GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Inhibition of Mast Cell-Derived Histamine Decreases Human Cholangiocarcinoma Growth and Differentiation via c-Kit/Stem Cell Factor—Dependent Signaling

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Accepted for publication
September 4, 2015.

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The tumor microenvironment of cholangiocarcinoma (CCA) is composed of numerous cells, including mast cells (MCs). MCs release histamine, which increases CCA progression and angiogenesis. Cholangiocytes secrete stem cell factor, which functions via the MC growth factor receptor c-Kit. Here, we show that cholangiocytes express histidine decarboxylase and its inhibition reduces CCA growth. MC recruitment in the tumor microenvironment increased CCA growth. MC infiltration and MC markers were detected by toluidine blue staining and real-time PCR in human biopsies and in tumors from athymic mice treated with saline, histamine, histidine decarboxylase inhibitor, or cromolyn sodium. Tumor growth, angiogenesis, and epithelial-mesenchymal transition (EMT)/extracellular matrix (ECM) markers were measured in mice treated with cromolyn sodium. *In vitro*, human CCA cells were treated with MC supernatant fluids before evaluating angiogenesis and EMT/ECM expression. Migration assays were performed with CCA cells treated with the stem cell factor inhibitor. MC supernatant fluids increased CCA histidine decarboxylase, vascular endothelial growth factor, and MC/EMT/ECM expression that decreased with pretreatment of cromolyn sodium. MCs were found in human biopsies. In mice treated with cromolyn sodium, MC infiltration and tumor growth decreased. Inhibition of CCA stem cell factor blocked MC migration and MC/EMT/ECM in CCA. MCs migrate into CCA tumor microenvironment via c-Kit/stem cell factor and increase tumor progression, angiogenesis, EMT switch, and ECM degradation. (*Am J Pathol* 2016, 186: 123–133; <http://dx.doi.org/10.1016/j.ajpath.2015.09.016>)

Cholangiocarcinoma (CCA) cancers are primary tumors that arise from the neoplastic transformation of cholangiocytes, the epithelial cells lining the intrahepatic and extrahepatic bile ducts of the liver.¹ CCA is the second most prevalent liver tumor after hepatocellular carcinoma and accounts for 10% to 20% of deaths caused by hepatobiliary malignancies.² Increased risk of developing CCA is associated with primary sclerosing cholangitis, liver fluke infestation, hepatitis C virus infection, and other diseases that lead to chronic biliary obstruction and inflammation.³ CCA is a metastatic cancer, and studies have found its potential to migrate outside of the biliary tract.⁴ We have found that histamine via the H4 histamine receptor (HR)

Supported in part by the Department of Veteran's Affairs, Biomedical Laboratory Research and Development Service career development award IK2 BX001760 (H.F.) and merit award 5I01BX000574 (G.A.), the PSC Partners Seeking a Cure (H.F. and S.D.), a Scott and White Research Mentor Award [H.F. (mentor) and C.J. (mentee)], the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Scott & White Healthcare (G.A.), the Texas Chapter of the Ladies Auxiliary to the Veterans of Foreign Wars (H.F. and G.A.), and the American Cancer Society grant RSC118760 (S.D.); in part by the Veterans Health Administration.

The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States government.

C.J. and V.H. contributed equally to this work.

Disclosures: None declared.

promotes epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) breakdown and that globally blocking histamine synthesis decreases tumor growth.^{4,5} Because of its clinically silent nature, CCA is typically diagnosed in advanced stages, when chemotherapy is often ineffective and radical surgery may be contraindicated.^{2,3} Thus, it is important to elucidate the mechanisms of CCA progression to provide alternative therapeutic targets and to allow for earlier detection.

The tumor microenvironment of CCA consists of numerous cells, including mast cells.⁶ These cells contribute to CCA pathogenesis by releasing mediators that support tumor growth and suppress immune responses.^{6,7} Although commonly associated with its role in mediating allergic response, mast cells play an important role in other disorders, although the exact mechanism by which they act is unknown.⁸ The role of mast cells in the tumor microenvironment is typically detrimental to the patient.⁹ On infiltration, mast cells release large amounts of inflammatory mediators into the tumor microenvironment, including histamine, ILs, and growth factors.^{9,10} These factors all contribute to the progression of tumor growth. Mast cells express numerous cellular ligands on their surface, including the stem cell factor (SCF) receptor, c-Kit that binds with SCF on other cell types.^{11,12} CCA cells secrete SCF⁵; thus, there is the potential for mast cells to be drawn to the tumor microenvironment via this interaction.

We have shown that histamine, via an autocrine pathway, increases CCA growth by increasing vascular endothelial growth factor (VEGF) expression and that blocking histamine with the use of an inhibitor for histidine decarboxylase (HDC) can substantially diminish tumor growth.⁵ However, no studies have been performed to determine the role that mast cells or mast cell-derived histamine might play in CCA tumor growth, the EMT switch, or ECM breakdown. Thus, we aimed to determine whether mast cells were present in the tumor microenvironment of CCA and whether the recruitment of mast cells into the tumor microenvironment via SCF/c-Kit interaction increases CCA growth, EMT, and ECM breakdown.

Materials and Methods

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Antibodies for immunoblots, immunofluorescence, and immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise stated. Cell culture reagents and media were obtained from Invitrogen Corporation (Carlsbad, CA). Human (for cell lines studies) and mouse (for tumor evaluation) sequenced primers and real-time PCR assay materials were obtained from SABiosciences (Frederick, MD). The following primers were used: Vimentin, Human-PPH00417F-200/Mouse-PPM04780B-200; Paxillin, Human-PPH02786A-200/Mouse-PPM25951B-200; s100A4, Human-PPH01313E-200/Mouse-PPM03811A-200; matrix metalloproteinase (MMP)2, Human-PPH00912B-200; MMP3, Human-PPH00235F-200;

MMP9, Human-PPH00152E-200; proliferating cell nuclear antigen (PCNA), Human-PPH00216B-200/Mouse-PPM03456F-200; VEGF-A, Human-PPH00251C-200/Mouse-PPM03041F-200; VEGF-C, Human-PPH00673D-200/Mouse-PPM03061F-200; HDC, Human-PPH07080F-200/Mouse-PPM04804A-200; H1HR, Mouse-PPM04806B-200; H2HR, Mouse-PPM04805A-200; H3HR, Mouse-PPM04893A-200; H4HR, Mouse-PPM04894A-200; E-cadherin, Human-PPH00135F-200/Mouse-PPM03652F-200; C-kit, Human-PPH00432A-200/Mouse-PPM05195A-200; Chymase, Human-PPH05676A-200/Mouse-PPM24635B-200; and Tryptase, Human-PPH19168B-200 (α/β 1)/Mouse-PPM24954A-200 (α/β 1). Both the iScript cDNA Synthesis Kit (catalog no. 1708891) and the iTaq Universal SYBR-Green Supermix (catalog no. 1725124) were obtained from Bio-Rad/Life Science Research Division (Hercules, CA).

In Vivo Studies

Tumor Model

We used tumors from xenograft mouse models to evaluate the presence of mast cells and expression of mast cell markers.^{5,13} Briefly, mice were injected with cells from an extrahepatic biliary cancer cell line (Mz-ChA-1) and, after tumor establishment, were treated by i.p. injections of 0.9% NaCl (vehicle), histamine (0.5 mg/kg in 100 μ L of 0.9% NaCl), or α -methyl-DL-histidine (HDC inhibitor; 150 mg/kg in 100 μ L of 0.9% NaCl).⁵ Tumors were collected after 52 days.⁵ In separate studies, we performed experiments to determine the effects of specifically blocking mast cell-derived histamine on tumor growth. Again, with the use of xenograft tumor models, we treated mice with 0.9% NaCl (saline) or cromolyn sodium (24 mg/kg body weight) for 38 days by i.p. injection three times per week. Tumor growth (length $\times$ width $\times$ height mm³) was measured every other day as described⁵; then, the presence of mast cells and mast cell markers was evaluated by immunohistochemistry and real-time PCR,⁵ respectively. All experimental procedures were conducted with approval from the Baylor Scott & White Institutional Animal Care and Use Committee.

Morphologic Analysis

Tumor samples were excised and fixed in 10% buffered formalin for 24 hours, embedded in low-temperature fusion paraffin, and sectioned (4 to 5 μ m) for immunohistochemistry analysis.^{13,14} Total mRNA was extracted with the Qiagen (Valencia, CA) RNeasy mini kit and, after amplification, a $\Delta\Delta$ CT (Δ threshold cycle) analysis was performed.¹⁵

Mast Cell Presence

In commercially available human biopsy tissue arrays (AccuMax; BioCarta LLC, San Diego, CA) we evaluated mast cell presence by toluidine blue staining and performed immunohistochemistry for the following mast cell markers: c-Kit (dilution 1:200; anti-c-Kit polyclonal; MBL International Corporation, Woburn, MA), chymase, and tryptase (dilution 1:50;

Figure 1 In human biopsy tissue arrays, the presence of mast cells was detected by toluidine blue staining. A representative image of a human CCA biopsy positive for toluidine blue staining indicates of mast cell infiltration (A). c-Kit, chymase, and tryptase expression increases in human CCA biopsies compared with nonmalignant tissue (B and C). Red arrows show mast cells. Green arrows show bile ducts. Data are expressed as means $\pm$ SEM. $n = 9$ experiments. * $P < 0.05$ versus nonmalignant controls. Original magnification: $\times 20$ (A); $\times 40$ (B and C). CCA, cholangiocarcinoma.

MC tryptase; Santa Cruz Biotechnology, Paso Robles, CA).^{16–18} The staining index was calculated by multiplying the staining intensity by abundance.¹⁵ Mast cell infiltration was measured by staining tumor sections from all animal groups for toluidine blue, which marks mature mast cells.¹⁶ Sections were visualized with a light microscope, and slides were scanned on Leica SCN400 (Wetzlar, Germany) and counted manually.

Mast Cell Marker Analysis

The expression of mast cell markers, including c-Kit, chymase, and tryptase, was measured by real-time PCR in tumor mRNA of the above-mentioned animal groups. To quantitatively measure the expression of histamine enzyme and receptor mRNA in CCA, we used the RT² real-time assay from SABiosciences.^{14,15} Glyceraldehyde 3-phosphate dehydrogenase was used as the housekeeping gene. In sections from vehicle or cromolyn-treated mice, we measured c-Kit, chymase, and tryptase expression by immunohistochemistry as described.⁵

Tumor Evaluation

We evaluated the effects of cromolyn sodium treatment *in vivo* by measuring tumor growth and the expression of PCNA and VEGF-C by immunoblots and real-time PCR in whole tumors from vehicle- and cromolyn sodium-treated mice as described.^{4,5} Tumor volume was measured as described⁵ after establishment of tumors (day 7). Mice were treated with either saline or cromolyn sodium (24 mg/kg of body weight), and measurements were taken every other day with the use of a digital caliper.

EMT and ECM Marker Analysis in Tumors

We measured the expression of EMT markers (paxillin, vimentin, E-cadherin, and s100A4) and ECM degradation

markers (MMPs) by real-time PCR in whole tumor mRNA from vehicle- and cromolyn-treated mice as described.^{4,5} By immunohistochemistry we measured the expression of the epithelial and mesenchymal markers, CK-7, E-cadherin (dilution 1:50; Santa Cruz Biotechnology), and vimentin (dilution 1:200; Cell Signaling Technology, Danvers, MA) in sections from tumors treated with saline and cromolyn sodium as reported.⁴

Evaluation of HDC and HR Expression in Tumor mRNA

By real-time PCR we evaluated the expression of tumor HDC and the HRs (H1 to H4) from vehicle- and cromolyn-treated mice as described.⁵

In Vitro Studies

Cultured Cell Lines

To evaluate the effects of mast cells *in vitro*, we used the extrahepatic biliary cancer cell line, Mz-ChA-1, derived from human gallbladder,¹⁹ obtained from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX) and cultured mast cells (derived from fetal mouse liver) that were obtained from ATCC (ATCC, Manassas, VA). All cultured lines were maintained as described¹⁹ or according to the supplier's protocol.

In Vitro Effects of Mast Cell Histamine on CCA

Mast cells were treated with 0.1% bovine serum albumin (basal) or cromolyn sodium (10 μ mol/L) for up to 30 minutes, and the conditioned medium was collected and frozen. Mz-ChA-1 cells were then stimulated for 72 hours with the following: 0.1% bovine serum albumin (basal), mast cell supernatant fluids, or supernatant fluids from mast cells treated

Figure 2 In tumors from mice treated with vehicle, histamine, the histidine decarboxylase inhibitor, α -methyl, or cromolyn sodium, mast cell presence was measured. Histamine treatment increases mast cell infiltration shown by toluidine blue staining compared with vehicle-treated mice (A). Mast cell infiltration is not seen in tumor sections from mice treated with α -methyl or cromolyn sodium (A). By immunohistochemistry in tumors from saline-treated mice, there is an increase of c-kit⁺ mast cells, whereas the number of c-kit⁺ mast cells is lower in cromolyn sodium-treated tumors (B). Immunohistochemistry and semiquantitative analysis show that the number of chymase⁺ mast cells decreases in tumors from mice treated with cromolyn sodium compared with mice treated with saline (C); similar results are found for tryptase⁺ mast cells (D). Red arrows show mast cells. Data are expressed as means $\pm$ SEM. $n = 9$ experiments. * $P < 0.05$ versus saline treatment. Representative images are shown for immunostaining. Original magnification, $\times 20$ (A, C, and D); $\times 40$ (B).

with 0.1% bovine serum albumin (basal) or cromolyn sodium (10 μ mol/L). Total mRNA was extracted as described above, and real-time PCR was performed for HDC, paxillin, and VEGF-C. The conditioned medium from Mz-ChA-1 cells treated as described was collected, and histamine release was measured by electroimmunoassay (Cayman Chemical, Ann Arbor, MI).⁵

Evaluation of the c-Kit/SCF Signaling Pathway

By immunofluorescence, we evaluated the expression of c-Kit in cultured mast cells and SCF in Mz-ChA-1 cells as described¹⁵ with the use of specific c-Kit and SCF antibodies from Abcam (Cambridge, MA). Antibodies were diluted 1:100 in phosphate-buffered saline that contained 1% bovine serum albumin. Species-appropriate nonimmune serum was used for negative controls. Images were visualized with an Olympus IX-71 confocal microscope (Tokyo, Japan).

To determine whether the effects of mast cells on CCA depend on c-Kit/SCF, we measured mast cell migration toward CCA cells with the use of Boyden Chambers and Transwell inserts.⁴ Mz-ChA-1 cells were plated into the

bottom of the chamber 24 hours before treatment and pre-treated with either 0.1% ETOH (basal) or the SCF inhibitor, ISCK03, 1 μ mol/L for 30 minutes before mast cells were added to the top of the Boyden Chamber insert. After 48 hours, mast cell presence was detected by toluidine blue staining, and the total percentage of migrating mast cells was manually calculated with a light microscope.

We evaluated the effects of inhibition of the c-Kit/SCF interaction on CCA proliferation and angiogenesis, EMT switch, and ECM breakdown by real-time PCR in cells treated as described in *Mast Cell Marker Analysis*.

Statistical Analysis

All data are expressed as means $\pm$ SEM. Groups were analyzed by the Student's unpaired t -test when two groups are analyzed, followed by an appropriate post hoc test. $P < 0.05$ was considered significant. For tumor analysis, we estimated missing values by using missing at random and multiple imputation procedures.²⁰ Predictive mean matching was used to impute volume. We obtained 24 multiple imputed data sets,

Figure 3 Tumor growth was measured starting 1 week (day 7) after injection with Mz-ChA-1 cells into the hind flanks. Tumors were measured three times a week, and animals were treated with either vehicle (0.9% NaCl) or cromolyn sodium (24 mg/kg of body weight) three times a week. **A:** Tumor growth reduces in mice treated with cromolyn sodium compared with vehicle treatment. At days 17 to 25, there is a significant decrease in tumor volume compared with vehicle. By immunoblotting (**B**) and real-time PCR (**C**) in tumors the expression of PCNA and VEGF-C significantly decreases in cromolyn sodium-treated mice compared with tumors from vehicle-treated mice. Data are expressed as means $\pm$ SEM. $n = 9$ experiments for real-time PCR. * $P < 0.05$ versus vehicle (saline) treatment. PCNA, proliferating cell nuclear antigen; VEGF-C, vascular endothelial growth factor C.

based on the rule of thumb that the number of imputed sets should approximate the percentage of missing values.²¹ We then estimated a multilevel mixed-effects regression model for volume with treatment type and days of treatment as fixed effects and the subject (mouse) as a random-effects variable. The model was fit by the restricted maximum likelihood method, and overall estimates of the regression coefficients, their standard errors, and significance were obtained with Rubin's rules.²² Both treatment ($P = 0.003$) and day ($P < 0.001$) were significant without evidence of a significant day by treatment interaction. Differences in volume between vehicle and cromolyn sodium were compared over days with the use of post-regression joint-hypothesis tests. Statistical analyses were performed with Stata 13.1 (StataCorp LP, College Station, TX).

Results

Mast Cells Infiltrate the Tumor Microenvironment of Human CCA

Figure 1A is a representative image of a CCA biopsy positive for toluidine blue staining. The expression of c-Kit, chymase, and tryptase was increased in human CCA biopsies compared with nonmalignant, uninvolved tissue (Figure 1, B and C).

Mast Cell–Derived Histamine Regulates Mast Cell Infiltration and Mast Cell Marker Expression

In tumors extracted from nude mice treated with histamine, mast cell infiltration increased as shown by toluidine blue staining, whereas in tumors from mice treated with the HDC

inhibitor, α -methyl, or cromolyn sodium, no detectable mast cells were found (Figure 2A). A closer examination of tumors from saline- and cromolyn sodium-treated mice shows a decrease in c-Kit⁺, chymase⁺, and tryptase⁺ mast cells in tumors from cromolyn sodium-treated mice shown by immunohistochemistry (Figure 2, B–D). By real-time PCR we found that histamine treatment increases tumor c-Kit, chymase, and tryptase expression compared with saline treatment (Supplemental Figure S1). In tumors extracted from mice treated with α -methyl, the expression of c-Kit, chymase, and tryptase was significantly decreased compared with histamine-treated mice (Supplemental Figure S1), suggesting that histamine regulates mast cell involvement in the tumor microenvironment. Finally, we found that treatment with cromolyn sodium significantly decreased the expression of c-kit, chymase, and tryptase, *in vivo* (Supplemental Figure S1). In correlation with our immunohistochemical analysis, the gene expression of chymase was also significantly down-regulated in tumors extracted from mice treated with cromolyn sodium (Supplemental Figure S1).

Effects of Blocking Mast Cell–Derived Histamine on Tumor Growth and Angiogenesis

Previously, we reported that by globally blocking histamine we can decrease tumor growth and angiogenesis.⁵ We evaluated these variables after blocking mast cell-derived histamine with the use of cromolyn sodium. In mice treated with cromolyn sodium we found a steady decrease in tumor growth (Figure 3A). Between days 17 and 25, there was a substantial reduction of tumor mass compared with the saline-treated mice. Toward the end of the treatment it is

Figure 4 Evaluation of epithelial-mesenchymal transition markers and extracellular matrix degradation. Treatment with cromolyn sodium decreases the expression of paxillin, vimentin, and s100A4 compared with tumors from vehicle-treated mice, whereas E-cadherin expression significantly increases in cromolyn sodium tumors compared with saline (A). Immunohistochemistry and semiquantitative analysis are shown for the epithelial marker CK-7 (B). In mice treated with cromolyn sodium, the expression of CK-7 increases compared with saline-treated mice (B). Immunohistochemistry for vimentin shows that this mesenchymal marker decreases in cromolyn sodium tumors compared with saline (C), whereas the epithelial marker E-cadherin strongly increases in tumors from mice treated with cromolyn sodium compared with saline treatment (D). Representative images are shown. Data are expressed as means $\pm$ SEM. $n =$ at least 6 experiments for real-time PCR. * $P < 0.05$ versus saline treatment. Original magnification, $\times 40$.

likely that our tumors were oversaturated and that our drug was no longer working; however, cromolyn sodium-treated tumors were consistently smaller throughout the duration of the treatment. We found a decrease in PCNA expression and VEGF-C expression (Figure 3, B and C), suggesting that blocking mast cell-derived histamine diminishes tumor proliferation and angiogenesis. Finally, we evaluated the effects of cromolyn sodium treatment on apoptosis in our tumors by real-time PCR. We found that cromolyn sodium increased the expression of cleaved caspase-3 compared with saline treatment [1.0 (saline) versus 1.45 ± 0.067 (cromolyn sodium)], suggesting that cromolyn sodium may reduce tumor growth by increasing apoptosis.

EMT and ECM Regulation by Mast Cell–Derived Histamine

We evaluated the effects of blocking mast cell-derived histamine on EMT and ECM. With the use of tumors extracted from mice treated with saline and cromolyn sodium, we measured markers of EMT and ECM integrity by real-time

PCR. In tumors extracted from mice treated with cromolyn sodium, the expression of mesenchymal markers (paxillin, vimentin, and s100A4) was significantly down-regulated compared with vehicle-treated tumors (Figure 4A). Further, the gene expression of the epithelial marker, E-cadherin, was significantly increased in cromolyn sodium tumors compared with saline-treated tumors (Figure 4A). Evaluation of ECM breakdown (MMP-2, MMP-3, and MMP-9) found that treatment with cromolyn sodium decreased the expression of these enzymes compared with saline-treated tumors (Supplemental Figure S2). It was found that histamine is a growth-promoting agent,^{4,5,13} and our data support this and also indicate the potential treatment strategy of blocking mast cell-derived histamine to inhibit EMT and ECM degradation. Figure 4B shows representative images and semiquantification for immunostaining of CK-7 expression. In tumors from mice treated with cromolyn sodium, there is increased expression of the epithelial marker, CK-7, suggesting that blocking mast cell-derived histamine reduces the EMT switch to allow for more epithelial-type cells within the tumor microenvironment. In addition, the protein expression of vimentin was

Figure 5 Evaluation of HDC and HR expression. Treatment with cromolyn sodium decreases the expression of HDC, H1HR, and H2HR (but not H3HR or H4HR) compared with tumors from vehicle-treated mice. Data are expressed as means $\pm$ SEM. $n =$ at least 3 experiments for real-time PCR. * $P < 0.05$ versus vehicle (saline) treatment. HDC, histidine decarboxylase; HR, histamine receptor.

significantly decreased in tumors from cromolyn sodium-treated mice compared with saline-treated mice (Figure 4C), whereas the expression of E-cadherin was significantly increased in tumors from cromolyn sodium-treated mice compared with saline-treated mice (Figure 4D). Taken together, these data indicate that inhibition of mast cell-derived histamine diminishes the mesenchymal transition found in CCA and supports an epithelial stroma.

HDC and HR Expression after *in Vivo* Cromolyn Sodium Treatment

In mice treated with cromolyn sodium, HDC expression and HRs, H1 and H2, were decreased (Figure 5). In contrast, cromolyn sodium did not have any significant effect on the H3 or H4 HRs. It has been demonstrated that the H1 and H2 HRs are stimulatory receptors that increase biliary hyperplasia and CCA growth,^{4,5,23} whereas the H3 and H4 HRs are inhibitory receptors and decrease CCA progression.^{4,5,23} Therefore, the present data suggest that cromolyn sodium may also function by reducing the availability and expression of H1 and H2 HRs on malignant cholangiocytes to drive down tumor growth.

Mast Cell Histamine Regulates CCA HDC and Histamine Release and Paxillin and VEGF Expression *in Vitro*

To begin to address the effects of mast cells on CCA proliferation, angiogenesis, and differentiation, we treated cultured mast cells with 0.1% bovine serum albumin (basal) or cromolyn sodium (to block mast cell histamine release) before being added to Mz-ChA-1 cells before measuring the gene expression of HDC, VEGF-C, and paxillin by real-time PCR and histamine secretion by electroimmunoassay. We found all were increased in Mz-ChA-1 cells stimulated with basal-treated mast cell supernatant fluids (Figure 6).

When Mz-ChA-1 cells were stimulated with mast cells treated with cromolyn sodium, there was a significant decrease in HDC and histamine secretion (Figure 6, A and B). We found that paxillin and VEGF-C expression decreased in Mz-ChA-1 cells treated after mast cells were pretreated with cromolyn sodium (Figure 6, C and D).

Figure 6 *In vitro* evaluation of mast cell-derived histamine Mz-ChA-1 cells. The gene expression of HDC and histamine secretion (by enzyme-immunoassay) increases in CCA cells stimulated with basal-treated mast cell supernatant fluids (A and B). These variables significantly decrease when mast cells were first treated with cromolyn sodium before being added to CCA cells (A and B). The expression of paxillin and VEGF-C significantly decreases in Mz-ChA-1 cells stimulated with supernatant fluids from mast cells treated with cromolyn sodium compared with basal treatment (C and D). Data are expressed as means $\pm$ SEM. $n = 9$ experiments for real-time PCR; $n = 12$ experiments for electroimmunoassay. * $P < 0.05$ versus 0.1% BSA (basal treatment); † $P < 0.05$ versus stimulation with mast cell supernatant fluid (basal treatment). BS, basal; BSA, bovine serum albumin; CCA, cholangiocarcinoma; Cr, cromolyn sodium treatment; HDC, histidine decarboxylase; MC, mast cell; VEGF-C, vascular endothelial growth factor C.

Figure 7 Mast cell migration depends on CCA SCF secretion. **A:** Migration of mast cells (red arrows) toward Mz-ChA-1 cells significantly decreases when Mz-ChA-1 cells were pretreated with 1 μ mol/L SCF inhibitor ISCK03 blocking the c-Kit/SCF interaction. **B:** When Mz-ChA-1 cells are stimulated with basal-treated mast cell supernatant fluids, PCNA, VEGF-A, and VEGF-C increase as shown by real-time PCR. In Mz-ChA-1 cells stimulated with mast cell supernatant fluids after SCF inhibitor treatment, these variables decrease. Data are expressed as means $\pm$ SEM. $n = 6$ experiments for migration studies and real-time PCR studies. * $P < 0.05$ versus basal treatment; † $P < 0.05$ versus all other groups. Original magnification, $\times 40$. BS, basal; BSA, basal serum albumin; CCA, cholangiocarcinoma; MC, mast cell; PCNA, proliferating cell nuclear antigen; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

Mast Cell Migration Depends on the c-Kit/SCF Pathway

The c-Kit/SCF pathway is a prominent signaling event that mast cells use to migrate toward other cells.^{24–26} To determine whether the c-Kit/SCF pathway is important in mast cell/CCA interactions, we first performed immunofluorescence to detect the presence of c-Kit in mast cells and SCF in Mz-ChA-1 cells. We found that mast cells express c-Kit and that Mz-ChA-1 cells express SCF as shown in Supplemental Figure S3.

With the use of Boyden Transwell chambers we evaluated the migratory capacity of mast cells toward CCA cells. Mz-ChA-1 were first treated with 0.1% ETOH (basal) or 1 μ mol/L SCF inhibitor ISCK03 before the addition of mast cells into the chamber. We found that the migratory capacity of mast cells toward Mz-ChA-1 cells was significantly reduced when Mz-ChA-1 cells were treated with the SCF inhibitor, indicating that their interactions may depend on

c-Kit/SCF (Figure 7A). Mast cell migration was detected by toluidine blue staining, and semiquantification was performed with a light microscope.

CCA Proliferation, Angiogenesis, EMT Switch, and ECM Breakdown Depends on the c-Kit/SCF Interaction between Mast Cells and CCA

By real-time PCR, CCA proliferation and angiogenic potential were substantially increased after treatment with mast cell supernatant fluids; however, when CCA cells were pretreated with 1 μ mol/L SCF inhibitor (ISCK03) and mast cell supernatant fluids were added, PCNA, VEGF-A, and VEGF-C expression significantly decreased (Figure 7B). Further, the expressions of vimentin, paxillin, and s100A4 were all down-regulated in CCA cells pretreated with the SCF inhibitor (Figure 8A). In addition, the expressions of

Figure 8 The expression of paxillin, vimentin, and s100A4 increases after treatment with basal mast cell supernatant fluids as shown by real-time PCR (A). When Mz-ChA-1 cells are pretreated with 1 μ mol/L SCF inhibitor ISCK03 and stimulated with mast cell supernatant fluids the expression of vimentin, paxillin, and s100A4 significantly decreases (A). The Mz-ChA-1 expression of MMP-2, MMP-3, and MMP-9 increases after stimulation with mast cell supernatant fluids and significantly decreases when Mz-ChA-1 are pretreated with the SCF inhibitor (B). Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus basal treatment; † $P < 0.05$ versus all other groups. BS, basal; BSA, bovine serum albumin; MC, mast cell; MMP, matrix metalloproteinase; SCF, stem cell factor.

MMP-2, -3, and -9 were all significantly decreased in CCA cells treated with the SCF inhibitor (Figure 8B), suggesting that the c-Kit/SCF interaction is key in regulating the paracrine effects of mast cells on CCA proliferation and potential metastasis.

Discussion

The CCA tumor microenvironment includes fibroblasts and myofibroblasts, ECM, and preexisting and newly forming vessels, along with innate and adaptive immune cells.⁶ Mast cells are unique in that they can be both beneficial and damaging to the host tissue. Mast cells accumulate in and around adenomatous polyps,²⁷ malignant melanoma,²⁸ gynecologic malignancies,²⁹ and colorectal carcinoma.³⁰ Here, we demonstrate that the infiltration of mast cells induces a chain reaction of increased histamine release, activation of mast cell markers, proliferation, angiogenesis, and EMT/ECM processes. Inhibition of mast cell histamine or disrupting the interaction between mast cells and CCA cells (via SCF/c-Kit) has beneficial effects on decreasing tumorigenesis.

Mast cells are known for their role in regulating allergic reactions; however, they are becoming more known for their role in numerous pathologic events, including tumor growth.^{16,31,32} Their presence was detected in pancreatic, gastric, and esophageal cancers, and their role in these cancers was mostly a detrimental one.⁹ Mast cell presence in the tumor microenvironment guarantees the increased release of inflammatory and protumorigenic factors such as histamine, cytokines, and growth factors that all contribute to increasing tumorigenesis.³³ Here, we have found increased infiltration of mast cells and mast cell markers in human CCA, and in support of our findings a study has shown that the density of mast cells is substantially higher in intrahepatic CCA tissues than in nonmalignant and mast cells found surrounding portal tracts.³⁴ In pancreatic ductal adenocarcinoma nongranulated mast cell density is substantially higher than in the control group, suggesting that mast cells may promote pancreatic tumor growth,³⁵ and, in a study that used colorectal cancer, mast cell presence and mast cell marker expression, including c-Kit (all up-regulated), were determined to be potential diagnostic markers for the early detection of colorectal cancer.³⁶

Our previous work found that histamine promotes CCA growth via an autocrine release of histamine from cholangiocytes.⁵ In our present study, we found that histamine treatment increased mast cell infiltration into the tumor along with increased proliferation, whereas blocking mast cell-derived histamine with the use of cromolyn sodium decreased mast cell infiltration and proliferation. Numerous studies have reported the positive effects of inhibition of histamine via specific HRs on decreasing tumorigenesis, including our previous work on CCA^{9,13}; however, few studies exist to demonstrate the potential that blocking mast cell-derived histamine has on tumor growth.⁵ Cromolyn sodium is a clinically relevant agent that is used to treat

asthmatic reaction, and this work gives rise to evidence that cromolyn sodium may have causative effects on tumor growth and potential metastasis.

Mast cell infiltration occurs by numerous signaling pathways that involve chemoattractants and various ligand/receptor interactions. One of the key signaling mechanisms is the c-Kit/SCF interaction. SCF, which is also termed steel factor or c-Kit ligand, is a growth factor that induces cellular survival, proliferation, and differentiation. SCF promotes mast cell recruitment into various tissues and also promotes the maturation of mast cells.²⁶ Studies that involve bone marrow mast cells and peritoneal mast cells demonstrate a dependence on the SCF/c-Kit interaction for mast cell recruitment.²⁶ Our evaluation of the SCF/c-Kit interaction demonstrates that, in CCA cells, blocking the interaction between mast cells and CCA decreases CCA proliferation, angiogenesis, mast cell marker presence; EMT switch; and ECM breakdown, pinpointing this pathway as a potential therapeutic avenue for CCA treatment. In studies that involve metastatic nasopharyngeal carcinoma it was found that activation of SCF/c-Kit signaling potentially contributes to carcinogenesis and progression of this cancer.³⁷ Finally, in human uveal melanoma the SCF/c-Kit axis was found to play a role in increasing melanoma transformation and proliferation,^{37,38} supporting our present studies that this axis may also be important in CCA proliferation.

CCA cells are able of undergoing EMT during the progression of the disease, leading to degeneration of ECM and increased metastasis. We have found that histamine, via the H4HR, regulates EMT switch and ECM breakdown,⁴ and, here, we demonstrate that mast cells and mast cell mediators may also play an important role in the metastatic potential of CCA. Our studies found that inhibition of mast cell-derived histamine disrupts EMT switch and ECM breakdown both *in vivo* and *in vitro*. We also demonstrated that interruption of the SCF/c-Kit pathway decreases the paracrine influence of mast cells on CCA EMT and ECM degradation. Numerous studies were performed to determine the potential signaling pathway that CCA cells use to undergo EMT and further degrade the ECM to allow for migration. These studies demonstrate that a multitude of players may be involved, including (but not limited to) transforming growth factor- β 1, SOX, tumor necrosis factor- α , and the epidermal growth factor—epidermal growth factor receptor axis,^{39–41} suggesting that our full understanding of CCA progression and migration is not completely defined. To date, our studies are the first to demonstrate that mast cells and the mast cell/CCA interaction may contribute to EMT and ECM degradation and CCA progression.

Clinical and diagnostic markers for detecting CCA are currently lacking, and, because the disease is typically advanced when found, the development of new, reliable markers is critical. Our study offers the potential that histamine concentrations (shown to be increased in nude mice injected with CCA cells⁵) or the detection of up-regulated mast cell markers such as c-Kit, chymase, and tryptase

may be alternative ways to detect the presence of tumorigenic cells. In support of this hypothesis, in hepatocellular carcinoma, SCF and CD34 were both reported to be promising prognostic markers for early detection.⁴² Mast cells themselves may be a potential diagnostic marker for prostate cancer, although there is discrepancy among scientists about the definitive role of mast cells in prostate cancer.⁴³ Finally, our studies elucidate that targeting the c-Kit/SCF interaction may be a beneficial treatment option to diminish tumorigenesis and progression.

In summary our findings provide evidence that mast cells participate in the progression and metastatic potential of CCA and that disruption of the interaction between mast cells and CCA may be a beneficial treatment option to patients experiencing this cancer. Histamine and mast cell markers may be beneficial diagnostic/prognostic tools for the early detection of CCA.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.09.016>.

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