Tumorigenesis and Neoplastic Progression

Adrenomedullin Is a Cross-Talk Molecule that Regulates Tumor and Mast Cell Function during Human Carcinogenesis

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We have previously demonstrated that adrenomedullin (AM) plays a critical role as an autocrine/paracrine tumor cell survival factor. We now present evidence that AM is an important regulator of mast cell (MC) function and that this modulation is potentially involved in tumor promotion. AM induced histamine or β -hexosaminidase release from rat and human MCs through a receptor-independent pathway. AM was chemotactic for human MCs and stimulated mRNA expression of vascular endothelial growth factor, monocyte chemoattractant protein-1, and basic fibroblast growth factor in this cell type. Differentiated but not undifferentiated human MCs responded to hypoxic insult with elevated AM mRNA/protein expression. Using confocal microscopy, we identified AM-producing MCs in tumor infiltrates of human breast and lung cancer patients. In mixed culture assays the AM-producing human MC line HMC-1 augmented

both anchorage-dependent and -independent growth of human lung cancer A549 cells, an effect that was suppressed by MC-targeted siRNA AM knockdown. Finally, HMC-1 cells induced *in vivo* angiogenesis as assessed by directed *in vivo* angiogenesis assay analysis; neutralizing anti-AM monoclonal antibody blocked this ability. Our collective data suggest a new role for AM as a cross-talk molecule that integrates tumor and MC communication, underlying a unique promotion mechanism of human cancers. (Am J Pathol 2006; 168:280–291; DOI: 10.2353/ajpath.2006.050291)

Our general concept of cancer has dramatically changed throughout the past 2 decades from the model of a single transformational event to one of a multistaged process involving complex interactions with the surrounding cellular microenvironment.^{1,2} Encompassed in these newly proposed cancer dynamics, chronic inflammation has been implicated as the driving force in many human malignancies.^{3,4} Recently, the mast cell (MC) has emerged as a primary candidate among the infiltrating cell population responsible for mediating tumor promotion.^{5–7} The first experimental evidence to demonstrate an important correlation between MC infiltration and tumor progression was generated in animal models of skin and breast cancer.^{8–10} Sequential tissue remodeling events leading to invasive carcinoma in a hamster model of oral cancer and the conversion of premalignant lesions to frank tumor in a rat model of mammary cancer were shown to be MC-dependent.^{9,10} Consistent with this MC/tumor progression correlate, compounds that blocked MC degranulation were shown to suppress rat mammary tumor growth.^{11,12} In addition, MC-deficient mice had a lower capacity for developing lung metastases than their wild-type controls when challenged with B16-BL-6 mela-

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noma cell line.¹³ Depending on its tissue of origin and surrounding stimuli, the MC has been shown to contain multiple bioactive factors including histamine, heparin, an assortment of interleukins (ILs), several cytokines, and a variety of growth factors.^{14,15} Regional release of these MC-derived bioactive substances can augment tumor cell growth, induce angiogenesis, inhibit apoptosis, and increase the metastatic potential of cancers.^{1,7,16} As a result of this activity, MC infiltrates have profound influence on tumor aggression that manifest as worsening clinical prognosis for patients with Hodgkin's lymphoma, esophageal squamous cell carcinoma, malignant melanoma, or pulmonary adenocarcinoma.¹⁷⁻²⁰

Since the discovery and isolation of adrenomedullin (AM), a 52-amino acid peptide amide, from a human pheochromocytoma,²¹ much has been done by our group and others to show its multifunctional nature and, most important for this study, its involvement in human carcinogenesis through diverse mechanisms.²² AM is elevated over normal levels in a variety of human malignancies of both neural and epithelial origin, including cancers of the brain, lung, colon, breast, ovary, uterus, prostate, skin, kidney, and eye.²³ Hypoxic insult and the resulting increase of hypoxia-inducible factor-1 have been implicated as one of the underlying pathways leading to AM overexpression in human tumors.^{24,25} Our group and others have shown that elevated AM expression in human cancer cells can stimulate autocrine/paracrine growth, augment angiogenesis, and reduce apoptosis, events that culminate in tumor proliferation.^{23,26,27} Disruption of the AM autocrine/paracrine loop mechanism in cancer cells from diverse tissue origins (lung, breast, pancreas, and brain), using neutralizing anti-AM antibodies, resulted in suppression of tumor cell growth *in vitro* and *in vivo*.^{28,29} In patients with prostate or ovarian carcinoma, higher AM expression in the tumor or in serum correlated with cancer aggression and poor clinical prognosis.^{30,31} Interestingly, in addition to its better known tumorigenic abilities, AM has been shown to regulate certain aspects of the immune function that include suppressing complement fixation³² and modulating macrophage³³ and MC activation,³⁴ features that are potentially involved with tumor promotion and progression. Hence our investigative interest in the role of AM as a cross-talk molecule regulating tumor and MC function as it relates to human carcinogenesis. Enhancing our understanding of the biological mechanisms surrounding cancer cells, their associated MCs infiltrates, and AM as a cross-talk communicator peptide between these cell populations may help to define new therapeutic targets for the clinical management of human malignancies.

Materials and Methods

Cell Culture

The human (H) MC leukemia line, HMC-1,³⁵ was a gracious gift from Dr. J.H. Butterfield, donated through a Material Transfer Agreement between the National Institutes of Health (NIH) (Bethesda, MD) and the Mayo Clinic

(Rochester, MN). Cells were routinely grown in 175-cm² flasks in Iscove's medium containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and 1.2 mmol/L monothioglycerol (Sigma-Aldrich, St. Louis, MO). For all HMC-1 experimental evaluations cited in this text, semiconfluent cells were fed 2 days before testing and then seeded at 10⁷ cells/175-cm² flask for procedure implementation. When indicated, HMC-1 cells were exposed to the differentiation reagent phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at 20 ng/ml for specified time periods.³⁶ For the remainder of this study, PMA-treated HMC-1 cells will be considered mature (differentiated) and untreated HMC-1 cells will be considered immature (undifferentiated).

Construction of Small Interfering RNA Expression Plasmids and Generation of Stable Transfectants

The mammalian expression vector pSEC(neo) (Ambion, Austin, TX) was used for expression of an AM-specific siRNA in lung carcinoma A549 and MC HMC-1 cell lines. An insert containing nucleotides 511 to 531 (5'-AAGCTGGCACACCAGATCTAC-3') of the AM gene was ligated into the pSEC(neo) backbone, and the final plasmid was referred to as pSEC(neo)-511. A control vector containing a scrambled sequence (5'-AAGGCGCCACTCGCCAAATAAT-3') served as a nonsilencing control (referred to as pSEC(neo)-SCR). All vectors were sequenced, in the forward and reverse directions, around the cloning boxes to verify the insertion of the AM sequences into the pSEC(neo) backbone. A549 and HMC-1 cells were transfected with plasmids pSEC(neo)-511 (A549-511 and HMC-1-511) and pSEC(neo)-SCR (A549-SCR and HMC-1-SCR) using FuGENE6 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After 72 hours, cells were exposed to the appropriate media containing 800 µg/ml of geneticin (Invitrogen). Stable transfectants were screened for AM expression by quantitative real-time polymerase chain reaction (PCR).

Conditioned Medium and Cell Harvest

Conditioned medium from appropriate test cells was aspirated from culture flasks, centrifuged, snap-frozen on dry-ice and stored at -80°C until used for quantitative AM radioimmunoassay or methylthiazolotetrazolium cell proliferation assays. Cells were harvested by washing once in serum-free RPMI 1640 (Life Technologies), trypsinizing for 3 minutes, pelleting cells by low-speed centrifugation, lysing cells in RLT buffer (RNeasy mini kit; Qiagen Inc., Valencia, CA) containing 2-mercaptoethanol (Sigma-Aldrich), snap-freezing lysate on dry-ice, and storing frozen sample at -80°C until ready for RNA extraction.

Quantitative Real-Time PCR Analysis

Total RNA (3.5 µg) from all samples was reverse-transcribed in a final volume of 21 µl using the SuperScript

First-Strand Synthesis system (Invitrogen) following the manufacturer's instructions. The quantitative real-time PCR reaction was run in an Opticon cycler (MJ Research, Waltham, MA) using Sybr Green PCR master mix (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Thermocycling was performed in a final volume of 25 μ l containing 2 μ l of cDNA (1:10 dilution) and 400 nmol/L of primers (18S forward: 5'-ATGCTCTAGCTGAGTGTCCCG-3', 18S reverse: 5'-ATTCCTAGCTGCGGTATCCAGG-3'; AM forward: 5'-TTGGATGTCGCGTCGGAG-3', AM reverse: 5'-TAGCTGCTGGACATCCGCA-3'; vascular endothelial growth factor (VEGF) forward: 5'-TTCATGGATGCTATCAGCGCA-3', VEGF reverse: 5'-CCGCATAATCTGCATGGTGA-3'; basic fibroblast growth factor (bFGF) forward: 5'-CGACCCTCACATCAAGCTACAAC-3', bFGF reverse: 5'-CCAGTTCGTTTCAGTGCCACAT-3'; monocyte chemoattractant protein-1 (MCP-1) forward: 5'-AGATGCAATCAATGCCCCAGT-3', MCP-1 reverse: 5'-TGGCCACAATGGTCTTGAAGAT-3'). 18S rRNA was run for every sample as a normalizing housekeeping gene. For every sample, both the housekeeping gene and target gene were amplified in triplicates in the same run using the following cycle scheme: after initial denaturation of the samples at 95°C for 2 minutes, 46 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. Fluorescence was measured in every cycle and a melting curve was performed after the PCR by increasing temperature from 50 to 96°C (0.5°C increments). A defined single peak was obtained for all amplicons, thus confirming the specificity of the amplification.

Isolation of MCs from Rat Peritoneum or Cultured from Human Peripheral Blood

Rat peritoneal MCs were prepared according to our previously established protocol under approved NIH institutional review board guidelines.³⁷ Briefly, male Sprague-Dawley rats (240 to 270 g) were euthanized and the peritoneal cavity lavaged with 35 ml of PIPES AC buffer [119 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L piperazine-*N,N'*-bis-2-ethanesulfonic acid (NaOH to pH 7.3), 0.3 mmol/L CaCl₂, 5.6 mmol/L glucose, and 0.1% bovine serum albumin]. Harvested cells were purified to 95% MC homogeneity via Percoll gradient centrifugation and used for histamine release experiments. Human peripheral blood mononuclear cells were collected from normal donors after informed consent on an approved NIH institutional review board protocol and subsequently enriched to 85% CD34⁺ cells using affinity column apheresis as previously described.³⁸ Cells were placed in serum-free media (StemPro-34 SFM, Life Technologies) supplemented with 2 mmol/L L-glutamine, 50 mg/ml streptomycin, 100 IU/ml penicillin, and fortified with a human recombinant cytokine mixture (Pepro Tech, Rocky Hill, NJ) containing 100 ng/ml of stem cell factor, 100 ng/ml of IL-6, and 30 ng/ml of IL-3 (first week only of *in vitro* culture). Cultures were maintained at 37°C and 5% CO₂, and half the culture media was replaced every 7 days. Greater than 95% of the cells were confirmed to be

human cultured mast cells (HCMCs) after 10 weeks of *in vitro* incubation when assessed via Kimura staining.

Assessment of Histamine Release

Histamine release was assayed by an automated fluorometric method previously described.³⁹ In brief, histamine was extracted from cell samples and coupled to *o*-phthalaldehyde under alkaline conditions to give a fluorescent product. The histamine-*o*-phthalaldehyde complex was stabilized and fluorescent intensity increased by HCl addition. Fluorometric analysis of AM-induced histamine released into the media was determined by comparing observed fluorescence for each test sample to values generated by histamine standard curve. Spontaneous release in all experiments was <5% of the total histamine. Results were expressed as the percentage of maximum release obtained with MCs treated with 3% perchloric acid. For all histamine quantitations, given replicates of each sample showed <8% variation between values. The luteinizing hormone-releasing hormone (LHRH) and its peptide antagonist [*N*-Ac-D-Nal(2)¹,D-pF-Phe²,D-Trp³,D-Arg⁶]LHRH (Nal-Arg) were obtained from Dr. J. Rivier (Salk Institute, La Jolla, CA). Substance P (SP), AM, and AM peptide fragments (AM₁₋₁₂, AM₃₄₋₅₂, and AM₂₂₋₅₂) were obtained from Phoenix Pharmaceutical, Inc. (Belmont, CA). The AM serum-binding protein human complement factor H was purchased from Quidel Corp. (San Diego, CA). Anti-AM monoclonal antibody MoAb-G6 was developed in-house and was previously shown to block a variety of AM-mediated biological effects.^{28,40}

Measurement of β -Hexosaminidase Release

Analysis of peptide-induced β -hexosaminidase (β -hex) release from human MCs was accomplished following our previously established protocol.³⁸ HCMCs (10⁴ cells) were suspended in HEPES-Tyroses buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L Na₂HPO₄, 5.6 mmol/L D-glucose, 1.8 mmol/L CaCl₂, 1.3 mmol/L MgSO₄) containing 0.025% bovine serum albumin. Appropriate molar concentrations of AM were added to MC suspensions and peptide/cell mixtures incubated for 30 minutes at 37°C to stimulate release of β -hex. Reactions were stopped by centrifugation at 4°C, supernatant removed, snap-frozen on dry ice, and stored at -80°C until ready for β -hex analysis. Deionized water (150 μ l) was added to cell pellets, frozen on dry ice, and either thawed for immediate β -hex quantitation or stored at -80°C for future measurements. Supernatant or cell lysate samples (50 μ l) were incubated in 0.04 mol/L citric acid with 0.02 mol/L Na₂HPO₄ containing 10 mmol/L *p*-nitrophenyl *N*-acetyl- α -D-glucosaminide (pNAG, β -hex substrate; Sigma) for 90 minutes at 37°C. The reaction was developed using 0.4 mol/L glycine and absorbance was determined at 405 nm. Resulting β -hex values (mean $\pm$ SD) were represented as: [(β -hex in supernatant $\times$ 2)/(β -hex in supernatant + {total β -hex in cell pellet $\times$ 4})]100%.

Migration Assay

A 96-well ChemoTx microplate (NeuroProbe, Inc., Gaithersburg, MD) was used to study MC motility. Upper and lower chambers of the microplate were separated by a porous membrane (8 μm in diameter) precoated with 10 $\mu\text{g}/\text{ml}$ of fibronectin (Sigma-Aldrich). Varying concentrations (0.01 nmol/L to 500 nmol/L) of synthetic AM diluted in RPMI 1640 plus 10% fetal calf serum (Life Technologies) were added to assigned wells of the lower chamber. HMC-1 cells were diluted in RPMI 1640 plus 10% fetal calf serum to a concentration of 5×10^5 cells/ml and 25 μl of this suspension (12,500 cells) was added to each well of the upper chamber. VEGF (R&D Systems, Minneapolis, MN), at a concentration of 10 nmol/L, was used as a positive control for MC migration. After a 4-hour incubation at 37°C, the membrane was fixed and stained with Hema3 (Biochemical Sciences, Inc., Swedesboro, NJ) as recommended by the manufacturer. HMC-1 cells that had traversed the membrane and were attached to the side facing the lower well were photographed through a $\times 25$ microscope objective, and the number of cells per photographic field were counted. For each test measurement four fields were examined and resulting values reported as the mean $\pm$ SD.

Hypoxia and Hypoxia Mimetic Exposure

HMC-1 cells were incubated under hypoxic conditions according to a previously described protocol.²⁴ In brief, cells were subjected to reduced oxygen tension using a seamless hypoxia chamber maintained at 37°C and flushed to equilibration with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂ (Roberts Oxygen, Co., Gaithersburg, MD). At appropriate incubation times, cells were removed from the chamber and the chamber re-equilibrated with the hypoxia gas mixture through repeated flushing. In parallel studies using an iron chelator/hypoxia mimetic, cells were exposed to 260 $\mu\text{mol}/\text{L}$ desferrioxamine mesylate (Sigma-Aldrich) and incubated for appropriate time schedules at 37°C under normal atmospheric conditions (21% O₂). Ferrous ethylenediammonium sulfate (300 $\mu\text{mol}/\text{L}$) (Sigma-Aldrich) was added to desferrioxamine mesylate cultures as a negative control to saturate iron chelate conditions and restore hypoxia-inducible factor-1 levels to normoxic values.

AM Radioimmunoassay

The concentrations of AM found in conditioned media of test samples were determined using a commercially available radioimmunoassay kit (Phoenix Pharmaceuticals, Inc., Mountain View, CA). Samples (1 ml) were initially diluted in an equal volume of 0.1% alkali-treated casein in phosphate-buffered saline (pH 7.4) and applied to prewashed reverse-phase Sep-Pak C-18 cartridges (Waters Corp., Milford, MA). The peptide fraction was eluted from the C18 matrix with 3 ml of 80% isopropanol containing 0.125 N HCl and freeze-dried overnight. AM levels found in lyophilized extracts were then determined

by radioimmunoassay following the manufacturer's instructions. Data were statistically evaluated by a two-tailed Student's *t*-test using Prism 3.0 software. Differences were regarded as significant when $P < 0.05$.

MTT Cell Proliferation Assay

HMC-1 was treated with PMA (20 ng/ml) for 3 days and its proliferation capability compared to untreated HMC-1 by MTT assay throughout a time course. In brief, a single cell suspension of 2×10^5 cells/ml (50 $\mu\text{l}/\text{well}$) was seeded into 96-well polyvinylchloride plates. The assay was performed in RPMI 1640 with 10% fetal calf serum. Cells grew at 37°C, 5% CO₂, in a humid incubator and the dye and solubilization solutions (Promega Proliferation Assay; Promega, Madison, WI) were added every day for 5 days to separate plates. The Spectra Rainbow (Tecan, Raleigh, NC) plate reader and software was used to determine changes in the number of viable cells from dye reduction measured by absorbance at 570 nm. To assess the influence of HMC-1-secreted AM on the anchorage-independent growth of tumor cells, A549-511 (1×10^5) was grown in the presence of 3-day-old conditioned media from HMC-1-SCR or HMC-1-511 (4×10^6 cells/175-cm² flask). Growth capabilities were evaluated as indicated above.

Soft Agar Clonogenic Assay

The anchorage-independent growth of A549-511 in the presence of HMC-1-SCR or HMC-1-511 was examined by soft agar clonogenic assay. Both HMC-1-SCR and HMC-1-511 were exposed to PMA (20 ng/ml) for 3 days before seeding them in the clonogenic assays (differentiated HMC-1 cells lack the ability to grow as evaluated by MTT assay; see Figure 5). Cells were trypsinized and 5×10^3 A549-511 and either HMC1-SCR or HMC1-511 were resuspended in 1.5 ml of the culture media with 10% FBS and 0.3% agarose and then plated in six-well plates containing 1.5 ml of presolidified culture media in 0.5% agar containing 10% FBS. The plates were incubated at 37°C in 5% CO₂ atmosphere for 4 weeks, and colonies >0.1 mm in diameter were counted.

In Vivo Angiogenesis Assay

Quantitative analysis of AM induction/enhancement of angiogenesis was performed using directed *in vivo* angiogenesis assay (DIVAA).⁴¹ In brief, 10-mm-long surgical-grade silicone tubes with only one opened end (angioreactors) were filled with HMC-1 cells (10^4 cells/angioreactor) alone or in combination with MoAb-G6 (0.8 $\mu\text{g}/\text{ml}$) or an indifferent isotypic immunoglobulin control (0.8 $\mu\text{g}/\text{ml}$, IgA; Sigma, St. Louis, MO). After Matrigel (catalog no. 354230, not supplemented with growth factors; BD Bioscience, San Jose, CA) solidified, the angioreactors were implanted into the dorsal flanks of athymic nude mice (National Cancer Institute colony). After 11 days, the mice were injected intravenously with 25 mg/ml of fluorescein isothiocyanate-dextran (100 $\mu\text{l}/\text{mouse}$,

Sigma) 20 minutes before removing the angioreactors. Quantitation of neovascularization in the angioreactors was determined as the amount of fluorescence trapped in the implants and was measured in a HP Spectrophotometer (Perkin-Elmer, Emeryville, CA). This animal protocol was approved by the NIH institutional review board and performed under Association for Assessment and Accreditation in Laboratory Animal Care guidelines. Some implants were formalin-fixed and paraffin-embedded for morphological analysis. In agreement with the original study describing the DIVAA assay,⁴¹ visual inspection and microscopic examination of the angioreactors confirmed that the fluorescence obtained in negative controls is background and not due to neovascularization. Some sections were immunostained for human MC tryptase using the R.T.U. Vectastain kit (Vector, Burlingame, CA) following the manufacturer's instructions.

Double Immunofluorescence and Confocal Microscopy

Paraffin-embedded breast and lung tumor tissues were obtained from the Cooperative Human Tissue Network (Philadelphia, PA). Tissue specimens were collected under an approved institutional review board protocol and in accordance with NIH guidelines. Sections (7 μ m thick) were microwave treated for 10 minutes for antigen retrieval, then blocked with 3% normal goat serum (Jackson ImmunoResearch, West Grove, PA) for 30 minutes and exposed to a mixture of two primary antibodies obtained in different species, overnight at 4°C. The mixture consisted of a previously characterized rabbit antibody against human AM⁴² at a dilution of 1:1000 and a murine monoclonal antibody against human MC tryptase (DAKO, Glostrup, Denmark) at a dilution of 1:100. The next day the sections were incubated for 1 hour with a mixture of Bodipy-goat anti-mouse and Texas Red-goat anti-rabbit (Molecular Probes, Eugene, OR), both at a final concentration of 1:200. Counterstaining with 4,6-diamidino-2-phenylindole (Molecular Probes) was performed for 10 minutes. After thorough washes, the slides were mounted in GelMount (Biomedex, Foster City, CA) and observed with a Zeiss 510 laser-scanning microscope (Carl Zeiss, Thornwood, NY) equipped with four lasers.

Results

AM Induces Histamine and β -Hexosaminidase Release from Rat and Human MCs

As expected from previous reports in the literature,³⁴ AM-induced histamine release from rat peritoneum-isolated MCs followed a classic sigmoid curve that showed a dose-dependent relationship with the amount of histamine released (Figure 1A). On a molar concentration basis, AM proved to be a less potent secretagogue than SP or Nal-Arg in inducing histamine release. Because the primary goal of this study relates to the relationship be-

tween MCs and human cancers, we extended this analysis to show a similar effect of AM on HCMC activation, this time using β -hex as a marker of degranulation (Figure 1B). Interestingly, none of the fragmentary peptides of AM, which lack various segments of the amino or carboxy end of the molecule (AM₁₋₁₂, AM₃₄₋₅₂, and AM₂₂₋₅₂), were able to mediate rat (Figure 1A) or HCMC (data not shown) degranulation. Additionally, neither MoAb-G6 nor complement factor H, throughout a broad dose range, showed any modulatory effects on AM-induced histamine or β -hex release from rat MCs or HCMCs (Figure 1, A, C, and D).

AM Induces MC Chemotaxis

A classic biphasic migration curve was produced when cells were exposed to varying amounts of AM demonstrating the ability of this peptide to regulate human MC (hMC) movement throughout a wide concentration range (Figure 2, A–C). Statistically significant hMC movement could be achieved at subnanomolar concentrations of AM, and a maximum migratory effect was observed at 10 nmol/L, comparable on a molar basis to the effect induced by VEGF (10 nmol/L), used as the positive control for the assay. At AM concentrations exceeding this peak, hMC motility began to diminish back to baseline values. Additional proof of AM as a chemoattractant for MCs comes from the microscopic examination of the neovascular network elicited by exogenous AM in the DIVAA model (Figure 2D; see below). Red tryptase staining demonstrates multiple infiltrating murine MCs among the newly formed blood vessels suggesting that AM has the ability to attract MCs to the site of its release *in vivo*.

AM Induces MC Expression of Proangiogenic Factors

As determined by real-time PCR, 100 nmol/L AM (which is below the threshold required to induce MC degranulation) was shown to induce the expression of a variety of angiogenic factors in both undifferentiated and differentiated HMC-1 when assessed in a time-course study. AM up-regulated VEGF, MCP-1, and bFGF mRNA expression in hMCs within 3 to 12 hours after treatment, and these augmented values returned to baseline levels or below by 48 hours (Figure 3, A–C). The same relative trend of induction of these angiogenic factors was observed in both undifferentiated and differentiated hMCs.

AM mRNA and Peptide Regulation in hMC by Hypoxia and Hypoxia Mimetic

We have previously shown the ability of hypoxia to up-regulate AM expression on several cell types of epithelial origin.²⁴ When differentiated HMC-1 cells were cultured in a low-oxygen atmospheric environment, a dramatic increase in AM mRNA was observed throughout the next 48 hours (Figure 4A). Interestingly, this hypoxic response

Figure 1. Peptide-induced histamine (HA) or β -hex release from MCs. **A:** The resulting dose-response curves measure the ability of different peptides to induce histamine release from isolated rat peritoneal MCs. The neutralizing anti-AM MoAb-G6 (10 μ g/ml; filled inverted triangles) had no effect on AM-mediated histamine release (filled triangles). Peptide fragments AM1-12 (diamonds), AM34-52 (circles), and AM22-52 (open squares) had virtually no effect in eliciting MC degranulation. Increases in net charge tracks with the ability of a given peptide to induce histamine release as demonstrated by comparing Nal-Arg (filled squares) to LHRH (open triangles) results. Each point represents the mean and SD of five independent repeats. **B:** Dose-response curve of AM-induced β -hex release from HMCs. **C:** Effect of varying concentration of anti-AM MoAb-G6 on AM (100 μ mol/L)-mediated HMC degranulation. **D:** Complement factor H effect on AM (100 μ mol/L)-induced β -hex release in HMCs. Each bar represents the mean and SD of four independent repeats. ns, Not statistically significant; *** P < 0.001.

was severely attenuated in undifferentiated HMC-1 cells (Figure 4A). Under normoxic conditions, neither undifferentiated or differentiated HMC-1 showed changes in AM mRNA within the first 24 hours of the experiment (Figure 4A). A similar trend in AM expression was noted when differentiated or undifferentiated HMC-1 cells were challenged with the hypoxia mimetic/iron chelator, desferrioxamine mesylate and this response was blocked by the addition of ferrous ethylenediammonium sulfate (data not shown). Peptide levels in the HMC-1-conditioned media followed the trend observed for AM mRNA expression (Figure 4B). Differentiated HMC-1 cells exposed to reduced oxygen tension showed approximately threefold increase (P < 0.001) in AM released to the conditioned media by 48 hours whereas undifferentiated HMC-1 cells

showed no statistically significant augmentation throughout the same time period (Figure 4B).

Human Tumor MC Infiltrates Express Immunoreactive AM

Confocal microscopy on archival material of human breast cancer (Figure 5, A–D) and human lung adenocarcinoma (Figure 5, E–H) shows an AM-producing MC infiltrate in the connective tissue surrounding the human cancers. Presence of such cells is assessed by co-localization of the MC marker tryptase (green deposition; Figure 5, B and F) and AM (red fluorescence; Figure 5, A and E).

Figure 2. AM functions as a chemotactic factor of human MC. **A:** Dose-dependent modulation of hMC migration by AM. Each bar represents the mean and SD of three independent repeats. * $P < 0.05$ and ** $P < 0.01$. **B:** HMC-1 control migration through porous filter. **C:** HMC-1 migration through porous filter in the presence of 1 nmol/L AM. **D:** Cross-section of Matrigel plug containing AM from DIVAA analysis, which identifies MC infiltrates (red stain) at the site of newly formed blood vessels. Scale bar, 50 μ m; 25 μ m (inset).

HMC-1 AM Effects on Anchorage-Dependent and -Independent Tumor Growth

To discriminate the role of AM in the interaction between MCs and tumor cells we generated HMC-1 and A549 transfectants expressing siRNA for either AM or scramble sequences. AM mRNA was reduced in both cell types by more than 65% as assessed by quantitative real-time PCR (Figure 6A). We subsequently performed MTT assays on AM-knockdown A549 cells (A549-511) in the presence of conditioned media from control MCs (HMC-1-SCR) or AM-depleted HMC-1 cells (HMC-1-511). A549-511 cells were able to proliferate at faster rates when grown in conditioned media from HMC-1-SCR than in conditioned media from HMC-1-511 throughout a 5-day time course (Figure 6B). Additionally, mixed culture experiments of A549-511 and PMA-treated HMC-1-SCR or HMC-1-511 were performed in soft agar. HMC-1 was unable to grow after exposure to PMA (Figure 6C). In

these experiments A549-511 showed a higher anchorage-independent growth capability when grown in the presence of HMC-1-SCR than HMC-1-511 (Figure 6D).

AM Secreted from MCs Induces Angiogenesis in Vivo

Using DIVAA analysis we found that HMC-1 cells induced an *in vivo* neovascularization response in the nude mouse model (Figure 7). This hMC-mediated blood vessel proliferative effect was inhibited by the AM-neutralizing MoAb-G6 monoclonal antibody (Figure 7). An unrelated isotypic control immunoglobulin, at the same concentration as MoAb-G6, did not suppress the HMC-1-induced effect. Thus, the MoAb-G6 data confirms, that at least in part, HMC-1-driven angiogenesis was modulated through MC-derived AM.

Figure 3. AM stimulates the expression of MC proangiogenic factors. **A:** AM (100 nmol/L) exposure causes a time-dependent increase in VEGF mRNA expression in differentiated (+PMA, **diamonds**) and undifferentiated (-PMA, **squares**) HMC-1 cells. **B:** AM-induced MCP-1 expression in HMC-1 cells. **C:** AM-mediated bFGF expression in HMC-1 cells. The y axis represents relative mRNA induction for a given angiogenic factor when comparing plus/minus AM treatment.

Figure 4. Hypoxia up-regulation of AM mRNA/peptide expression in human MCs. **A:** Reduced oxygen tension (1.0% O₂) caused a dramatic increase in AM mRNA expression in differentiated HMC-1 cells (**squares**) throughout a 48-hour interim while having no effect on undifferentiated HMC-1 cells (**circles**) throughout the same time period. AM mRNA normoxic response data for differentiated (**diamonds**) and undifferentiated (**triangles**) HMC-1 cells is also presented. **B:** Time-dependent release of immunoreactive AM detected in the conditioned media of HMC-1 cells incubated under normoxic or hypoxic conditions (see symbols above for treatment identification).

Discussion

Recent studies have suggested that the MC may be involved in major human diseases such as arteriosclerosis, liver cirrhosis, osteoporosis, fibrosis, and cancer.^{15,43,44} A better understanding of tumor and/or MC-derived factors modulating mechanisms such as MC migration and degranulation, cancer cell growth, angiogenesis, or apoptosis resistance at the tumor body may prove to be rational biological targets for the therapeutic intervention of human cancers. Here we explore the hypothesis that AM is a peptide link between tumor cells and MC infiltrates, regulating the function of both cell types and resulting in enhanced tumor promotion and progression.

AM has been previously shown to induce histamine release from rat peritoneal MCs at concentrations exceeding 1 μ mol/L.³⁴ We have now extended this initial observation to include human MCs based on β -hex release as an indicator of AM-induced degranulation. Polycationic peptides such as SP, at micromolar concentrations, have been shown to induce MC histamine release through a receptor-independent mechanism.⁴⁵ AM is also a highly charged peptide (pI = 9.7) and therefore may mediate its regulatory control on MC histamine release via a similar receptor-independent mechanism. There are several experimental observations that support this line of thinking. First, even though transcriptional products encoding AM receptor components have been identified in rat peritoneal MCs,⁴⁶ the concentration of AM required to induce MC histamine release far exceeds the binding constant for the cognate AM receptor by several orders of magnitude.⁴⁷ Second, we have demonstrated that neither MoAb-G6 (antagonist) nor complement factor H (superagonist) has any effect on AM-induced MC degranulation, both reagents having been shown previously to be dynamic modulators of AM/receptor biology. Based on our collective findings, there is compelling evidence to support a receptor-independent mechanism as the basis of AM-induced histamine release in MCs.

We have demonstrated that AM mediates a biphasic response on human MC migration similar to the one observed with other chemotactic factors such as VEGF, bFGF, and MCP-1.^{48,49} Hence, variability in the rate of MC movement directly relates to the concentration of AM exposure and a parallel situation would be expected to take place in a tissue environment. As MCs traverse a radiating AM gradient toward the ligand source (ie, the

Figure 5. Immunohistochemical detection of AM-containing MCs in human cancer tissue. **A–D:** Archival material of human breast cancer; **E–H:** pathological specimens of human lung adenocarcinoma. Red fluorescence is indicative of AM immunostaining (**A** and **E**), green deposition represents localization of tryptase-containing MCs (**B** and **F**), blue coloration is 4,6-diamidino-2-phenylindole staining of cellular nuclei (**C** and **G**), and yellowish staining indicates co-localization of AM and tryptase in MCs (**arrows** in **D** and **H**). tc, Tumor cell; br, bronchiolus. Scale bar, 15 μ m.

Figure 6. MC-derived AM enhances A549 anchorage-dependent and -independent growth. **A:** AM mRNA evaluation by quantitative real-time PCR of HMC-1 and A549 cells expressing a siRNA for AM or scrambled sequence. A significant down-regulation of at least 65% was observed in both cells lines. **B:** MTT growth assay showing the effect of conditioned media from HMC-1-SCR and HMC-1-511 on A549-511 growth. A549-511 was able to grow significantly faster in the presence of conditioned media from HMC-1-SCR (diamonds) than HMC-1-511 (squares). **C:** MTT growth assay showing complete absence of growth in HMC-1 after treatment with PMA. **D:** Comparison of the anchorage-independent growth of A549-511 in co-culture with PMA-treated HMC-1-SCR or HMC-1-511 in clonogenic assays. A549-511 cloned better in the presence of HMC-1-SCR than when co-cultured with HMC-1-511. ** $P < 0.01$ and *** $P < 0.001$.

tumor cell), a rise or fall migratory response would be put into play, ultimately resulting in MC infiltrates localizing at the tumor body or periphery. This model is consistent with the *in vivo* data generated in this study showing the presence of MC infiltrates in AM-producing tumors and migration of murine MCs toward the Matrigel containing synthetic AM in the mouse DIVAA assay.

MC infiltrates and the hMC line HMC-1 have the ability not only to respond to AM but also to produce this peptide. MC-derived AM released at a primary cancer site could enhance the growth capacity of the tumor. Focal areas of low oxygen availability are inherent to the carcinogenesis process,⁵⁰ and we have previously shown that hypoxic insult elevates AM expression in human epithelial tumors.²⁴ However, our studies with HMC-1 have revealed undifferentiated MCs (–PMA) to have a severely

attenuated AM response compared to that observed for mature MCs (+PMA) when challenged by 1% O₂ or hypoxia mimetic. Our findings seem to parallel what would be expected for MC precursors (immature/undifferentiated state) circulating in an oxygen-rich bloodstream versus the mature MC (differentiated state) found in an oxygen-poor environment of the loose connective tissue. Hence, the observed AM/hypoxia response profile of MCs is dependent on the cell's maturation state.

It has been previously described by Theoharides and Conti⁵¹ that MCs can have diametric roles in their ability to regulate tumor cell growth depending on the cellular factors they release. These authors demonstrate that total granule release during MC degranulation would result in a massive proinflammatory response at the tumor site that is detrimental for cell survival. Alternatively, selective

Figure 7. MC-derived AM functions as an *in vivo* angiogenic factor. HMC-1 (10^4 cells/angioreactor) induces a statistically significant increase ($P < 0.001$) in new blood vessel formation as compared to the empty angioreactor when evaluated by DIVAA analysis. The addition of $0.8 \mu\text{g/ml}$ of MoAb-G6 markedly suppressed ($P < 0.001$) HMC-1-induced angiogenesis demonstrating AM dependence of vessel formation. An indifferent isotopic control antibody (MOPC315) was ineffective in suppressing the response. Relative fluorescence unit (RFU) values are directly proportional to the amount of new blood vessels formed for a given experimental sample. Each bar represents the mean and SD of six independent repeats. *** $P < 0.001$.

release of critical granules containing mitogenic/trophic factors would establish an enriching milieu to drive tumor proliferation. As we demonstrated earlier in the Results section, AM at submicromolar concentrations is incapable of eliciting MC degranulation but will, however, induce proangiogenic factor expression in this cell type and on selective release will induce neovascularization events at the tumor body. Given that AM is rapidly degraded by tumor cell/MC matrix metalloproteinase-2,⁵² it is highly unlikely that this peptide reaches the micromolar concentration threshold required for MC degranulation to occur within the tissue stroma surrounding the tumor. Hence, due to inherent enzymatic properties at the tumor cell/MC interface, it is much more likely that AM levels conducive to selective granule release would be attained at this anatomical site, thereby favoring a tumor proliferative response.

Immunohistochemical analysis of pathological tissue from patients with lung or breast cancer reveals the presence of AM-producing MC in infiltrates of tumors. This clinical finding further implicates AM-mediated autocrine/paracrine interactions between tumor and MCs. AM is a potent angiogenic factor with similar molar activity to VEGF and bFGF.⁵³ Furthermore, transfected cancer cells that overexpress AM were shown to induce a larger neovascularization response in nude mice than did the wild-type transfected cells.²⁶ Thus, it seems reasonable that AM coming from either the tumor cell or surrounding stromal components would mediate new blood vessel formation. In support of this logic, we have shown that HMC-1 cells can induce an *in vivo* angiogenic response using DIVAA and that this effect was suppressed by MoAb-G6. Therefore, MCs induce angiogenesis at the

Figure 8. Proposed model of the AM/tumor cell/MC relationship in human carcinogenesis. Tumor-derived AM is released into the microenvironment setting up a concentration gradient of peptide that attracts distal MCs to infiltrate the tumor site. As MCs migrate up the peptide gradient, higher AM concentrations are reached, slowing MC migration and stimulating MC-derived angiogenic factor expression and ultimately MC release at the tumor site. Given that the microenvironment around tumor cells tends to be hypoxic, this reduced oxygen state would also augment MC-derived AM expression/release. MC-derived AM could mediate a paracrine tumor survival effect (direct mitogen, angiogenic factor, and anti-apoptosis), initiate an autocrine loop-based relationship, or function as a paracrine recruitment factor drawing additional MCs to the area, thus perpetuating the inflammatory process and enhancing tumor promotion.

site of the neoplastic lesion via AM release. In addition, using a mixed culture strategy and targeted AM siRNA suppression, we have shown that AM of MC origin can directly simulate the growth of human lung tumor cells *in vitro*, again reinforcing the ability of MC infiltrates to regulate tumor cell growth through an AM cross-talk mechanism.

Taken collectively our data reveals AM to be a common peptide mediator linking tumor/MC communication and serving to enhance the tumorigenic capability of human cancers. An overview of our proposed model on the AM/MC/tumor interactive relationship is summarized in Figure 8. Given the hypoxic condition of most human tumors and the ability of this environmental insult to up-regulate AM expression,^{24,25,50} we hypothesize that AM is released from the tumor cell and establishes a concentration gradient within the surrounding microenvironment. Subnanomolar amounts of AM initiate MC migration toward the tumor cell, ultimately generating MC infiltrates in and around the tumor body. AM expression would also be up-regulated in these differentiated MCs in response to the hypoxic milieu establishing an autocrine/paracrine interaction with the surrounding cellular microenvironment (including the tumor cells). As AM concentrations approach nanomolar levels and higher within the MC/tumor complex, expression of angiogenic factors (VEGF, MCP-1, and bFGF) is up-regulated in MCs, and these factors are released into the surrounding microenvironment, driving tumor promotion and angiogenesis. In addition, AM produced by MCs would enhance the growth and clonogenic capabilities of tumor cells. One could easily envision a scenario in which cancer cells and infiltrating MCs contribute to the overall expression of AM at the tumor body, ultimately driving neoplastic growth in this region through AM direct/indirect mechanisms.

Based on our previous findings on AM as an important tumor cell survival factor and our present revelations on the AM/MC/tumor interactive relationship, it becomes increasingly clear that reagents that suppress the biological activity of AM (neutralizing monoclonal antibodies, peptide antagonists, or small molecule inhibitors^{23,54}) and/or the tumor/MC relationship may prove to have therapeutic relevance in the clinical management of human cancers.

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