SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment

Bo Huang,1 Zhang Lei,1 Gui-Mei Zhang,1 Dong Li,1 Chuanwang Song,1 Bo Li,1 Yanyan Liu,1 Ye Yuan,1 Jay Unkeless,2 Huabao Xiong,2 and Zuo-Hua Feng1

1Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, The People’s Republic of China; and 2Immunology Institute, Mount Sinai School of Medicine, New York, NY

Despite the evidence for the role of inflammation in cancer initiation, promotion, and progression, the precise mechanism by which the inflammation within tumor is orchestrated by inflammatory cells remains to be determined. Here, we report that tumor-infiltrating mast cells remodel tumor microenvironment and promote tumor growth. Mast cell infiltration and activation in tumors were mainly mediated by tumor-derived stem cell factor (SCF) and its receptor c-Kit on mast cells. Low concentrations of SCF efficiently induced the chemotactic migration of mast cells. Tumor-infiltrating mast cells, activated by higher concentrations of SCF, expressed multiple proinflammatory factors and increased IL-17 expression in tumors. The activity of NF-κB and AP-1 in tumor cells was intensified in the mast cell-remodeled inflammatory microenvironment. SCF-activated mast cells also exacerbated tumor immunosuppression by releasing adenosine and increasing T regulatory cells, which augmented the suppression of T cells and natural killer cells in tumors. These findings emphasize that the remodeling of the tumor microenvironment can actually be initiated by tumor cell–released SCF and suggest that mast cells are not only a participator but also a critical regulator of inflammation and immunosuppression in the tumor microenvironment.

Introduction

Chronic inflammation, a “promoting force” in the tumor microenvironment, has long been known to be commonly braided with the initiation, promotion, and progression of tumorigenesis.1-5 To date, however, it is still incompletely understood how the inflammation in the tumor microenvironment is orchestrated by inflammatory cells. Recently, mast cells were highlighted as not only a major participant but also an important regulator of inflammation,6,7 and their accumulation in tumors has also been well documented,8-13 implying that mast cells may possibly play an important role in orchestrating the inflammation in tumors.

The tumor microenvironment is regarded as a “smoldering” inflammation site in which a lot of cytokines, chemokines, and enzymes mediate the inflammatory process and drive malignant progression.14,15 Among them, TNF-α, IL-6, VEGF, iNOS, Cox-2, and MMP-9 are of particular interest.15,18 Coincidentally, all of them can be produced by mast cells. However, the tumor microenvironment is also characterized by its immunosuppression from immunosurveillance to immunosuppression.19 Mast cells have been found to play a critical role in the suppression of immune reactions.20 They not only produce inhibitory cytokine IL-10,21 but they also are essential for the immune tolerance mediated by regulatory T (Treg) cells.22 Thus, mast cell infiltration into tumor may possibly remodel tumor microenvironment and profoundly influence tumor behavior by participating and regulating inflammatory and immune reactions. However, although some studies have shown that mast cells promote tumor angiogenesis and tumor growth because of their properties as inflammatory cells,23-25 the roles of mast cells in tumor progression have been incompletely understood so far. Several key questions remain unclear, especially how mast cells are recruited into the tumor site and whether they can remodel the tumor microenvironment.

Mast cell migration to the tumor site and the following activation may be the prerequisite for their promoting effect on tumors. In this regard, stem cell factor (SCF) is possibly involved, because SCF triggers the c-Kit signaling pathway for the differentiation, migration, maturation, and survival of mast cells.26 In the present study, we investigated the relation of mast cells and SCF in tumor progression and showed that SCF recruited and activated mast cells, the activated mast cells remodeled the tumor microenvironment by intensifying inflammation and immunosuppression, the tumor cell NF-κB and AP-1 activities were augmented, and the suppression of T cells and natural killer (NK) cells was exacerbated in such remodeled microenvironments. These findings provide a new insight into the role of mast cells in tumors and the relation among inflammation, immunosuppression, and tumor.

Methods

Animals and cell lines

BALB/c and C57BL/6 mice, 6 to 8 weeks old, were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China) and the Center of Experimental Animals of the Chinese Academy of Medical Science (Beijing, China) for studies approved by the Animal Care and Use Committee of Tongji Medical College. Mouse tumor cell lines H22 (hepatocarcinoma), colon-26 (colon cancer), 4T1 (breast cancer), RM1 (prostate cancer), B16 (melanoma), and LLC1 (lung cancer), and human tumor cell lines HepG2 (liver cancer), SNU1 (gastric cancer), Caco2 (colon cancer), A549 (lung cancer), MCF7 (breast cancer), SKOV3 (ovarian cancer), and EC9706 (esophageal...


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cancer) were purchased from the ATCC (Manassas, VA) and the China Center for Type Culture Collection (CCTCC, Wuhan, China) and cultured according to their guidelines.

**Generation of bone marrow–derived mast cells**

Bone marrow cells were harvested from femurs of mice and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, 50 μM 2-ME, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in the presence of 10 ng/mL IL-3 (PeproTech, Rocky Hill, NJ), and the nonadherent cells were passed every 3 days. Four weeks later, the cells were used as mast cells for experiments and referred to as bone marrow–derived mast cells (BMMCs).

**Tumor growth experiment**

BALB/c mice were inoculated with H22 tumor cells by subcutaneous injection of 10^5 cells to the left flank. Twelve days later, the mice (n = 8 per group) with tumors approximately 5 × 5 mm^2 received 10^5 BMMCs by intratumor injection or 5 × 10^5 BMMCs by intravenous injection. When indicated, the mice received intraperitoneal injection of 100 μg of goat anti–mouse SCF neutralizing antibody (IgG; R&D Systems, Minneapolis, MN) or goat IgG isotype control 24 hours and 1 hour before BMMC injection, or they received the intravenous injection of BMMCs mixed with 50 μg rat anti–mouse c-Kit blocking antibody (eBioscience, San Diego, CA) or rat IgG2b isotype control. Tumor growth was monitored by measuring the length (L) and width (W) of tumors. The volume (V) of the tumor was calculated by the formula V = (L × W^2)/2.

**Analysis of the infiltration of mast cells in tumor tissues**

BALB/c mice were inoculated with H22 tumor cells by subcutaneous injection of 10^5 cells. Twelve days later, 5 × 10^5 CFSE-labeled BMMCs, with or without antibodies as above, were injected into tumor-bearing mice through the tail vein. The peripheral tumor tissues were surgically excised from mice 24 hours after the injection, and frozen sections were prepared and analyzed by fluorescence microscopy (200×, Leica DMi6000B, Wetzlar, Germany), using HC Plans objective lens and a Leica DFC300 FX camera. Image acquisition and processing were performed using Leica Application Suite software, version 2.3.4.R2.

**In vitro migration assay**

Fresh H22 tumor tissues were set in the lower chamber, and 6 × 10^4 BMMCs were set in the upper chamber, in triplicate, of a 24-well transwell apparatus (BD Biosciences, San Jose, CA) and incubated at 37°C for 6 hours. Here, we used the uncoated filter, not the filter coated with fibronectin or other ECM proteins as commonly used, to avoid the possibility that some tumor-derived factors might affect mast cell migration by influencing the interaction of mast cells with fibronectin or other ECM proteins. Thus, the efficiency of mast cell migration might be not high, but the result was significant and could be used to evaluate the chemotactic effect of SCF on mast cells. BMMCs migrating into the lower chamber were enumerated under microscopy, and the migrated cell percentage was calculated. SCF (PeproTech) was used as positive control. Anti-SCF neutralizing antibody, anti–c-Kit antibody, and isotype controls (10 μg/mL) were used in the inhibition test.

**Analysis of gene expression by conventional RT-PCR and real-time RT-PCR**

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA) or from tissues homogenized in TRIzol according to the manufacturer’s instructions. The relative quantity of mRNA was determined by reverse transcription–polymerase chain reaction (RT-PCR: 28 cycles; One-step RT-PCR kit, Qiagen, Valencia, CA). The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences are shown in Table S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article).

For real-time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from the NCBI database. The primers were designed with the Oligo Primer Analysis 4.0 software, and the sequences were blasted (http://www.ncbi.nlm.nih.gov/BLAST/). The primer sequences are shown in Table S2. Total RNA (100 ng) was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μL. Then, 2 μL of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. For sample analysis, the threshold was set based on the exponential phase of products, and the CT value for samples was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against the housekeeping gene GAPDH.

**Western blot analysis**

Cell lysates or tumor tissue homogenates (30 μg of total protein) and precast molecular weight markers were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by the transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% Triton X-100) containing 5% nonfat milk and probed with anti–mouse or –human SCF antibodies (R&D Systems). After incubation with the secondary antibody conjugated with horseradish peroxidase, membranes were extensively washed, and the immunoreactivity was visualized by enhanced chemiluminescence according to the manufacturer’s protocol (ECL kit; Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA).

**Assay of soluble SCF and IFN-γ by enzyme-linked immunoabsorbent assay**

For the assay of SCF, tumor cells were cultured with or without BMMCs for 24 hours, or 400 mg of tumor tissue was cut into small pieces and cultured in 24-well plate in 1 mL RPMI 1640 medium for 24 hours. The soluble SCF in the supernatants was assessed by Mouse SCF Quantikine ELISA (enzyme-linked immunosorbent assay) Kit (R&D Systems) according to the manufacturer’s protocol.

For the analysis of the effect of MMP-9 inhibitor on the production of soluble SCF, MMP-9 inhibitor 2-[(4-biphenylsulfonyl) amino]-3-phenylpropionic acid; Calbiochem, La Jolla, CA) was injected into mice (1 mg/kg, intraperitoneally) 30 minutes before mast cell injection. The control mice received an equal amount of vehicle (1% DMSO). In the in vitro experiments, the inhibitor was added to the culture medium to a final concentration of 0.2 mM.

For the assay of IFN-γ, NK cells were cultured in the presence of IL-2 (50 U/mL) for 24 hours, and IFN-γ in the supernatant was measured by the mouse IFN-γ ELISA Kit (R&D Systems).

**Construction of H22 tumor cell line expressing SCF-siRNA**

SCF sense and antisense siRNAs were generated using the Silencer siRNA construction kit according to the manufacturer’s instruction (Ambion, Austin, TX). After hybridization and purification, the different double-stranded SCF-siRNAs and control siRNAs were transiently transfected into H22 tumor cells using GeneSilencer siRNA transfection reagent (Gene Therapy Systems, San Diego, CA). The most efficient SCF-siRNA sequence (cagcagtcgctcagagg) and its control siRNA (ctgctagctcaga-gg) were verified by RT-PCR detection of SCF mRNA 24 hours after transfection and inserted into pSIREN-RetroQ–expressing vector with the U6 promoter (Clontech, Mountain View, CA). The recombinant SCF siRNA-expressing plasmids and control plasmids were transfected into H22 tumor cell using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) for stable expression after selection.

**MMP-9 assay by gelatin zymography**

Active MMP-9 mediates the shedding of membrane-associated SCF. To evaluate the influence of mast cell–derived MMP-9 on the release of SCF, the production of MMP-9 by mast cells was analyzed by gelatin zymography as described previously. Briefly, proteins prepared from mast cells were separated by 7.5% SDS-PAGE containing 1% gelatin. The gels were
incubated in MMP activation buffer containing 50 mM Tris (pH 8.0) and 10 mM CaCl2 at 37°C overnight, and then stained with 1% Coomassie Brilliant Blue R-250 for 3 hours and destained in 10% (vol/vol) methanol and 5% (vol/vol) acetic acid.

**Clinical tumor specimens and murine tumor specimens**

Clinical tumor specimens were acquired by surgery from untreated cancer patients, which was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained in accordance with the Declaration of Helsinki from all subjects. Seven types of tumor specimens were collected, including breast cancer, gastric cancer, colon cancer, lung cancer, ovarian cancer, liver cancer, and esophageal cancer. For each type of tumor, specimens were acquired from 4 to 5 patients.

To obtain different murine tumor specimens, mice were inoculated with tumor cells by subcutaneous injection of 106 cells to the left flank. BALB/c mice were inoculated with H22 cells, colon-26 cells, or 4T1 cells, respectively. C57BL/6 mice were inoculated with RM1 cells, B16 cells, or LLC1 cells, respectively. Tumors were surgically dissected for the experiments when tumor size reached approximately 5 × 5 mm2.

**Flow cytometric analysis**

Mouse tumor cells or human tumor cells were incubated with biotin-conjugated goat anti–mouse SCF or goat anti–human SCF and the corresponding isotype control IgG for 30 minutes at 4°C. After washing, the cells were further incubated with PE-conjugated streptavidin for flow cytometric analysis. All antibodies were purchased from R&D Systems. Variables were acquired on a fluorescence-activated cell sorting Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

For IL-17 intracellular staining, tumor-infiltrating immune cells were isolated from tumor tissue,59 and then fixed and permeabilized with Fix/Perm solution (BD Biosciences). The cells were then resuspended in Perm buffer and incubated with FITC-labeled anti-IL-17 antibody or isotype control (ebioscience) at room temperature in the dark for 20 minutes.

For intracellular staining of Foxp3, tumor-infiltrating immune cells were stained with PE-cy3-conjugated anti–mouse CD3. After cellular surface staining, the cells were treated with Fix/Perm solution and restained with FITC-labeled anti–mouse Foxp3 antibody or isotype control (ebioscience).

**Assay of the activities of NF-κB and AP-1**

BMCCs (5 × 106), with or without anti–c-Kit antibody as indicated, were injected by the tail vein into mice (n = 5 per group) bearing WT H22 tumor or SCF-knockdown H22 tumor. The mice were killed 72 hours later, and tumor cells were isolated from peripheral tumor tissues.65 The nuclear extract was prepared with Nuclear Extraction Kit (Millipore, Billerica, MA). The activities of NF-κB and AP-1 in nuclear extract were determined by NF-κB Assay kit and AP-1 Assay kit (Millipore), respectively, according to the manufacturer’s protocol.

**Isolation of T cells and NK cells**

Tumor tissues from different groups were digested with collagenase and hyaluronidase for 1 hour at 37°C and minced. After lysis of red blood cells, the dissociated cells were underlaid with 5 mL of Lymphocyte-M solution and centrifuged at 11 000 g for 20 minutes. Tumor-infiltrating lymphocytes were harvested from the interface. T cells were isolated on a T-cell enrichment column (R&D Systems), and NK cells were purified by magnetic sorting with biotinylated-DX5 antibody. T cells and NK cells were also isolated from spleen of normal mice and used in the related experiments.

**T-cell proliferation assay**

The isolated T cells (104) were cultured in the presence or absence of anti-CD3 and anti-CD28 antibodies (1 μg/mL each) in 96-well culture plates. [3H]-thymidine was added during the last 10 hours of the 72-hour culture, and then the incorporation of [3H]-thymidine was measured to determine T-cell proliferation. The results were expressed as stimulation index (cpm of stimulated cells/cpm of unstimulated cells).

**Assay of adenosine**

The peripheral tumor tissues were frozen and powdered in liquid nitrogen. The tissue powder (1 mg) was mixed with 1 mL of 0.6 M perchloric acid by vigorous vortexing and sonicated with a microsonicator for 10 seconds at 45 W. Then, the mixture was centrifuged at 11 000g for 20 minutes at 4°C. The supernatant was neutralized with 5 M K2CO3 to pH 7.2, centrifuged at 11 000g to remove precipitates, and stored at −80°C until high-performance liquid chromatography (HPLC) analysis. HPLC analysis of adenosine was performed as previously described.66

For the assay of adenosine in the culture supernatant, 1 mL of supernatant was mixed with 30 μL of perchloric acid (4.4 M), and centrifuged at 16 000g for 10 minutes at 4°C. The supernatant was neutralized to pH 7.2 and analyzed by HPLC.

**Statistics**

Results were expressed as mean values plus or minus SD and interpreted by repeated-measure ANOVA. Differences were considered to be statistically significant when the P value was less than .05.

**Results**

**Mast cells promote tumor growth on the basis of SCF/c-Kit–mediated migration into tumor**

To verify whether the chemotactic migration of mast cells into tumor is the prerequisite for their tumor-promoting activity, we first investigated whether SCF is the main chemokine mediating the migration of mast cells into tumor. The result from transwell assay showed that the migration of BMCCs was induced by the cultured tumor tissues, and the migration was significantly inhibited by SCF neutralizing antibody and c-Kit–blocking antibody (Figure 1A). When CFSE-labeled BMCCs were injected into mice bearing H22 hepatocarcinoma through the tail vein, both anti-SCF and anti–c-Kit antibodies effectively impaired the infiltration of BMCCs into the tumor (Figure 1B), indicating that the migration is mainly mediated by SCF released from tumor tissues. We then injected BMCCs into tumor-bearing mice to analyze the effect of mast cells on tumor growth. The intravenous injection of BMCCs markedly accelerated the growth of the tumor (Figure 1C) and shortened the survival of mice (Figure 1D), both anti-SCF and anti–c-Kit antibodies abolished the tumor-promoting effect of the intravenously injected BMCCs (Figure 1E). These data suggest that SCF/c-Kit axis–triggered mast cell migration into tumor is the prerequisite for their tumor-promoting activity.

**Tumor cell–derived SCF is responsible for the infiltration of mast cells into the tumor**

Next, we tested whether SCF is mainly produced by tumor cells. By detecting the expression of SCF, we found that the cultured H22 cells expressed both mRNA and protein of SCF, whereas SCF was not detectable in the culture supernatants (Figure 2A), suggesting that SCF is a membrane-associated protein on tumor cells and might be released in tumor tissue. To confirm this, we additionally detected SCF expression in another 5 murine tumor cell lines, 7 human tumor cell lines, and the corresponding mouse and human tumor tissues. SCF expression was very high in tumor cells and tumor tissues, but very low in the adjacent tissues around the tumor (Figure 2B). All of these tumor cells expressed membrane-associated SCF (Figure 2C), whereas SCF
was only detectable in the supernatants of the cultured tumor tissues but not in the supernatants of either tumor cells or adjacent tissues around the tumor (Figure 2D). Thus, we constructed a H22 tumor cell line stably expressing SCF siRNA (Figure 2E). Both SCF expression and SCF release were strikingly decreased in siRNA-expressing tumors (Figure 2E), indicating that the soluble SCF in tumor tissue is mainly released from tumor cells. More importantly, SCF-knockdown tumors could not efficiently attract BMMCs either in vitro or in vivo (Figure 2F), suggesting that mast cell infiltration into the tumor is mainly induced by tumor cell–produced SCF.

Activation of mast cells by SCF is necessary for their tumor-promoting effect

We then investigated the role of SCF in mast cell–mediated tumor promotion. Compared with WT tumors, the inoculation of SCF-knockdown tumor cells generated a much retarded tumor growth. The SCF-knockdown tumor on day 40 after inoculation reached similar size as that of WT tumor on day 25 (Figure 3A), indicating that SCF and mast cells are crucial for tumor development. As expected, the intravenous injection of BMMCs had no effect on the growth of SCF-knockdown tumors (Figure 3B). Unexpectedly, the intratumor injection of BMMCs, untreated or pretreated with low concentration of SCF (0.2 ng/mL or 10 ng/mL), to the SCF-knockdown tumor did not promote tumor growth either (Figure 3B), whereas the injection of BMMCs pretreated with higher concentration of SCF (200 ng/mL) promoted the growth of the tumor (Figure 3C). This finding implies that SCF not only mediates the chemotactic migration of mast cells into tumor but it also activates, at higher concentration, mast cells to generate the tumor-promoting effect.

SCF-stimulated mast cells augment the release of SCF from H22 tumor cells

Active MMP-9 can release membrane-bound SCF.33 Coincidently, mast cells have been found to produce MMP-9 for their migration.37 Therefore, we wondered whether the SCF/c-Kit signal can regulate the expression of MMP-9 in mast cells, therefore promoting the release of SCF from tumor cells. The cultured BMMCs expressed a low level of a latent form of MMP-9. However, as low as 2 ng/mL SCF could stimulate BMMCs to produce more latent MMP-9 and active MMP-9 (Figure 4A). SCF-promoted production of MMP-9 by mast cells could be inhibited by anti–c-Kit antibody (Figure 4A), suggesting that SCF/c-Kit signal regulates the production and activation of MMP-9 in mast cells. In addition, SCF-treated BMMCs significantly increased the concentration of SCF in the supernatant of H22 cells, whereas this effect was inhibited by MMP-9 inhibitor. Moreover, the release of SCF was not increased if BMMCs were pretreated with SCF in the presence of anti-c-Kit (Figure 4B). Under each of the above conditions, SCF mRNA levels were not significantly influenced (Figure 4B). Consistent with the in vitro data, a much higher level of SCF was detected...
in tumor tissue after intratumor injection or intravenous injection of mast cells (Figure 4C), but SCF mRNA levels were not changed (Figure 4C). In these situations, the increased release of SCF was also suppressed by MMP-9 inhibitor and anti–c-Kit (Figure 4C). Taken together, these data suggest that SCF/c-Kit signal can stimulate mast cells to produce active MMP-9, and the latter then increases the level of soluble SCF in tumor by promoting the release of SCF from tumor cells, thereby favoring the activation of mast cells in tumor.

SCF-activated mast cells remodel tumor inflammatory microenvironment

The activated mast cells can release a large number of proinflammatory factors. Here, we found that the expressions of proinflammatory genes encoding IL-6, TNF-α, VEGF, Cox-2, iNOS, and CCL2 were up-regulated in BMIMCs in the presence of SCF at high concentration (200 ng/mL) but not low concentration (5 ng/mL; Figure 5A). This up-regulation could be abolished by
anti–c-Kit antibody (Figure 5A), indicating that SCF/c-Kit signal activates mast cells. Consistent with in vitro data, the intravenous injection of BMMCs resulted in the significant increase of mRNA levels of the above proinflammatory genes in WT tumor, which was abolished by anti–c-Kit antibody (Figure 5B). The intravenous injection of BMMCs into SCF-knockdown tumor did not significantly change the mRNA levels of these genes in the tumor (Figure 5B). More importantly, the intravenous injection of BMMCs also increased the transcription of IL-17 gene and the amount of IL-17–producing cells in the WT tumor.

Figure 3. Activation of mast cells by SCF is necessary for their tumor-promoting effect. (A) SCF-knockdown retards tumor growth. Mice (n = 8 per group) were inoculated with SCF-knockdown H22 cells and control WT H22 cells, respectively. The growth of tumor was monitored. (B,C) Mice (n = 8 per group) were inoculated with SCF-knockdown H22 tumor cells. When tumor size reached approximately 5 × 5 mm², the mice received BMMCs either by intravenous (i.v.) injection or by intratumor (i.t.) injection (B), or received the intratumor injection of BMMCs pretreated with different concentrations of SCF and anti–c-kit antibody (20 μg/mL) as indicated (C). The growth of the tumor was promoted only by the intratumor injection of MCs pretreated with a higher concentration of SCF (200 ng/mL), which was abolished by anti–c-kit antibody. Error bars represent SD.

Figure 4. SCF-stimulated mast cells augment the release of SCF from tumor cells. (A) SCF stimulates the production of active MMP-9 by mast cells (MCs). BMMCs were cultured for 24 hours in the presence of different concentrations of SCF and anti–c-Kit (10 ng/mL). The production of MMP-9 was detected by RT-PCR and gelatin zymography. (B) MC-derived MMP-9 increased the release of SCF from H22 tumor cells. BMMCs were treated with 5 ng/mL of SCF for 4 hours in the absence or presence of 10 μg/mL anti-c-Kit antibody. H22 cells and SCF-treated BMMCs were cultured alone or in 2 chambers separated by semipermeable membrane. SCF in the supernatants was detected by ELISA (left). SCF mRNA was detected by real-time RT-PCR (right). *P < .05, compared with the H22/MC group. (C) Assay of SCF in tumor tissues. Tumor-bearing mice received the intraperitoneal injection of MMP-9 inhibitor and the intratumor (i.t.) injection of MCs, or received the intravenous (i.v.) injection of MCs with anti–c-Kit antibody. The tumor tissues were excised 48 hours after MC injection and cultured in vitro. SCF in the supernatants was detected by ELISA (left). The mRNA level of SCF in tumor tissues was detected by real-time RT-PCR (right). *P < .05, compared with the MC injection groups. Error bars represent SD.
tumor but not in the SCF-knockdown tumor (Figure 5C,D). These data suggest that SCF-activated mast cells remodel tumor inflammatory microenvironment. Because the activities of NF-κB and AP-1, 2 key transcription factors, in tumor cells can be increased by the proinflammatory factors such as TNF-α and others,38 we then further analyzed the influence of the mast cell–remodeled environment on tumor cells by detecting the activities of NF-κB and AP-1 in tumor cells. The result showed that the activities of p65/p50 and c-Jun/c-Fos were significantly increased in the tumor cells isolated from the peripheral tumor tissue after mast cell injection (Figure 5E). Anti–c-Kit antibody hindered the increase of such activities, and the injection of BMMCs into SCF-knockdown tumor did not significantly alter the activities of NF-κB and AP-1 (Figure 5E).

SCF-activated mast cells remodel tumor microenvironment by intensifying immunosuppression

To investigate whether SCF-activated mast cells contribute to tumor immune suppression, we analyzed the expressions of immune-associated genes in tumors. Among them, IL-2 is related to immune activation; IL-10, TGF-β, and Foxp3 are related to immune suppression. After intravenous injection of mast cells into mice, IL-2 mRNA in the tumor was decreased, and the levels of IL-10, TGF-β, and Foxp3 mRNAs were increased (Figure 6A). Consistent with the increased expression of TGF-β and Foxp3, the percentage of Treg cells in total T cells in the tumor was also increased (Figure 6B). In line with this, the intravenous injection of mast cells intensified the suppression of T lymphocytes and NK cells, 2 major types of cytotoxic effector cells, in tumor microenvironment, evaluated by the further decreased response of T cells to the stimulation of anti-CD3 and anti-CD28 antibodies (Figure 6C) and the reduced production of IFN-γ by NK cells (Figure 6D). The administration of anti–c-Kit antibody suppressed the effect of mast cell injection on the gene expression pattern and the activities of T cells and NK cells in the tumor microenvironment (Figure 6A,C,D). However, the injection of mast cells into SCF-knockdown tumors produced a much less effect on the gene expression pattern and the activities of T cells and NK cells (Figure 6A,C,D). Taken together, these data indicate that SCF-activated mast cells can exacerbate the immunosuppression in the tumor microenvironment.

SCF-activated mast cells release adenosine in the tumor microenvironment

The cultured BMMCs may release adenosine after stimulation.36 Recently, adenosine has been reported to inhibit the production of IL-2 and IFN-γ by T cells.39,40 Thus, SCF-activated mast cells might release adenosine to directly suppress T cells and NK cells. To test this, we first detected the release of adenosine by BMMCs in the presence of SCF. SCF could stimulate BMMCs to release adenosine in a dose-dependent manner (Figure 7A). However, the
intravenous injection of mast cells into mice bearing WT tumor increased the level of adenosine in tumor, which was suppressed by anti–c-Kit antibody, whereas the intratumor injection of mast cells did not increase the level of adenosine in the SCF-knockdown tumor (Figure 7B). We next investigated the effect of mast cell–released adenosine on T-cell proliferation driven by anti-CD3 and anti-CD28 antibodies and the production of IFN-γ by NK cells in response to IL-2 stimulation. Both T-cell proliferation and the production of IFN-γ by NK cells were inhibited in the presence of the supernatants of mast cells stimulated with 100 ng/mL of SCF (Figure 7C). The inhibitory effect was significantly relieved by adenosine receptor A2A antagonist SCH-58261 (Sigma-Aldrich, St Louis, MO; Figure 7C). We then used SCH-58261 to interfere with adenosine signaling pathway in vivo. The results showed that SCH-58261 not only relieved the suppression of T cells and NK cells after the injection of mast cells, but it also augmented the activities of T cells and NK cells in control tumor (Figure 7D).

Discussion

Our present findings strongly suggest that mast cells infiltrating into tumor produce a protumor effect by remodeling tumor microenvironment. Given that the expression of SCF was found in all the tumor cell lines or tumor tissues of different tumor types tested in this study, such remodeling process may be general in most types of tumors.

During the development of tumor, circulating mast cells migrate into tumor and form one of the major stromal cell populations. Although various factors may induce the chemotactic migration of mast cells, our data in this report suggest that SCF/c-Kit signal is mainly responsible for the migration of mast cells into tumor, because mast cells failed to migrate into SCF-knockdown tumor, and anti–c-Kit antibody abolished the migration of mast cells into WT tumor. Except for mast cell migration and adhesion to extracellular matrix components, the SCF/c-Kit signal is also critical for the survival and functional activation of mast cells and the secretion of mediators from mast cells. Importantly, our data show that SCF acts on mast cells in a dose-dependent manner. Low dose of SCF induces both the chemotactic migration of mast cells and the production of active MMP-9 by mast cells, whereas higher concentration of SCF is required for the activation and mediator-secretion of mast cells. The accumulation and activation of mast cells in tumor can be intensified by a vicious feedback circle between mast cells and SCF. SCF-stimulated mast cells produce MMP-9 which not only facilitates the migration of mast cells into tumor but also augments the release of SCF from tumor cells. The increased release of SCF is in favor of both the accumulation and further activation of mast cells.

Inflammation is a fundamental character of the tumor microenvironment. Numerous proinflammatory factors existent in the tumor microenvironment that influence the growth and metastasis of tumors. Many of them are produced by mast cells. Mast cells were thought to be detrimental to tumor cells by...
releasing IL-4, TNF-α, and others to induce the apoptosis of tumor cells. However, much evidence obtained in recent years indicates that these inflammatory cytokines are beneficial to tumors. In addition, other proinflammatory factors and inflammation-related enzymes also benefit tumor development. Our previous studies have shown that IL-6, iNOS, and CCL2 have the protumor effects through different mechanisms.

The increased expression of these genes is obviously beneficial to tumor development, the established tumors primarily induce immune tolerance, resulting in the shift of the immune balance from activation to tolerance induction. An absolute immune suppression is usually generated in the tumor microenvironment at the later stage of tumor. Our data in this report indicate that mast cells play important roles in such immune balance shifting. The infiltration of mast cells into the tumor significantly increased the expressions of TGF-β, IL-10, and Foxp3 in the tumor. Consistently, Treg cells in the tumor microenvironment was also increased, because CD4+CD25+ T cells can be converted into CD4+CD25+ regulatory T cells by TGF-β-induced expression of transcription factor Foxp3. Both the infiltration of mast cells and the increase of Treg cells can explain the increased expression of IL-10, a cytokine-mediating complex immunosuppressive effect. Given that Treg cells release IL-9 to activate mast cells for immune suppression, here we propose a novel partnership between mast cells and Treg cells in the tumor microenvironment: they activate each other and cooperate to suppress immune responses.

Adenosine release from the stimulated mast cells was found 23 years ago. However, its connection with tumor progression has not yet been elucidated. Our data show that mast cells augment the release of SCF by tumor cells, and then the increased SCF in turn stimulates the release of adenosine by mast cells. Adenosine not only inhibits the production of IFN-γ and IL-2 by CD4+ T cells but also suppresses the migration of mast cells. The latter may partly explain the accumulation of mast cells around the vasculature in the peripheral region of tumor tissue. Thus, mast cells localizing around tumor vasculature may form the first layer of barrier, where the tumor-infiltrating T cells initially accept the immunosuppressive signal, adenosine, leading to the attenuation of immune attack on tumor cells.
In summary, the findings in this report disclose the complex relation between mast cells, tumor cells, and other immune cells in the tumor microenvironment. The remodeling of the tumor microenvironment can actually be initiated by tumor cell-released SCF. The following events include the mutual influence between mast cells and tumor cells, mast cells and other immune cells. Therefore, the SCF/Kit pathway is not only very important for the remodeling of tumor microenvironment but also a very important target for tumor therapy.

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References


Authorship

Contribution: B.H. designed and conducted research, analyzed data, and wrote the paper; Z.L., G.-M.Z., D.L., C.S., B.L., Y.L., and Y.Y. conducted research and analyzed the data; J.U., H.X., and Z.-H.F. designed the research, analyzed the data, and wrote the paper. All authors have reviewed and contributed to the manuscript.

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Correspondence: Zuo-Hua Feng or Bo Huang, Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, The People’s Republic of China; e-mail: fengzhg@public.wh.hb.cn or tjhuangbo@hotmail.com.


SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment

Bo Huang, Zhang Lei, Gui-Mei Zhang, Dong Li, Chuanwang Song, Bo Li, Yanyan Liu, Ye Yuan, Jay Unkeless, Huabao Xiong and Zuo-Hua Feng