Antitumor Activity of Targeting Src Kinases in Endothelial and Myeloid Cell Compartments of the Tumor Microenvironment

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Abstract

Purpose: Several Src family kinase (SFK) inhibitors have entered clinical trials based on their direct effects against tumor cells. Here, we characterize the effects of targeting Src kinases on the tumor microenvironment and how these effects influence tumor growth.

Experimental Design: Human cancer cells grown in cell culture or in mice were treated with dasatinib, a small-molecule inhibitor of SFKs. Tumor cell, endothelial cell, and myeloid cell compartments within the tumor microenvironment were analyzed. Primary human endothelial cells and freshly isolated CD11b+/CD11c− myeloid cells from mice were treated with dasatinib in cell culture. Cellular functions and signaling pathways affected by dasatinib were evaluated.

Results: Dasatinib was not cytotoxic in cell culture against the human cancer cell lines investigated here. However, dasatinib administration in human tumor-bearing mice suppressed tumor growth associated with increased tumor cell apoptosis, decreased microvessel density, and reduced intratumoral CD11b+ myeloid cells. Dasatinib directly inhibited motility and other functions of endothelial and myeloid cells, accompanied by the inhibition of phosphorylation of SFKs and downstream signaling. Tumor-infiltrating myeloid cells were identified as the major source of matrix metalloproteinase (MMP)-9 in the tumor microenvironment. Dasatinib treatment reduced MMP-9 levels in the tumor microenvironment through the simultaneous inhibition of recruitment of MMP9+ myeloid cells and MMP-9 gene expression in tumor-infiltrating myeloid cells.

Conclusions: These findings suggest that Src kinase inhibitors such as dasatinib possess a previously unrecognized anticancer mechanism of action by targeting both host-derived endothelial and myeloid cell compartments within the tumor microenvironment. Clin Cancer Res; 16(3): 924–35. ©2010 AACR.

Recent work has indicated that stromal cells within the tumor microenvironment undergo phenotypic and epigenetic changes during tumor initiation, progression, and metastasis (1). The cross-talk between tumor cells and stromal cells, including endothelial cells, immune cells, fibroblasts, and pericytes, leads to enhanced tumor growth, metastasis, and altered response to anticancer therapy (2–4). These findings underscore the importance of developing therapeutics that target both tumor cells and the tumor microenvironment to treat cancer.

Angiogenesis, an important process in the tumor microenvironment and a valuable target for anticancer therapy, involves proliferation, migration, and differentiation of vascular endothelial cells, modification of extracellular matrix, and recruitment of accessory cells (5–10). Multiple proangiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are usually found upregulated within the tumor microenvironment and associated with tumor progression (11). High levels of VEGF or bFGF sustain the activation of their cognate receptors and downstream effectors in endothelial cells, resulting in aberrant endothelial cell functions (7).

It has been well established that tumor-recruited immune cells participate in cancer development and mediate response to anticancer therapy (9, 12–14). Increased tumor infiltration by some immune cell subsets, such as macrophages, myeloid-derived suppressor cells, and neutrophils, correlates with increased angiogenesis and/or poor prognosis (13). Several lines of evidence have also indicated that tumor-infiltrating myeloid cells are important sources of proangiogenic factors such as VEGF, bFGF, and matrix metalloproteinase (MMP)-9 within the tumor microenvironment (15–19).
Src family kinases (SFK) comprise a subset of nonreceptor protein tyrosine kinases that includes c-Src, Fyn, Yes, Lyn, Lck, Hck, Blk, Fgr, and Yrk. It has been shown that Src kinase activity and protein levels are significantly elevated in human cancers (20). Aberrant SFK function promotes tumor growth and metastasis by stimulating tumor cell proliferation, migration, and invasion (21). Besides their roles in tumor cells, numerous studies have suggested that SFKs in the stromal cells can contribute to tumor growth (22–24). For example, SFKs, expressed in endothelial cells, coordinate multiple signaling pathways involved in regulating endothelial cell function (25), making SFKs attractive targets for antiangiogenic therapy.

Dasatinib (BMS-354825) was originally described as a dual kinase inhibitor of SFKs and BCR/ABL and was later found to be a multiple kinase inhibitor of SFKs, BCR/ABL, c-kit, platelet-derived growth factor receptor (PDGFR), and EphA2 (26–29). Dasatinib is also an effective therapeutic for imatinib-resistant chronic myelogenous leukemia (27). Furthermore, dasatinib has exhibited activities against some solid tumors by inducing tumor cell apoptosis, inhibiting tumor cell proliferation, and blocking tumor cell migration and invasion (30–32). Recently, it has been reported that dasatinib inhibits multiple myeloma–derived angiogenesis by inactivating the PDGFRβ and Src signaling pathway (33). However, the in vivo mechanism underlying the action of dasatinib and whether this finding can be applied to solid tumors remain to be determined. In the present study, we characterized the effects of targeting SFKs by dasatinib on distinct cellular compartments in the tumor microenvironment and how these effects influence tumor growth.

**Materials and Methods**

**Animals and drug administration.** Athymic nude mice (NCR– nu/nu) were obtained from Taconic. All animal studies were done in accordance with the regulations of the Animal Resources Center of City of Hope Cancer Center. Mice were treated with dasatinib through oral gavage twice daily (B.I.D.). Control mice were given an equal volume of vehicle solution (propylene glycol/water, 1:1).

**Tumor model.** Nude mice were injected s.c. with 5 × 10⁶ tumor cells. Once tumor became palpable, mice were randomly assigned to two groups and were treated with vehicle solution or dasatinib for indicated days. Tumor volume was measured with a caliper every 3 or 4 d and was calculated according to the formula tumor volume = length × (width)² × 0.5.

**Immunohistology.** Tumor samples were fixed in 10% formalin followed by paraffin embedding, or were embedded in optimum cutting temperature compound (Sakura Finetek) and frozen in liquid nitrogen. Paraffin-embedded tumors were sectioned and processed in COH Pathology Core. Sections were stained with anti-CD31 (1:50), anti-Ki67 (1:200), anti–p-Src (Tyr419; 1:250), anti–Src (1:250), and anti–MMP-9 (1:100) antibodies. To quantify tumor microvessel density, CD31+ vessels were counted manually from 10 randomly selected fields at ×200 magnification by two observers who were blinded to the identity of the samples. The mitotic index of all cells in tumors was quantified by calculating the percentage of Ki67+ area relative to total field area using the Image-J software.

Frozen tumor sections were fixed in 2% paraformaldehyde, were permeabilized, were and stained with anti-CD31 (1:50), anti–F4/80 (1:50), anti–CD11b (1:50), and anti–MMP-9 (1:500) antibodies overnight at 4°C followed by incubation with Alexa Fluor 555– or Alexa Fluor 488–conjugated secondary antibodies (1:200; Invitrogen) for 30 min. After nuclear staining with Hoechst 33342, the slides were then examined under a Zeiss upright LSM510 confocal microscope or a Nikon Eclipse TE2000-U fluorescence microscope. To determine apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done using a DeadEnd Fluorometric TUNEL kit (Promega). The apoptotic index in tumor sections was quantified by calculating the number of apoptotic cells per field using Image-J software.

**Statistical analysis.** All the in vitro experiments were done thrice in duplicates or triplicates. In vivo mouse studies were repeated twice with similar results. The statistical significance of differences between control and drug treated groups was determined by a two-tailed t test. A value of \(P < 0.05\) was considered statistically significant.

Details about cell isolation and culture conditions, reagents and antibodies, cell viability assay, cell apoptosis assay, cell migration assay, in vitro tube formation assay, cell detachment assay, chick aortic ring assay, in vivo Matrigel plug assay, small interfering RNAs (siRNA) and
transfection, immunoblotting and immunoprecipitation, flow cytometry analysis, and real-time quantitative PCR are presented as supplementary information.

Results

Dasatinib inhibits endothelial cells but not tumor cells in culture. We first determined the effect of dasatinib on cell viability in vitro by using an MTS assay. After 48 hours of treatment, dasatinib inhibited VEGF- or bFGF-mediated human umbilical vascular endothelial cell (HUVEC) viability in a dose-dependent manner (Fig. 1A). In contrast, cell viability of two human cancer cell lines, DU145 and Colo205, was minimally affected at the same doses (Fig. 1A).

Next, we evaluated whether the dasatinib-mediated loss of endothelial cell viability was associated with the induction of apoptosis. Prolonged incubation of HUVECs in low serum medium (0.1% fetal bovine serum) induced profound (~60%) cell apoptosis, which could be reversed by VEGF or bFGF. Dasatinib treatment induced HUVEC apoptosis in the presence of VEGF or bFGF in a dose-dependent manner (Supplementary Fig. S1A; Fig. 1B). At 300 nmol/L, a clinically relevant concentration (34, 35), dasatinib treatment for 48 hours inhibited >90% of VEGF-mediated or 50% of bFGF-mediated HUVEC survival. To confirm that dasatinib induced apoptosis, we examined the activation of caspases by immunoblot analyses. Dasatinib treatment increased the cleavage of caspase-3, caspase-7, and poly ADP ribose polymerase, a substrate for caspases.

![Fig. 1] Dasatinib inhibited endothelial cell functions. A, DU145, Colo205, and HUVEC cells were treated with DMSO (control) or dasatinib for 48 h. HUVECs were serum starved and were treated with VEGF (HUVEC+VEGF) or bFGF (HUVEC+bFGF) to stimulate cell growth. Cell viability was measured by MTS assay. Average values in control (DMSO) wells were set as 100% viability. Columns, mean of viability relative to control (n = 3); bars, SEM. SS, serum starvation. B, HUVECs were treated with DMSO or dasatinib for 48 h with or without VEGF. Cell apoptosis was measured by TUNEL assay. Columns, mean of apoptotic cells (n = 3); bars, SEM. CM: complete medium. C, serum-starved HUVECs were treated with DMSO or dasatinib and allowed to migrate toward VEGF or bFGF for 6 h in a modified Boyden chamber assay. Average number of migrated cells in control (DMSO) wells was set as 100% migration. Columns, mean of migration relative to control (n = 4); bars, SEM. D, tube formation was quantified by counting the cord junctions of branches formed by endothelial cells. Average numbers of cord junctions in control wells (VEGF only or bFGF only) were set as 100% tube formation. Columns, mean of tube formation relative to control (n = 3); bars, SEM.
Dasatinib inhibits angiogenesis in vivo and in vitro. Endothelial cell growth supplement or VEGF stimulated vessel sprouting from chick aortic rings embedded in Matrigel. Treatment with 100 nmol/L dasatinib for 72 hours inhibited endothelial cell growth supplement or VEGF induced vessel sprouting (Supplementary Fig. S4A). This inhibitory effect was dose- and time-dependent (data not shown). The effect of dasatinib on in vivo neovascularization was evaluated in a mouse Matrigel assay. The endothelial cell content in the Matrigel plugs was determined by immunostaining for CD31. Plugs containing VEGF and bFGF showed robust infiltration of endothelial cells; however, dasatinib treatment for 7 days led to a significant (P < 0.001) reduction in the number of infiltrating endothelial cells (Supplementary Fig. S4B-C).

Dasatinib inhibits human tumor growth in mouse xenograft models. To test the hypothesis that dasatinib inhibits tumor growth through targeting the tumor microenvironment, Colo205 and DU145 human tumor cells were chosen for in vivo studies because their cell viability is relatively resistant to dasatinib in cell culture. In the Colo205 xenograft mouse model, dasatinib treatment (15 mg/kg, B.I.D.) for 21 days significantly (P < 0.001) inhibited tumor growth by 50% compared with that of vehicle-treated tumors (Fig. 3A). Dasatinib treatment showed no toxicity in these animals, as assessed by body weight loss as well as by histologic evaluation of the mouse livers (data not shown). Western blot analyses of whole tumor lysates revealed that dasatinib potently inhibited the phosphorylation of SFKs but not p38 (Fig. 3B). Immunostaining of tumor sections showed high levels of SFK phosphorylation in the endothelial cells and

for both caspase-3 and caspase-7, as early as 6 hours (Supplementary Fig. S1B). The broad-spectrum caspase inhibitor Z-VAD-FMK, when cotreated with dasatinib, was able to reverse dasatinib-induced apoptosis, indicating that dasatinib-induced HUVEC apoptosis is caspase dependent (Supplementary Fig. S1C).

During dasatinib treatment, we observed a concurrent loss of HUVEC adhesion along with the induction of cell apoptosis. Therefore, we examined the effect of dasatinib on HUVEC adhesion to collagen I in a cell detachment assay. We found that dasatinib interfered with established HUVEC adhesion in a dose- and time-dependent manner (data not shown; Supplementary Fig. S1D). Cotreatment with 40 μmol/L Z-VAD-FMK, which completely reversed dasatinib-induced apoptosis, did not prevent dasatinib-induced cell detachment (Supplementary Fig. S1D). Furthermore, DMSO- and dasatinib-treated HUVECs underwent cell death with the same kinetics when cells were maintained in suspension (Supplementary Fig. S1E). Together, these results suggest that the mechanism of dasatinib-induced apoptosis involves anoikis (36). However, the possibility that dasatinib-induced apoptosis and cell detachment were unrelated cannot be excluded based on these experiments.

The effect of dasatinib on HUVEC migration was measured by a modified Boyden chamber assay. Dasatinib inhibited HUVEC migration in response to VEGF or bFGF with IC50 values between 3 and 10 nmol/L (Fig. 1C). Endothelial cells plated on growth factor–reduced Matrigel formed tubular structures upon stimulation with VEGF or bFGF. Our data indicate that dasatinib blocked the tube formation of endothelial cells with IC50 values between 3 and 10 nmol/L (Supplementary Fig. S2; Fig. 1D).

Dasatinib selectively blocks Src downstream signaling. Dasatinib was originally identified as a potent SFK inhibitor in an in vitro Src kinase assay (26). Autophosphorylation of Tyr419 in c-Src (or equivalent in other SFK) in the kinase domain is required for catalytic activity (21). As expected, dasatinib blocked the VEGF-stimulated (Fig. 2A) or bFGF-stimulated (Supplementary Fig. S3A) phosphorylation of SFKs on Tyr419 with an IC50 of ∼3 nmol/L. Next, we investigated the effect of dasatinib on known substrates of SFKs. In particular, SFKs have been implicated in regulating VEGF- or bFGF-mediated activation of focal adhesion kinase (FAK) and other focal adhesion proteins such as p130CAS and paxillin (21). Activation of the SFKs/FAK/p130CAS/paxillin signaling pathway has been suggested to be involved in the regulation of cell adhesion, survival, proliferation, and migration (21). Our data indicated that dasatinib inhibited VEGF-induced (Fig. 2A) or bFGF-induced (Supplementary Fig. S3A) Src-dependent phosphorylation of FAK (Tyr576/577, Tyr861), p130CAS (Tyr410), and paxillin (Tyr118) but not autophosphorylation of FAK (Tyr397). The IC50 values were in the low nanomole range and were comparable with the IC50 values for the inhibition of SFKs. These findings, along with other reports (reviewed in ref. 21), suggest a critical role of SFKs, downstream to FAK autophosphorylation and other stimuli, in regulating activation of focal adhesion proteins. The reason for increased expression of SFKs and autophosphorylation of FAK on Y397 following dasatinib treatment is not clear, but suggests a compensatory mechanism involving negative feedback regulation by SFK kinase activity.

We next examined the effect of dasatinib on individual family members of SFKs (c-Src, Fyn, and Lyn) in HUVECs. Dasatinib inhibited the phosphorylation of c-Src, Fyn, and Lyn immunoprecipitated from HUVEC lysates at low nanomole concentrations (Supplementary Fig. S3B).

We also investigated other signaling pathways that could be associated with SFKs. VEGF induces VE-cadherin tyrosine phosphorylation in endothelial cells through Src kinases (37). Our data showed that the Tyr658 phosphorylation of VE-cadherin was inhibited by dasatinib at IC50 values comparable with those for inhibition of SFKs (Fig. 2B). Furthermore, dasatinib, at higher concentrations, partially inhibited the VEGF-induced phosphorylation of p38 mitogen-activated protein kinase in HUVECs (Fig. 2C).

Dasatinib inhibits HUVEC migration in response to VEGF or bFGF. Our data indicate that dasatinib blocked the tube formation of endothelial cells with IC50 values between 3 and 10 nmol/L (Supplementary Fig. S2; Fig. 1D).

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in the tumor cells of vehicle-treated tumors but not dasatinib-treated tumors (Fig. 3C). Total SFK protein expression was not inhibited by dasatinib treatment (Fig. 3B-C). Next, we investigated the mitotic and apoptosis index of vehicle- or dasatinib-treated tumors by immunostaining tumor sections with anti-Ki67 and TUNEL assays. Ki67 content was not affected by dasatinib treatment (Fig. 3D). However, dasatinib treatment led to a significant \((P = 0.004)\) increase in tumor cell apoptosis (Fig. 3D). To determine whether the antitumor activity of dasatinib could be the result of angiogenesis inhibition, we performed immunostaining for CD31 and found that dasatinib significantly \((P < 0.001)\) decreased CD31+ tumor microvesel density (Fig. 3D). The quantitative analysis of Ki67, TUNEL, and CD31 staining is summarized in Supplementary Table S1.

The antitumor and antiangiogenic activities of dasatinib were further confirmed in a DU145 xenograft mouse model. At a dose of 25 mg/kg (B.I.D., through oral gavage), dasatinib treatment for 22 days significantly \((P = 0.004)\) inhibited tumor growth, induced tumor cell apoptosis \((P = 0.019)\), and reduced tumor microvessel density \((P = 0.003)\;\text{Supplementary Fig. S5A-B})\). The quantitative analysis of TUNEL and CD31 staining is summarized in Supplementary Table S1. Consistently, dasatinib treatment potently blocked the phosphorylation of SFKs/FAK/p130CAS/paxillin but not p38 in DU145 tumors (Supplementary Fig. S5C).

The established role of tumor-associated myeloid cells in regulating tumor growth prompted us to investigate the effect of dasatinib on these cell populations. We performed immunostaining on tumor sections for F4/80, a macrophage-specific marker, and CD11b, a surface marker expressed by multiple myeloid cell subsets. We found that dasatinib administration significantly \((P < 0.01)\) reduced the tumor infiltration of F4/80+ macrophages or CD11b+ myeloid cells (Supplementary Fig. S6A-B; Fig. 4A). Suppressed recruitment of F4/80+ macrophages and CD11b+ myeloid cells by dasatinib was also confirmed by flow cytometry analyses (Fig. 4B). To verify the inhibition of Src kinases, CD11b+/CD11c− myeloid cells were isolated from

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**Fig. 2.** Dasatinib selectively inhibited Src downstream signaling in HUVECs. A, B and C, serum-starved confluent HUVECs were pretreated with DMSO or dasatinib for 1 h before stimulation with 50 ng/mL VEGF for another 30 min (for SFKs, FAK, p130CAS, paxillin, VE-cadherin, and Stat3) or 10 min (for VEGF receptor 2, Erk1/2, Akt, and p38). Cells were lysed and cell lysate was probed with indicated antibodies. β-Actin was used to show protein loading.
Fig. 3. Dasatinib inhibited human tumor growth in mouse models. Mice bearing Colo205 tumors were treated with vehicle solution or dasatinib (15 mg/kg, B.I.D., through oral gavage) for 21 d. A, tumor volume was shown as mean ± SEM; (\(\ast\), \(P < 0.001; n = 10\)). B, whole tumor lysate was isolated from four vehicle- or dasatinib-treated tumors and immunoblots were probed with indicated antibodies. C, tumor sections were immunostained for p-SFKs (left and middle) and total SFKs (right). Representative images were obtained with a ×40 objective. Regions surrounded by white lines were further amplified to show p-SFKs staining in endothelial cells; scale bars, 20 µm (left and right) and 5 µm (middle). D, tumor sections were immunostained for CD31, TUNEL (green)/Hoechst 33342 (blue), and Ki67. Scale bars, 50 µm.
Fig. 4. Dasatinib directly inhibited tumor-associated myeloid cells. 
A, top, Colo205 tumor sections were immunostained for CD11b (red) or F4/80 (red). Representative images were obtained with a ×20 objective. Scale bars, 50 μm. Bottom, quantification of CD11b+ cells (*, P < 0.01; n = 10) or F4/80+ cells per field (*, P < 0.01; n = 10). Results are shown as mean ± SEM. B, quantification of CD11b+ cells (*P < 0.01, n = 3) or F4/80+ cells (*P < 0.05, n = 3) by flow cytometry analyses. Columns, mean percentage infiltrating cells; bars, SEM. C, CD11b+/CD11c− cells were isolated from the spleens of Colo205 tumor–bearing mice treated with vehicle or dasatinib, and were subjected to Western blot analysis. Cell lysate was probed with indicated antibodies. D, CD11b+/CD11c− cells were isolated from the spleens of tumor-bearing mice before dasatinib treatment, treated with DMSO or dasatinib in vitro, and allowed to migrate toward 33% tumor cell conditioned medium (CM) for 18 h in a modified Boyden Chamber assay. Columns, mean of 1 × 10^3 migrated cells per milliliter medium (*, P < 0.001; n = 8); bars, SEM.
the spleens of tumor-bearing mice with or without dasatinib treatment, and were subjected to Western blot analysis. Dasatinib treatment potently inhibited the phosphorylation of SFKs, FAK, and paxillin but not p38, Erk1/2, Akt, and EphA2 in CD11b+/CD11c− myeloid cells (data not shown; Fig. 4C). The phosphorylation of c-Kit (Y719) or PDGFRβ (Y1021, Y751) in lysates of isolated myeloid cells from either control or drug-treated tumor-bearing mice was not detectable by Western blot analysis (data not shown).

To determine whether myeloid cells were direct targets for dasatinib, we isolated CD11b+/CD11c− myeloid cells from the spleens of tumor-bearing mice before dasatinib treatment. Tumor-driven migration of myeloid cells was evaluated in a modified Boyden Chamber assay. As shown in Fig. 4D and Supplementary Fig. S6C, tumor cell conditioned medium dramatically enhanced CD11b+/CD11c− myeloid cell migration, which was significantly (P < 0.001) inhibited by dasatinib treatment at low nanomole concentrations. Taken together, these results suggest that dasatinib inhibits tumor infiltration of myeloid cells at least partially by directly impairing their migratory capacity.

To delineate the mechanism underlying the migratory defects in dasatinib-treated myeloid cells, we transfected the RAW264.7 macrophage cell line with siRNAs that target c-Src or FAK. Expression of c-Src and FAK proteins were substantially inhibited by siRNA transfection (Fig. 5A). As shown in Fig. 5B, reduced expression of c-Src or FAK significantly (P < 0.01) inhibited tumor cell conditioned medium–stimulated RAW264.7 cell migration. Treatment with 30 nmol/L dasatinib inhibited the migration of control siRNA-transfected RAW264.7 cells to a comparable level; however, dasatinib was not able to further inhibit migration in cells transfected with siRNAs against c-Src or FAK. Consistently, dasatinib blocked the phosphorylation of SFKs in RAW264.7 cells stimulated with tumor cell conditioned medium (Fig. 5C). These results suggest that
dasatinib and the siRNAs against c-Src or FAK act on the same signaling pathway that regulates macrophage migration, indicating that the inhibition of Src and FAK by dasatinib is sufficient to block myeloid cell migration.

**Dasatinib reduces MMP-9 levels in the tumor microenvironment.** Previous studies have shown that the expression of MMP-9 in tumor-infiltrating leukocytes is critical for tumor vasculature and tumor growth (15, 18, 19). Deletion of MMP-9 in myeloid-immune suppressor cells completely abolishes their tumor-promoting ability (16). To assess the effect of dasatinib on tumor MMP-9 levels, we first performed immunohistochemical analysis to identify the intratumoral expression pattern of MMP-9. In Colo205 xenograft tumors, we detected different expression patterns: although most the cells displayed a weak and diffuse cytoplasmic staining of MMP-9, a small fraction of cells showed a stronger and granular staining (Fig. 6A).

To identify the MMP-9–expressing cells, Colo205 tumor sections were double-stained with MMP-9 and CD11b or F4/80. The majority of MMP-9+ cells were F4/80 negative (data not shown). In contrast, almost all the MMP-9+ cells are also CD11b positive (Fig. 6B). Because our results showed that dasatinib inhibited the tumor infiltration of CD11b+ cells, we investigated whether dasatinib influenced intratumoral MMP-9+ cells. Our data showed that dasatinib treatment led to a significant decrease of MMP-9+ cells (P = 0.025) and MMP-9+/CD11b+ cells (P = 0.032) in the tumor microenvironment (Fig. 6A-B).

We performed real-time PCR to investigate whether MMP-9 gene expression was affected by dasatinib in the tumor microenvironment. Primers designed specifically for human or mouse MMP-9 were used to identify MMP-9 mRNA levels in human Colo205 tumor cells or mouse stromal cells. Our results showed that dasatinib administration, which partially reduced MMP-9 mRNA in human tumor cells (~29%), reduced mouse MMP-9 mRNA to a greater extent in both host-derived stromal cells (~53%) and CD11b+/CD11c− tumor-infiltrating myeloid cells isolated from Colo205 xenografts (~48%; Fig. 6C). Taken together, these results suggest that dasatinib reduces MMP-9 levels in the tumor microenvironment through simultaneous inhibition of recruitment of MMP-9–producing tumor-associated myeloid cells as well as their MMP-9 gene expression.

**Discussion**

In this study, we assessed the effects of targeting SFKs by dasatinib on distinct cell populations within the tumor microenvironment and how these effects influence tumor growth. Our studies using in vivo human tumor xenograft mouse models show that SFK inhibition by dasatinib suppresses tumor growth, associated with increased tumor cell apoptosis, decreased microvessel density, and reduced intratumoral myeloid cells. It is notable that the viability of these tumor cell lines in culture is relatively resistant to dasatinib. By contrast, dasatinib displays potent activity against endothelial cell and myeloid cell functions that are essential for supporting tumor cell growth in vivo, suggesting that dasatinib inhibits tumor growth at least in part by directly targeting endothelial and myeloid cell compartments in the tumor microenvironment.

Another study recently reported that dasatinib, by targeting PDGFRβ and SFKs in both tumor cells and tumor-associated endothelial cells, inhibits multiple myeloma tumor growth (33). Although these data support our conclusion on the importance of SFKs in endothelial cells, we detected no expression of PDGFRβ in either HUVECs (data not shown) or the endothelial cell compartment of our tumor models (Supplementary Fig. S7). Furthermore, in our solid tumor models, SFK inhibition was not sufficient to directly induce cytotoxicity in tumor cells (Fig. 1A), which suggests the tumor microenvironment including endothelial cells and myeloid cells is an important target that mediates the anticaner activity of dasatinib in vivo.

Tumor-associated myeloid cells recently gained attention due to their important roles in supporting tumor growth and generating resistance to anticancer therapy. Our finding that dasatinib inhibits intratumoral infiltration of myeloid cells suggests a potential use of dasatinib to target myeloid cells in the tumor microenvironment. Dasatinib directly inhibited tumor cell conditioned medium–driven CD11b+/CD11c− myeloid cell migration, suggesting that the deficiency in myeloid cell migration is a consequence of a functional breakdown in the cell migration machinery rather than a secondary effect due to tumor growth inhibition. We also showed that siRNAs that specifically targeted c-Src or FAK reduced tumor cell conditioned medium–driven RAW264.7 cell migration. These results are consistent with previously published results showing that SFKs, together with their substrates FAK and paxillin, regulate the motility of inflammatory cells (monocytes, macrophages, granulocytes, etc.) and their recruitment to inflammation sites (38–40). Our hypothesis that inhibition of Src and its downstream signaling by dasatinib is sufficient to block myeloid cell migration is supported by data showing that dasatinib selectively inhibited the SFKs/FAK/paxillin signaling pathway in CD11b+/CD11c− myeloid cells, and combination of siRNA against c-Src or FAK and dasatinib treatment did not achieve additional inhibition of RAW264.7 cell migration.

Although our studies focus on the effect of dasatinib on cell motility, we cannot rule out the possibility that targeting SFKs may affect other properties of tumor-associated myeloid cells. A recent publication indicated that dasatinib inhibits cell proliferation of tumor-associated macrophages in vivo, although it is not clear whether this effect is Src dependent (41). Furthermore, it has been reported that decreased phosphorylation of VE-cadherin prevents transendothelial migration of leukocytes (42). It will be of interest to determine whether inhibition of VE-cadherin phosphorylation in endothelial cells by dasatinib contributes to reduced recruitment of myeloid cells.

Numerous studies have suggested an essential role of MMP-9 in myeloid cell–mediated oncogenic events...
The mechanism of action involves increased bioavailability of VEGF, mobilization of bone marrow–derived cells to the peripheral blood, and recruitment of pericytes (43). We identified CD11b+ tumor–associated myeloid cells as the major source of MMP-9 within the tumor microenvironment. Our results further show that targeting SFKs by dasatinib reduces MMP-9 levels within the tumor microenvironment by simultaneous inhibition of

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**Fig. 6.** Dasatinib reduced MMP-9 levels in the tumor microenvironment. A, top, MMP-9 immunostaining in Colo205 xenograft tumors treated with vehicle or dasatinib. Representative images were obtained with a ×40 objective. Regions surrounded by white lines were further amplified to show different MMP-9 expression pattern. Scale bars, 20 μm (left and right) and 5 μm (middle). Bottom left, negative control for MMP-9 staining. Bottom right, quantification of MMP-9+ cells per field (*, \( P = 0.025; n = 10 \)). Columns, mean; bars, SEM. B, top, immunostaining of tumor sections for CD11b (red) and MMP-9 (green). Confocal fluorescence images were obtained with a ×20 objective. Scale bars, 50 μm. Bottom, quantification of CD11b+/MMP-9+ cells per field (*, \( P = 0.032; n = 10 \)). Columns, mean; bars, SEM. C, normalized MMP-9 mRNA expression in human tumor cells, mouse stromal cells, and CD11b+/CD11c– tumor–associated myeloid cells. Columns, mean (\( n = 3 \)); bars, SEM.
tumor recruitment of CD11b+/MMP-9+ myeloid cells and also their MMP-9 gene expression. Previous work has shown that SFKs/FAK signaling regulates integrin-mediated MMP-9 expression in tumor cells and T lymphocytes (44, 45). It is possible that dasatinib treatment inactivates the SFKs/FAK signaling pathway and thereby inhibits MMP-9 gene expression in tumor-infiltrating myeloid cells.

Although the VEGF–VEGF receptor 2 signaling axis is still the most recognized target for antiangiogenic therapy, refractory or progressive resistance to anti-VEGF agents has been widely reported (46, 47). One possible mechanism of action involves induction of other proangiogenic factors such as bFGF that compensate for the lack of VEGF (48). Our results show that dasatinib inhibits the angiogenic potential of endothelial cells in response to a wide variety of proangiogenic stimuli including VEGF, bFGF and endothelial cell growth supplement, which further confirms SFKs as key downstream effectors of multiple angiogenic signaling pathways. These findings provide a rationale for combining dasatinib with anti-VEGF agents to test for potential synergistic antiangiogenic effects. Moreover, the accumulation of myeloid-derived suppressor cells, a subset of myeloid cells defined by the expression of cell surface markers CD11b and Gr1, in tumors has been shown to suppress antitumor immunity and render tumors refractory to anti-VEGF agents (14). We show that dasatinib directly inhibits the tumor-driven migration of CD11b+/CD11c− myeloid cells. It will be of interest to determine whether intratumoral recruitment of myeloid-derived suppressor cells is affected by dasatinib.

Several SFK inhibitors including dasatinib are currently in clinical trials to treat solid tumors based on their direct effects on tumor cells (49). Our results show another important property of dasatinib in that it can target both endothelial and myeloid cell compartments within the tumor microenvironment to inhibit tumor growth. Therefore, combining dasatinib treatment with other anticancer therapeutics may achieve additional clinical benefit.

Disclosure of Potential Conflicts of Interest

F. Lee, employment and collaborative research agreement, Bristol-Myers-Squibb. The other authors disclosed no potential conflicts of interest.

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References

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Antitumor Activity of Targeting Src Kinases in Endothelial and Myeloid Cell Compartments of the Tumor Microenvironment

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