time PCR

RNA extraction, cDNA synthesis, and real-time PCR were described previously (16). The primers for mouse vegf, il1β, mmp9, fibroblast growth factor factor 2 (fgf2), s100a8, s100a9, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Super Array Bioscience.

Evaluation of lung metastasis

Each mouse were stained with hematoxylin–eosin (H&E) and evaluated the effect of targeting the JAK/STAT3 signaling pathway with AZD1480 on tumor-associated myeloid cells. CD11b+/Gr1+ myeloid cells [myeloid-derived suppressor cells (MDSC)] in spleens and tumors were quantified by flow cytometric analyses in Renca tumor–bearing mice after 21 days of treatment. We observed a 2- to 3-fold reduction of MDSCs in AZD1480-treated groups compared with vehicle groups (P < 0.01), as shown in Fig 1C. It has been shown that constitutively activated STAT3 not only plays a critical role in tumor cell signaling but also stimulates the accumulation of tumor-associated myeloid cells (7). Therefore, we evaluated whether STAT3 signaling could be regulated by AZD1480 in myeloid cells. Tumor-infiltrating CD11b+/CD11c− myeloid cells isolated from tumor-bearing mice after 14 days of treatment were analyzed. STAT3 phosphorylation was potently inhibited in AZD1480-treated group, and STAT3-dependent, angiogenic, and metastasis-promoting factors, VEGF, IL-1β, FGF-2, and MMP9, were downregulated in tumor-infiltrating CD11b+/CD11c− myeloid cells (Fig. 1D). Furthermore, immunostaining of Renca tumor sections for CD11b also indicated a dramatic reduction of CD11b+ myeloid cell infiltration after AZD1480 administration (Fig. 1E). To identify whether AZD1480 directly affects myeloid cell tumor–promoting functions, we conducted an ex vivo migration assay to examine the effect of AZD1480 on myeloid cell motility. Splenic CD11b+/CD11c− myeloid cells isolated from Renca tumor–bearing mice were subjected to a Transwell migration assay. The percentage of migrated myeloid cells was significantly (P < 0.05) inhibited by AZD1480 treatment in a dose-dependent manner (Supplementary Fig. S2B, left), and a reduction of p-STAT3 by AZD1480 treatment in CD11b+/CD11c− myeloid cells was also observed (Supplementary Fig. S2B, right).

Intravital multiphoton microscopy

Implantation of 786-O-pRC-vector or 786-O-pRC-STAT3C tumor cells into nude mice and treatment with AZD1480 or vehicle were described previously (14). Tumor vasculature, apoptosis, and extracellular matrix were visualized by dextran–rhodamine (red; Invitrogen), Annexin V (green; BioVision), and Hoechst 33342 (blue; Sigma).

Statistics

The 2-tailed Student t test was used for statistical analysis. Differences were considered statistically significant when P < 0.05. ***, P < 0.001; **, P < 0.01; and *, P < 0.05.

Results

AZD1480 inhibits Renca tumor growth in vivo with a reduction in tumor myeloid cell infiltration

Our previous studies indicated that although AZD1480 could induce tumor growth inhibition and tumor cell apoptosis in vivo, in certain tumor cell lines it did not effectively inhibit tumor cell proliferation and induce apoptosis in vitro (14). Consistent with this observation, we found that AZD1480 treatment of 786-O human renal cancer cells and mouse Renca cells in vitro had only limited reduction in cell viability (Fig. 1A, left and Supplementary Fig. S1A), although p-JAK2 and p-STAT3 were inhibited (Supplementary Fig. S1B and Fig. 1A, right). These findings prompted us to investigate the in vivo antitumor effects of AZD1480 on Renca, a syngeneic murine renal carcinoma model. Renca tumor cells were subcutaneously injected into BALB/c mice and treated with AZD1480 (50 mg/kg/d) or vehicle for 21 days. We observed a significant (P < 0.001) inhibition of tumor growth in AZD1480-treated group compared with vehicle-treated group (Fig. 1B, top). Western blot analyses of the whole tumor lysates revealed a dramatic inhibition of p-STAT3 by AZD1480 treatment (Fig. 1B, bottom). These results suggest that AZD1480 has significant antitumor effects in vivo, with inhibition of STAT3 signaling.

The tumor microenvironment is a complex system composed of many types of cells, many of which play crucial roles in tumor progression (17). In particular, tumor-associated myeloid cells are an important component of the tumor microenvironment that regulates tumor growth and responses to anticancer therapies (18, 19). We investigated the effect of targeting the JAK/STAT3 signaling pathways with AZD1480 on tumor-associated myeloid cells. CD11b+/Gr1+ myeloid cells [myeloid-derived suppressor cells (MDSC)] in spleens and tumors were quantified by flow cytometric analyses in Renca tumor–bearing mice after 21 days of treatment. We observed a 2- to 3-fold reduction of MDSCs in AZD1480-treated groups compared with vehicle groups (P < 0.01), as shown in Fig 1C. It has been shown that constitutively activated STAT3 not only plays a critical role in tumor cell signaling but also stimulates the accumulation of tumor-associated myeloid cells (7). Therefore, we evaluated whether STAT3 signaling could be regulated by AZD1480 in myeloid cells. Tumor-infiltrating CD11b+/CD11c− myeloid cells isolated from tumor-bearing mice after 14 days of treatment were analyzed. STAT3 phosphorylation was potently inhibited in AZD1480-treated group, and STAT3-dependent, angiogenic, and metastasis-promoting factors, VEGF, IL-1β, FGF-2, and MMP9, were downregulated in tumor-infiltrating CD11b+/CD11c− myeloid cells (Fig. 1D). Furthermore, immunostaining of Renca tumor sections for CD11b also indicated a dramatic reduction of CD11b+ myeloid cell infiltration after AZD1480 administration (Fig. 1E). To identify whether AZD1480 directly affects myeloid cell tumor–promoting functions, we conducted an ex vivo migration assay to examine the effect of AZD1480 on myeloid cell motility. Splenic CD11b+/CD11c− myeloid cells isolated from Renca tumor–bearing mice were subjected to a Transwell migration assay. The percentage of migrated myeloid cells was significantly (P < 0.05) inhibited by AZD1480 treatment in a dose-dependent manner (Supplementary Fig. S2B, left), and a reduction of p-STAT3 by AZD1480 treatment in CD11b+/CD11c− myeloid cells was also observed (Supplementary Fig. S2B, right).

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AZD1480 inhibits tumor growth in vivo by inhibition of STAT3 and reduction of tumor-associated myeloid cells. A, analysis of tumor cell viability after treatment with AZD1480 in vitro. MTS assay showing viability of 786-O and Renca tumor cells 48 hours after AZD1480 treatment at indicated doses (left; bars, SD; n = 3). Western blotting evaluating p-STAT3 level in the tumor cells 2 hours after treatment with AZD1480 (right). B, top, AZD1480 inhibits Renca tumor growth. Volumes of Renca tumors in mice treated with AZD1480 or vehicle for 21 continuous days (bars, SEM; n = 10). Bottom, western blotting using whole tumor lysates showing total and p-STAT3 levels 2 hours after dosing. C, AZD1480 reduces tumor-associated MDSCs in Renca tumor–bearing mice. Flow cytometric analysis quantifying CD11b<sup>+</sup>/Gr1<sup>+</sup> myeloid cells in spleens and tumors collected 14 days of treatment with AZD1480 or vehicle. Bars show mean ± SEM; n = 3 (3 independent experiments with 4 mice per group). D, AZD1480 inhibits p-STAT3 and expression of STAT3-regulated genes in myeloid cells. Western blotting and real-time PCR detecting p-STAT3 and expression levels of the indicated genes in tumor-infiltrating CD11b<sup>+</sup>/CD11c<sup>−</sup> myeloid cells isolated from 4 pooled tumors after 14 days of treatment. All gene expression levels in vehicle-treated group were set as one. The experiments were repeated twice with similar results. E, AZD1480 treatment reduces tumor myeloid cell infiltration. Representative images of immunofluorescent staining showing CD11b<sup>+</sup> cells in tumor tissues harvested after 10 days of treatment. The numbers of CD11b<sup>+</sup> cells per field based on 6 fields per slide, 3 slides per tumor, with total of 4 tumors per mice per group were shown. Bars, SEM. DAPI, 4',6-diamidino-2-phenylindole.
AZD1480 inhibits tumor angiogenesis in Renca tumor model

We next investigated the antiangiogenic effect of AZD1480 on Renca tumors. Following 10 days of treatment, tumors were collected and immunostained for endothelial cell marker CD31. We observed a more than 3-fold reduction of CD31⁺ tumor blood vessels in AZD1480-treated mice compared with vehicle treated (Fig. 2A), along with downregulation of VEGF and MMP9 in whole tumor lysates (Fig. 2B). Emerging evidence has indicated that tumor-associated myeloid cells are important sources of proangiogenic factors in the tumor microenvironment (20, 21), and our group has previously shown that constitutively activated STAT3 in tumor-associated myeloid cells plays a crucial role in promoting tumor angiogenesis (10). We therefore analyzed the effect of AZD1480 on myeloid cell–induced angiogenesis in a modified Matrigel angiogenesis assay. Matrigel plugs containing a mixture of Renca tumor cells and CD11b⁺/CD11c⁻ myeloid cells (a ratio of 1:10) enriched from spleens of tumor-bearing mice were implanted into BALB/c mice and analyzed by immunostaining for CD31. We found a potent reduction of neovasculature in AZD1480 treatment group (Fig. 2C, left). Quantified results indicated a more than 7-fold reduction in CD31⁺ vasculature comparing AZD1480 with vehicle-treated group (Fig. 2C, middle). Measurement of hemoglobin content of Matrigel plug also showed that AZD1480 significantly reduced neovascularization (P < 0.01; Fig. 2C, right). Taken together, these data suggest that AZD1480 inhibits STAT3 signaling and tumor angiogenesis, at least, in part, by targeting tumor-associated myeloid cells, in the Renca tumor model. Furthermore, inhibition of vascularization of Matrigel plugs and tumor growth has also been observed in the Calu-6 lung carcinoma xenograft model and in association with inhibition of p-STAT3 and induction of apoptosis (Supplementary Fig. S3). The extent of antiangiogenic effect is comparable with that observed with VEGFR inhibitors (22).

To examine whether targeting STAT3 by AZD1480 directly inhibits the function of endothelial cells, we analyzed tube formation activity of both mouse endothelial cells and HUVECs in the presence or absence of AZD1480. AZD1480 inhibited both mouse and human endothelial cell tube formation induced by Renca tumor–conditioned medium in a dose-dependent manner (Fig. 3A). In addition, the effect of AZD1480 on mouse endothelial cell migration was measured by a
AZD1480 inhibits spontaneous lung metastasis and modulates the metastatic environment

We also investigated the effect of AZD1480 on 4T1, a syngeneic mouse mammary carcinoma model that spontaneously develops lung metastasis. 4T1 tumor cells were orthotopically implanted into the mammary glands of mice, and AZD1480 or vehicle was orally administered 3 days after tumor challenge. The number of lung metastatic nodules was significantly (P < 0.05) reduced after 21 days of AZD1480 treatment compared with vehicle treatment (Fig. 5A). Meanwhile, we examined lung myeloid cell infiltration in 4T1 tumor-bearing mice by flow cytometry. We observed a 2- to 4-fold reduction of CD11b<sup>+</sup>/Gr1<sup>-</sup> myeloid cells in the lungs as early as 4 days after initial AZD1480 treatment (P < 0.05; Fig. 5B, left). Lung tissue sections were subjected to immunofluorescent staining for CD11b antibody. A reduction of lung myeloid cell infiltration after 8 days of AZD1480 treatment was shown (Fig. 5B, right). Furthermore, we examined STAT3 signaling in pulmonary CD11b<sup>+</sup>/CD11c<sup>-</sup> myeloid cells by either western blotting or real-time PCR. As shown in Fig 5C, p-STAT3 along with VEGF and MMP9, as well as s100a8 and s100a9, all of which have been shown to be important in myeloid cell–mediated distant site metastasis, were inhibited after treatment with AZD1480 compared with vehicle group. To further address the effects of AZD1480 on the ability of myeloid cells to attract 4T1 tumor cells, we conducted an ex vivo migration assay. CD11b<sup>+</sup>/CD11c<sup>-</sup> myeloid cell–conditioned medium was used to induce 4T1 tumor cell migration. The number of migrated tumor cells was significantly decreased in AZD1480 treatment group (P < 0.05; Supplementary Fig. S4). Taken together, these results suggest that AZD1480, by targeting STAT3 signaling, potently reduced the infiltration of myeloid cells into the lung, which could inhibit tumor cell distant colonization.

Antiangiogenic and antimetastatic effects of AZD1480 on a human renal cell carcinoma xenograft

Previous study indicated the ability of AZD1480 to inhibit growth of various human tumors, including 786-O human renal

AZD1480 inhibits lung metastasis and factors important for premetastatic niche formation

STAT3 has been implicated in tumor migration and metastasis (9, 23, 24). Therefore, we tested the effect of AZD1480 on an experimental lung metastasis model. Renca cells were injected into BALB/c mice, and AZD1480 or vehicle was given orally 3 days after implantation. As shown in Fig 4A, the number of metastatic lung nodules was significantly reduced on day 21 by AZD1480 treatment compared with vehicle treatment (P < 0.01). Western blot analysis of whole lung lysates revealed reduced p-STAT3, VEGF, and MMP9 (Fig. 4B). It has been shown that the primary tumor influences the lung environment before metastasis occurs (25), and infiltration and accumulation of tumor-associated myeloid cells into the lung play a crucial role in the development of metastasis (26). Thus, we examined whether subcutaneous primary tumor influenced myeloid cell infiltration into the lung and whether AZD1480 treatment blocked this process. We analyzed lung myeloid cell infiltration by immunofluorescent staining in subcutaneous Renca tumor model and found a significant (P < 0.05) reduction of CD11b<sup>+</sup> myeloid cells in the lungs after 14 days of treatment with AZD1480 (Fig. 4C). These results indicate that AZD1480 can inhibit Renca tumor metastasis.

Figure 3. AZD1480 inhibits endothelial cell function in vitro. A, AZD inhibits tube formation of endothelial cell in vitro. Numbers of tube-like structure formed by AZD1480-treated mouse (left) or human (right) endothelial cells per well were shown (bars, SD; n = 3). B, AZD1480 inhibits endothelial cell migration. Wound-healing migration assay to detect the migration of mouse endothelial cells induced by Renca tumor cell conditioned medium. Western blotting showing the effect of AZD1480 on p-STAT3 levels in endothelial cells exposed to the tumor cell–conditioned medium. We observed a significant reduction in the number of cells that migrated into the wound area (P < 0.01; Fig. 3B). The doses required to inhibit endothelial cell tube formation and migration were noticeably less than those that affect the viability of mouse and human endothelial cells (Fig. 3A and B and Supplementary Fig. S2A). Moreover, p-STAT3 was evaluated in mouse endothelial cells after treatment of AZD1480 for 2 hours followed by 30 minutes stimulation of Renca tumor–conditioned medium. We observed that 0.5 μmol/L of AZD1480 potently inhibited STAT3 phosphorylation induced by Renca tumor–conditioned medium (Fig. 3C).

Figure 3A shows the effect of AZD1480 on tube formation of endothelial cells. Figure 3B demonstrates the effect of AZD1480 on endothelial cell migration. Figure 3C illustrates the effect of AZD1480 on p-STAT3 levels in endothelial cells exposed to the tumor cell–conditioned medium.
cell carcinoma, in xenograft models (14). We determined here whether AZD1480 could also inhibit tumor growth through antiangiogenesis or antimetastasis in 786-O human renal cell carcinoma xenografts. Western blot analyses of the whole tumor lysates showed a dramatic inhibition of p-STAT3 by AZD1480 treatment (Fig. 6A). Tumor sections were immunostained with CD31 antibody to detect tumor vessels after AZD1480 or vehicle treatment for 35 days. As shown in Fig 6B, AZD1480 treatment led to a 2- to 2.5-fold reduction in CD31$^+$ blood vessels in 786-O xenografts ($P < 0.05$). We also examined infiltrating myeloid cells in tumors by immunostaining for CD11b$^+$. The number of tumor CD11b$^+$ myeloid cells was significantly decreased after AZD1480 treatment ($P < 0.05$; Fig. 6C). To determine whether the reduction in myeloid cells correlated with inhibition of lung metastasis, we investigated the effect of AZD1480 on experimental pulmonary metastasis model induced by 786-O tumor cells. Lung tissue was collected and analyzed for metastasis after 2 months of treatment. Seven of 8 mice in vehicle group developed metastasis on histologic examination, whereas only 3 of 7 mice in AZD1480 group developed metastases. The number of micrometastatic nodules per field in the vehicle group was also significantly higher ($P < 0.05$) than that of AZD1480-treated mice (Fig. 6D). These results further indicate that AZD1480 inhibits angiogenesis and metastasis in 786-O xenografts, which is associated with inhibition of myeloid cells by AZD1480 treatment.

Because AZD1480 also inhibits JAK2/STAT3 in tumor cells, we investigated the effect of constitutive STAT3 within tumor cells signaling on the tumor stromal angiogenic environment. We stably transfected 786-O cells with either STAT3C (27) or control vector, challenged the tumor cells into athymic nude mice, and observed the effects of AZD1480 on angiogenesis. Intravital multiphoton laser microscopy was used to visualize tumor vasculature in living mice. As shown in Fig 6E, 786-O xenografts expressing STAT3C showed resistance to AZD1480-induced angiogenesis inhibition compared with vector control. These data indicate that despite the antiangiogenic activity of AZD1480 within the tumor microenvironment, tumor autonomous STAT3 signaling can interact with stroma to promote tumor angiogenesis.

Discussion

Previous work has established the importance of JAK1/2 in STAT3-dependent tumorigenesis, and inhibition by AZD1480 resulted in blockage of tumor growth, although direct inhibitory effects on tumor cells were not evident in vitro in some cell lines (14). Moreover, AZD1480 treatment of myeloma cells resulted in decreased tumor proliferation and the induction of apoptosis, which could be seen in the presence of bone marrow stromal cells (28). Our current work shows the effects of AZD1480 on modulating JAK/STAT3 signaling in the tumor microenvironment and reducing tumor angiogenesis and metastasis.

A complex multidirectional interaction exists between tumor cells, surrounding stroma and the microenvironment at metastatic sites (19, 29). The accumulation of myeloid cells has been shown to create a permissive environment at distant organs for metastasis to occur (25, 29–31). In the premetastatic niche, recruited myeloid cells in concert with endothelial cells and stromal cells produce a milieu of chemokines, growth factors, extracellular matrix proteases, and proteins essential for tumor cell invasion to facilitate metastasis (25). It has been shown that STAT3 promotes

Figure 4. AZD1480 inhibits experimental lung metastasis of mouse syngeneic tumors. A, metastatic nodules were visualized to show the inhibitory effect of AZD1480 on Renca tumor metastasis 21 days after treatment in an experimental lung metastasis model. The number of metastatic nodules were shown as mean ± SEM ($n = 10$). B, Western blotting showing p-STAT3 and STAT3-regulated genes in whole lung lysates at day 21, 2 hours after the last dosing. C, AZD1480 treatment reduces lung myeloid cell infiltration in subcutaneous Renca tumor model. Immunofluorescent staining for CD11b$^+$ cells quantifying numbers of myeloid cells per field in the lungs of subcutaneous Renca tumor–bearing mice after 14 days of treatment with AZD1480 or vehicle (bars, SEM; $n = 4$).
crosstalk within the tumor stroma allowing tumor cells to interact with myeloid and endothelial cells, and STAT3 within myeloid cells then stimulates endothelial cells resulting in tumor growth, migration, and angiogenesis (10), thereby playing an important role in metastatic potential. Our study provides evidence that JAK/STAT3 signaling within the primary tumor microenvironment is critical for myeloid cell infiltration and the formation of tumor vasculature. Furthermore, inhibition of STAT3-mediated myeloid infiltration and angiogenesis with AZD1480 dramatically decreased the formation of metastases. In addition, when a constitutively activated mutant form of STAT3 was introduced into the tumor cells, treating mice with AZD1480 was not able to inhibit tumor angiogenesis. These results support the importance of factors produced by tumor cells in promoting tumor angiogenesis, and suggest that the antiangiogenic effects of AZD1480 are partly mediated by blocking JAK/STAT3 in tumor cells, highlighting a tumor autonomous mode of antiangiogenic activity distinct from that of VEGFR inhibitors. Taken together, blocking JAK/STAT3 activity with AZD1480 may have promise in the treatment of solid malignancies by
inhibiting tumor growth at the primary site and preventing invasion and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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