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Guanylyl Cyclase C Prevents Colon Cancer Metastasis by Regulating Tumor Epithelial Cell Matrix Metalloproteinase 9

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Abstract

Matrix metalloproteinase 9 (MMP-9) produced by colorectal cancer cells is a critical determinant of metastatic disease progression and an attractive target for antimetastatic strategies to reduce colon cancer mortality. Cellular signaling by cGMP regulates MMP-9 dynamics in various cell systems and the bacterial enterotoxin receptor guanylyl cyclase C (GCC), the principle source of cGMP in colonocytes which is overexpressed in colorectal cancers, inhibits tumor initiation and progression in the intestine. Here, we demonstrate that ligand-dependent GCC signaling through cGMP induces functional remodeling of cancer cell MMP-9 reflected by a compartmental redistribution of this gelatinase, in which intracellular retention resulted in reciprocal extracellular depletion. Functional remodeling of MMP-9 by GCC signaling reduced the ability of colon cancer cells to degrade matrix components, organize the actin cytoskeleton to form locomotory organelles and spread, and hematogenously seed distant organs. Of significance, GCC effects on cancer cell MMP-9 prevented establishment of metastatic colonies by colorectal cancer cells in the mouse peritoneum *in vivo*. Since endogenous hormones for GCC are uniformly deficient in intestinal tumors, reactivation of dormant GCC signaling with exogenous administration of GCC agonists may represent a specific intervention to target MMP-9 functions in colon cancer cells. The notion that GCC-mediated regulation of cancer cell MMP-9 disrupts metastasis, in turn, underscores the unexplored utility of GCC hormone replacement therapy in the chemoprevention of colorectal cancer progression.

Keywords

Colorectal cancer; guanylyl cyclase C; cyclic GMP; matrix metalloproteinase 9; metastasis

INTRODUCTION

The majority of patients (~60%) with colorectal cancer, the third most common neoplasm in men and women in developed nations, exhibit metastatic disease at the time of diagnosis (1). Progress in postsurgical adjuvant chemotherapy is effectively improving clinical management by prolonging disease-free intervals in patients with metastatic spread confined at regional sites (2,3). However, advanced metastatic disease with tumor colonization of distant organs remains untreatable (3), reflected by high disease-specific mortality rate (~50%) (4). In that regard,

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colorectal cancer cell dissemination is the result of a complex progression regulated by universal metastatic mediators encompassing extracellular matrix (ECM) remodeling, invasion through tissue boundaries and seeding of target organs (5). The vulnerability of the metastatic cascade, including a coordinated series of rate-limiting steps (5), predicts that disruption of one or more of these underlying mechanisms may greatly improve the prognosis of patients with colon cancer by interrupting disease progression.

Matrix metalloproteinase 9 (MMP-9) promotes key processes underlying tumor cell propagation, from migration and invasion to angiogenesis and tumor growth (6,7). A zinc-dependent metalloendopeptidase, MMP-9 cleaves specific factors constituting or present in the ECM, including collagen IV in basement membranes and angiogenic molecules (7), thereby creating a physical and biochemical path for tumor invasion and neovascularization (8). MMP-9 confers invasive abilities to cancer cells by regulating the function of dynamic membrane regions (9,10). In turn, actin cytoskeleton remodeling associated with membrane protrusion (lamellipodia, invadopodia) formation induces polarized MMP-9 release (9,11). In colorectal cancer, expression of tumor epithelial cell MMP-9 positively correlates with disease progression (12), suggesting that cancer cell MMP-9 controls essential functions mediating metastasis. Indeed, colorectal cancer cells secrete MMP-9, which promotes ECM degradation and tumor cell seeding (13). Thus, targeting tumor epithelial cell MMP-9 may represent a novel therapeutic strategy for colorectal cancer by disrupting fundamental processes underlying metastasis. However, an obstacle to this approach is the lack of selective therapeutics that inhibit MMP-9 mediating metastasis in cancer cells, without perturbing the physiological activity of MMP-9 in normal tissues (6,14,15).

Beyond inhibition of catalytic activity (6), regulation of transcription, translation or intracellular trafficking through tumor-specific signal transduction mechanisms represent attractive modalities to decrease the pro-metastatic function of MMP-9 in the pericellular space (16,17). In that context, the guanylyl cyclase (GC)/cGMP transduction system reduces MMP-9 mRNA levels in rat renal mesangial cells (18), and inhibits the secretion of MMP-9 in vascular smooth muscle cells (19). Intriguingly, intestinal epithelial cells selectively express the transmembrane receptor GCC, a particulate GC promoting GTP to cGMP conversion upon binding to endogenous ligands guanylin and uroguanylin and diarrheagenic bacterial heat-stable enterotoxins (STs) (20–22). Normally confined to apical membranes of intestinal mucosa cells (23), GCC is uniformly overexpressed in human colon tumors including those at extra-intestinal sites (24–26), where it behaves as a selective cell surface receptor for targeted drug delivery (27).

The present study demonstrated GCC and cGMP induce a compartmental redistribution of cancer cell MMP-9 which resulted in suppression of the proteolytic activity of that gelatinase in the pericellular space and inhibition of tumor seeding and metastasis. These observations underscore the potential of GCC-targeted strategies inhibiting cancer cell MMP-9 for metastatic colorectal cancer chemoprevention.

METHODS

Reagents

ST, the inactive analog TJU 1–103 (28) and human recombinant pro-MMP-9 (29) were prepared as described. Uroguanylin and guanylin were from Peninsula Laboratories (Belmont, CA), while purified human recombinant active MMP-9 (67-kDa form) from Calbiochem (San Diego, CA). Antibodies to human MMP-9 and MMP-2 were from Biomol (Plymouth Meeting, PA). Antibody to human GAPDH was from SantaCruz Biotechnology (Santa Cruz, CA). Alexa fluor 555 anti-rabbit IgG, Oregon Green 488 phalloidin and Slowfade Gold with DAPI were

obtained from Molecular Probes (Eugene, OR). Cyclic GMP analogs (8-br-cGMP and 8-pCPT-cGMP) and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell culture

T84 and Caco-2 human colon carcinoma cells (American Type Culture Collection, Manassas, VA) were cultured (37°C, 5% CO₂) with 10% fetal bovine serum in DMEM/F12 or DMEM, respectively. Unless otherwise indicated, treatments were conducted for 24 h in serum-free media.

Cell transductions

Full length MMP-9 cDNA (Invitrogen) was sub-cloned into Xho1-EcoR1 multiple cloning site of mouse stem cell virus (MSCV)-puro retroviral vector employing forward (5'-ATCTCGAGATGAGCCTCTGGCAGCCC-3') and reverse (5'-ATGAATTCCTAGTCCTCAGGGCACTGC-3') primers. HEK 293T cells were transfected with 1 µg of either purified MSCV-puro or MSCV-puro-MMP-9 plasmid plus 1 µg of the packaging vector pCl-Ampho employing Fugene (Roche, Basel, Switzerland). Viral supernatants supplemented with 4 µg/ml polybrene were used to transduce T84 (for 72 h). Cells transduced with MMP-9 or empty vector were selected with 5 µg/ml puromycin (for 1 week) and maintained in 1 µg/ml puromycin.

Immunofluorescence

Cancer cells were fixed (15 min) with ice-cold paraformaldehyde (4%, in PBS) and permeabilized (10 min) with ice-cold acetone. Following overnight incubations (4°C; in PBS containing 2% bovine serum albumin) with rabbit anti-MMP9 antibody (1:100) and 25 µl/ml Oregon Green 488 phalloidin, cells were incubated (60 min at room temperature) with Alexa fluor 555 anti-rabbit IgG. Specificity of antigen/antibody reactions was assessed employing the secondary antibody alone. To identify cell nuclei, preparations were counterstained with Slowfade Gold anti-fade reagent with DAPI. Images were acquired with a confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany).

Real-time reverse transcription (RT)-PCR

Total RNA was isolated from cancer cells with RNA Easy kit (Qiagen, Valencia, CA) and from mouse diaphragms with TRI Reagent (Molecular Research Center, Cincinnati, OH). RNA was subjected to one-step RT-PCR on a 7000 Sequence Detection System for 45 cycles (95°C, 5 min; 94°C, 20 sec; 62°C, 1 min) using TaqMan EZ RT-PCR core reagents (Applied Biosystems Inc., Foster City, CA). The mRNAs for human GCC, MMP-9 and β -actin, and for mouse β -actin were quantified employing specific fluorescently labeled primer/probe sets (Assay on Demand, Applied Biosystems). Data were analyzed using Sequence Detection Software (Applied Biosystems) with thresholds set at 0.2. Template-negative controls were run on each PCR plate. MMP-9 or GCC mRNA levels were normalized to human or mouse β -actin mRNA, respectively.

Immunoblot analysis

Protein samples (in SDS loading buffer) were separated by electrophoresis on 10% acrylamide Tris-Glycine gels, transferred to nitrocellulose membranes, and probed with rabbit polyclonal antibodies against MMP-9, MMP-2 or GAPDH (1:1,000 each) in TBS-Tween (3.5% milk) overnight at 4°C. After washing the primary antibody, membranes were probed with goat anti-rabbit IgG antibody (1:2,000) (SantaCruz Biotechnology) for 1 h at room temperature. Immunostained bands were visualized by autoradiography using horseradish peroxidase and quantified by densitometry.

Gelatin zymography

Tumor cell conditioned media (500 μ l) were concentrated using Centricon-30 filters (Millipore Corporation, Bedford, MA) and resolved on 0.1% gelatin, 10% acrylamide electrophoretic gels under denaturing conditions. After renaturation (for 1 h), proteins were incubated (18 h; 37°C) in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM CaCl_2 . Gelatinolytic activity was quantified by densitometry following Coomassie blue staining (30).

Flow cytometry

T84 cells were harvested with 0.05% Trypsin-EDTA, pelleted by centrifugation and fixed (20 min) in ice-cold 2% paraformaldehyde. After washing, cells ($\sim 1 \times 10^6$) were either permeabilized with 90% methanol or incubated with 1% BSA in PBS (4°C, 20 min). Cells were washed again and incubated (4°C, 30 min) with rabbit anti-MMP9 (1:100) in the presence or absence of a specific blocking peptide (Biomol). After additional washes, cells were incubated with AlexaFluor 488 anti-rabbit secondary antibody (1:200) and analyzed employing a Fluorescent Activated Cell Sorting (FACS) Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Cell spreading (13)

Cancer cells ($\sim 5 \times 10^4$) were plated in T25 flasks. After 6 h, the number of cells that formed distinct protrusions was scored as the fraction of total cell number per microscopic field (≥ 100 cells) of an inverted microscope.

Metastatic lung seeding (31)

Following treatments, cancer cells were fluorescently labeled (30 min, 37°C) with 0.2 μ M MitoTracker Red CMXRos (Molecular Probe Inc.), washed and harvested with 0.05% trypsin-EDTA. Cells ($\sim 6 \times 10^5$ per ml) were resuspended in serum-free medium and injected into the inferior vena cava of anesthetized (Nembutal) female *nu/nu* mice (4–6 weeks old; Taconic Farms, Germantown, NY), artificially ventilated by a pressure-controlled ventilator through a tracheotomy. After 5 min, the pulmonary vasculature was cleared of blood and lungs were excised, placed onto a slide chamber with ports for the tracheal cannula and inflated with 5% CO_2 in air (31). Fluorescent tumor cells attached to pulmonary microvasculature in the left lobe were detected using inverted fluorescent microscopy, and quantified by scanning 45 consecutive fields (1.1 mm^2 of field/picture) employing OpenLab (Improvision, Boston, MA) (31).

Peritoneal metastasis

Following treatments, cells ($\sim 1 \times 10^7$ per ml) were injected into the lower-right peritoneal cavity of lightly anesthetized male Cr:NIH-*bg-nu-Xid* mice (4–6 weeks old; NCI, Frederick, MD). Two weeks after injections mice were sacrificed by CO_2 inhalation, the diaphragmatic peritoneum excised and either fixed with 3.7% formalin or placed in RNA-Later for RT-PCR analysis (see above). Paraffin-embedded sections (5 μ m) of diaphragmatic peritoneal rolls were stained with H&E and examined with a computer attached to a light microscope. All animal procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Statistical analysis

Unless otherwise indicated, data are mean $\pm$ SEM of ≥ 3 independent experiments. Statistical analyses were performed employing the unpaired Student's *t* test.

RESULTS

GCC and cGMP signaling inhibits MMP-9 release into the extracellular compartment by colon cancer cells

Induction of GCC signaling with two membrane-permeant cGMP analogs or the bacterial enterotoxin ST, a potent GCC agonist inducing cytostasis by increasing intracellular cGMP (28), significantly diminished the accumulation of the MMP-9 pro-form (pro-MMP-9, 92 kDa) in the media conditioned by human colon cancer cells (Fig. 1A). Pro-MMP-9 depletion in the extracellular space did not reflect increased cleavage of the inhibitory pro-peptide and pro-MMP-9 conversion into the active MMP-9 form (84 kDa) (7), since accumulation of active MMP-9 was also reduced in the media conditioned by tumor cells with induced cGMP signaling, as assessed by gelatin zymography (Fig. 1B, *upper panel*). Rather, cGMP-mediated MMP-9 depletion was associated with reduced MMP-9-dependent gelatinolytic activity in tumor cell conditioned media (Fig. 1B, *lower panel*). Moreover, cGMP effects on MMP-9 did not reflect changes in gene expression, as MMP-9 mRNA levels by RT-PCR were nearly identical in cancer cells treated with the vehicle PBS or agents inducing cGMP signaling (Fig. 1C). However, flow cytometry analyses revealed that ST induces a dramatic increase (≥ 10 fold) in intracellular MMP-9 compared to the vehicle control (Fig. 2A, *upper, middle panel*; cells were methanol-permeabilized). In contrast, the total amount of membrane-bound MMP-9 protein was not altered by ST (Fig. 2A, *lower, middle panel*; cells were not permeabilized). The specificity of these observations was demonstrated by omitting the primary antibody (Fig. 2A, *left panels*) or administering the specific blocking peptide (Fig. 2A, *right panels*), procedures that eliminated any detectable MMP-9 signal. Immunoblot analyses of total cell lysates (Fig. 2B) and confocal microscopy (Fig. 3A, *MMP-9 panels*) with anti-MMP-9 polyclonal antibody confirmed that cancer cells exposed to ST exhibit intracellular accumulation of MMP-9. Importantly, intracellular MMP2 levels were not affected by ST (Fig. 2B). Thus, GCC activation induces a selective compartmental redistribution of MMP-9 in colorectal cancer cells, wherein MMP-9 retention in the intracellular compartment results in MMP-9 depletion in the extracellular space associated with reduced MMP-9-dependent proteolytic activity.

GCC-mediated regulation of cancer cell MMP-9 prevents tumor cell spreading

The catalytic activity of MMP-9 secreted by cancer cells promotes tumor cell locomotion through enhanced formation of membrane protrusions (filopodia, lamellipodia) (13). Colon cancer cells extend membrane protrusions to spread and migrate along 2-dimensional surfaces (arrows in *upper, left panel* of Fig. 3A). However, formation of these locomotory organelles was disrupted by ST treatment (Fig. 3A, *lower, left panel*). Indeed, compared to the vehicle control, tumor cells with ST-stimulated GCC activity displayed a rounded morphology in the absence of actin-rich filopodia and lamellipodia (Fig. 3A, *β -Actin panels*). Moreover, rounded ST-treated cells exhibited intracellular MMP-9 accumulation (Fig. 3A, *MMP-9 panels*), consistent with the hypothesis that in colon cancer cells GCC signaling inhibits membrane protrusion formation, in part, by inducing intracellular MMP-9 retention. Accordingly, ST significantly reduced membrane protrusion-driven cell spreading, an effect mimicked by the endogenous GCC ligands guanylin and uroguanylin but not an inactive ST analog (Fig. 3B). GCC effects on cell spreading were cGMP- and MMP-9-dependent, since a membrane-permeant cGMP analog mimicked ST actions whereas administration of active MMP-9 blocked inhibition of cell spreading by ST or cGMP (Fig. 4A). To confirm that MMP-9 is the downstream molecular target of impaired tumor cell locomotion by GCC, a stable colon cancer cell line which over-expressed (Fig. 4B) and over-secreted MMP-9 (Fig. 4C) was generated. As expected, MMP-9 over-expressing, but not empty vector control, cells were resistant to ST-induced inhibition of cell spreading (Fig. 4D).

GCC-mediated regulation of cancer cell MMP-9 reduces hematogenous metastatic seeding

Metastatic seeding of the mouse pulmonary microvasculature, an MMP-9-dependent process (13), can be visualized by fluorescence microscopy following intracaval injection of fluorescently labeled tumor cells (Fig. 5A) (13,31). Colon cancer cells treated *in vitro* with ST exhibit a reduced ability to attach to the lung microvasculature *in vivo* (Fig. 5A). The endogenous GCC ligand uroguanylin or a membrane-permeant cGMP analog mimicked, while addition of purified human pro-MMP-9 blocked ST effects on metastatic seeding (Fig. 5B). Moreover, a broad inhibitor of MMP-dependent catalytic activity restored sensitivity to ST-induced inhibition of hematogenous seeding in tumor cells supplemented with pro-MMP-9 (Fig. 5B), confirming that the proteolytic activity of endogenous MMP-9 in the pericellular space confers seeding abilities (13). Finally, whereas it significantly reduced metastatic seeding by tumor cells stably expressing the empty vector, ST had no effect on MMP-9 overexpressing tumor cells (Fig 5A–B), demonstrating that GCC inhibits metastatic cell dissemination by regulating MMP-9.

Targeting GCC signaling suppresses peritoneal metastasis by colon cancer cells

Treatment with ST *in vitro* prevented the establishment of metastatic tumors in the mouse peritoneum *in vivo* by human colorectal cancer cells (Fig. 6). Whereas cancer cells treated with the vehicle control produced substantial diaphragmatic micrometastases 2 weeks after intra-peritoneal injection (arrows in *left panel* of Fig. 6A), human cancer colonies from ST-treated cells could not be detected by H&E on mouse peritoneal rolls (Fig. 6A, *right panel*). Quantification of metastatic tumor burden by RT-PCR for human GCC mRNA revealed that ST significantly reduced the amount of persistent colon cancer cells which resided in the mouse peritoneum, compared to the vehicle controls (Fig. 6B). Importantly, ST-effects on peritoneal metastasis were abolished in MMP-9 overexpressing cancer cells (Fig. 6B), indicating that MMP-9 mediates GCC-dependent inhibition of metastasis formation. Further, ST reduced (~30–40%) the incidence of peritoneal metastasis, defined as the percentage of mice with any detectable human GCC mRNA in peritoneal specimens, in animals injected with either wild type colon cancer cells (PBS, 60% vs ST, 42.8%) or isogenic clones expressing the empty vector (PBS, 62.5% vs ST, 35%), but was ineffective in cells overexpressing MMP-9 (PBS, 67% vs ST, 66%). Together, these observations suggest that ligand-dependent GCC signaling suppresses the ability of colorectal cancer cells to invade and establish metastatic colonies in target organs by regulating the function of cancer cell MMP-9.

DISCUSSION

GCC and cGMP have emerged as key physiological regulators of intestinal crypt-villus homeostasis (27). Mice with loss of GCC signaling exhibit dysregulated intestinal proliferation and differentiation (32) and are susceptible to colorectal tumorigenesis (33). Protective effects by GCC depend on discreet cGMP-mediated signaling events, including protein kinase activation and ion channel currents (20,32,34). The present findings that ligand-induced GCC signaling negatively regulates MMP-9 and its dependant functions in colon cancer cells reveal a previously unappreciated arm of the GCC/cGMP signalome, probably contributing to antitumorigenesis (27). Importantly, GCC is uniformly overexpressed during transformation and may be exploited as a selective therapeutic target for metastatic colorectal cells in extra-intestinal sites (24,25,35), including the use of GCC ligands to regulate cancer cell MMP-9.

Although significant, the role of MMP-9 in metastasis is controversial. MMP-9 is a fundamental component of invadopodia, dynamic membrane structures directing ECM degradation and tumor cell invasion (11,36). Increased expression and secretion of MMP-9 at invadopodia tips conferred metastatic behavior to CT26 murine colon adenocarcinoma cells (37). Moreover, MMP-9 promotes the angiogenetic switch supporting tumor progression by

activating ECM-embedded VEGF (38). Since it regulates most of the rate-limiting steps underlying metastasis (39), MMP-9 could represent an ideal target for effective disruption of the metastatic cascade. However, MMP-9 also mediates wound healing and inflammation (14,15) and opposes tumor growth by mobilizing angiogenetic inhibitors angiostatin and endostatin (40). The dual role in pathological and physiological processes may underlie the disappointing results obtained in cancer clinical trials with MMP inhibitors (6). Of significance, mechanisms of tissue repair and tumor suppression primarily depend on the MMP-9 contributed by stromal cells, including cancer-associated fibroblasts, which are principle sources of MMPs within tumors (41). Conversely, MMP-9 contributed by colorectal cancer cells specifically mediates pathological processes promoting metastasis (13). Further, relative overexpression of MMP-9 in tumor epithelial, but not stromal, cells compared to matched normal adjacent tissues, correlates with lymph node metastasis and disease progression in colon cancer (12). Confinement of GCC expression and signaling exclusively to epithelial cell populations within both normal and transformed intestinal tissues (24,25,35), in the context of the GCC-mediated inhibition of MMP-9 functions described here, offers the unrecognized therapeutic opportunity to selectively target the pathological MMP-9 species mediating metastasis in colon cancer employing specific GCC ligands.

Beyond physicochemical differences, unique pro-metastatic activities by colon cancer cell MMP-9 probably reflect stringent spatiotemporal requirements of its functional regulation. Indeed, successful lamellipodia- or invadopodia-driven migration and invasion relies on the polarized release of MMP-9 at critical membrane microdomains, an obligatory step toward productive MMP-9 activation at the pericellular space (9,11). Here, GCC signaling in cancer cells induced a compartmental shift of MMP-9, reflected by intracellular MMP-9 retention and decreased enzyme activity in the extracellular compartment. Arguably, GCC effects on MMP-9 are intimately linked to spatiotemporal remodeling of MMP-9 functional dynamics imposed by changes in the cellular/pericellular MMP-9 balance affecting surface localization, productive membrane binding and catalytic activity (7). Accordingly, GCC-mediated compartmental redistribution of cancer cell MMP-9 was associated with reduced MMP-9-dependent membrane protrusion formation, motility and metastatic seeding.

Functional remodeling of cancer cell MMP-9 by GCC and cGMP signaling is reminiscent of other tumor-based mechanisms which inhibit the release and pro-metastatic activity of MMP-9 in the extracellular environment. In that context, integrins induce MMP-9 expression and secretion in various cell types (42,43), and inhibition of selected integrin-dependent signaling at adhesion complexes has been suggested to disrupt MMP-9-dependent metastasis in colon cancer (44). Moreover, caveolins (45) and gangliosides (17) regulate the appropriate delivery and intracellular localization of MMP-9 to specialized tumor cell surface domains mediating MMP-9 secretion and activation, and potentiation of their dependant intracellular signaling suppresses metastasis in various cancer models. Thus, functional remodeling of MMP-9 by altering its intracellular trafficking, secretion and compartmental distribution represents a general strategy for metastasis prevention. Specifically in colorectal cancer, tumor epithelial cell MMP-9 is the key promoter of discreet processes defining metastatic disease progression (12,13), and GCC-mediated functional MMP-9 remodeling critically compromises those pathological processes impeding invasion, seeding and establishment of ectopic colonies (present results). These observations suggest a model (Fig. 6C) in which the catalytic activity of secreted MMP-9 promotes colorectal cancer cell metastasis by driving both invadopodia-mediated ECM degradation, migration and invasion at the primary tumor site, and attachment of intravasated tumor cells to exposed basement membrane components in the microvasculature of target organs (31). MMP-9 effects on metastasis can be independently regulated, contributing to the ordered sequence of distinct pathological processes, or mutually reinforcing, as each individual process may promote the proper execution of other processes comprising the metastatic sequence (Fig. 6C). Indeed, MMP-9 independently regulates ECM

degradation, cell spreading and tumor seeding (13), but ECM remodeling by MMP-9 may be a prerequisite for proper cytoskeleton-driven membrane extension, cell migration and cell attachment to the host microvasculature (5,31). In turn, GCC signaling through cGMP disrupts cancer cell MMP-9 functions at the primary tumor, decreasing local tissue invasion, and in intravasated metastatic cells, preventing productive seeding (Fig. 6C). As a result, primary tumors are contained within tissue boundaries, tumor cells are cleared from the circulation and colon cancer metastatic progression is interrupted by inhibition of underlying mutually reinforcing mechanisms (Fig. 6C).

An emerging paradigm suggests colorectal cancer is a disease of paracrine hormone insufficiency in which loss of GCC endogenous hormones guanylin and uroguanylin early in tumorigenesis promotes tumor formation and progression (27,46,47). In the context of the data presented here, loss of GCC signaling at the onset of colorectal carcinogenesis may be permissive to the emergence of the metastatic phenotype imposed by tumor epithelial cell MMP-9. Importantly, reactivation of the silent, but conserved, GCC signalome in cancer cells with exogenous administration of specific GCC ligands may oppose tumorigenesis and metastasis (27). Antimetastatic therapy with GCC ligands is tumor-specific, because it targets only metastatic colon cancer cells uniquely overexpressing GCC at extraintestinal sites (24, 35), and mechanism-based, since it inhibits pathological functions of colon cancer cell MMP-9 by activating cGMP-dependent signaling. Thus, GCC hormone replacement therapy represents an unexplored approach to reduce colon cancer mortality by disrupting metastasis and its component processes in patients.

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Figure 1.

GCC signaling depletes extracellular MMP-9 proteolytic activity. *A*, T84 and Caco-2 colon carcinoma cells expressing GCC (28) were treated for 24 h in serum-free DMEM/F12 media with PBS, 8-pCPT-cGMP (1 mM), 8-br-cGMP (5 mM) or ST (1 μ M ST). Then, cell conditioned media were subjected to immunoblotting. *B*, Gelatin zymography of conditioned media from T84 cells treated as in *A*. Immunoblots (*A*, upper panel) and zymograms (*B*, upper panel) show representative experiments repeated ≥ 3 times. Bar graphs (lower panels) reflect quantification of bands in immunoblots (*A*) or zymograms (*B*) by densitometry, and represent percentages of respective vehicle (PBS)-treated control cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ versus respective control. *C*, Levels of MMP-9 mRNA (per μ g RNA) by RT-PCR, relative to the PBS control.

Figure 2.

GCC signaling induces intracellular MMP-9 retention. T84 cells were treated (24 h in serum-free DMEM/F12) with PBS or ST (1 μ M) and subjected to flow cytometry (A) or immunoblot analysis (B). In A, intracellular MMP-9 protein was analyzed in cells permeabilized with methanol, while membrane-bound MMP-9 was quantified employing non-permeabilized cells. Histograms of MMP-9-stained cells are representative of three independent experiments. In B, upper panels show a representative experiment, while the lower panel reflects mean $\pm$ SEM of MMP-9 or MMP-2 levels in total cell lysates, normalized to the respective loading (GAPDH) and vehicle (PBS) controls, from three independent experiments as quantified by densitometry. *, $P < 0.05$ versus respective control (PBS).

Figure 3.

GCC activation suppresses actin cytoskeleton-based locomotory organelles. **A**, Representative images (magnification $\times 63$) from Differential Interference Contrast (DIC) and confocal microscopy of PBS- (*upper panels*) and ST-treated (*lower panels*) T84 cells. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI, for nuclei in *blue*), phalloidin (*green*) and anti-MMP-9 antibody (*red*). *Yellow* in merged images indicates β -actin and MMP-9 colocalization. **B**, Cell spreading was quantified as the ratio of cells extending membrane protrusions (filopodia, lamellipodia) versus the total cell number per microscopic field (magnification, $20\times$) of an inverted microscope (13). TJU, the inactive ST analog Thomas Jefferson University 1–103. GCC ligands and the inactive analog were employed at $1\ \mu\text{M}$ each for 24 h. Data represent percent inhibition of cell spreading obtained with PBS-treated control. *, $P < 0.05$ versus control.

Figure 4.

GCC signaling inhibits cell spreading by regulating MMP-9. *A*, Cell spreading of T84 cells treated for 24 h with ST (1 μ M) or 8-br-cGMP (5 mM), in the presence or absence of the active form of MMP-9 (aMMP-9, 500 ng/ml). *B–D*, T84 cells were transduced with either MSCV-empty vector (T84-V) or an MSCV carrying MMP-9 (T84-MMP-9), and cell cultures stably expressing the engineered nucleic acid were selected in puromycin-supplemented media. *B*, Levels of MMP-9 mRNA (per μ g RNA) by RT-PCR, relative to the empty vector control. *C*, Representative immunoblot of the MMP-9 pro-form (proMMP-9) in media conditioned by tumor cells. *D*, Effects of ST treatment (1 μ M, 24 h) on the spreading of MSCV-transduced tumor cells. Cell spreading in *A* and *D* was quantified as described in Fig. 3B, and data represent percent inhibition of cell spreading obtained with the respective vehicle-treated control. *, $P < 0.05$; **, $P < 0.01$ versus respective control.

Figure 5.

GCC signaling reduces hematogenous seeding of mouse lung. *A*, Representative inverted fluorescence microscope fields of MitoTracker-stained wild type T84 cells and isogenic clones stably expressing the MSCV-empty vector (T84-V) or the MSCV-MMP-9 vector (T84-MMP-9). Cells were treated *in vitro* (24 h in serum-free media) with PBS (*upper panels*) or ST (1 μ M, *lower panels*). Bar, 200 μ m. *B*, Tumor cell seeding of mouse lung *in vivo* was quantified as described in Methods. Results are percentages of respective vehicle-treated controls. Uroguanylin, 1 μ M; 8-pCPT-cGMP, 1 mM; MMP-9, 500 ng/ml of purified human pro-MMP-9; BB94, a broad MMP-9 inhibitor (60 nmol/L). MMP-9 and BB94 were added to cell cultures during the last 2 h of incubations. With the exception of uroguanylin (N=2), at least 3 animals per condition were examined. *, $P < 0.05$; ***, $P < 0.005$ versus respective control.

Figure 6.

Ligand-dependent GCC signaling suppresses peritoneal metastasis. *A*, Representative H&E slides of diaphragmatic rolls from mice injected with T84 cells treated *in vitro* (24 h, in serum-free media) with PBS or ST (1 μ M). *B*, Metastatic tumor burden in the mouse peritoneum 2 weeks after intra-peritoneal injection of wild type T84 cells, or T84 cells stably expressing either the MSCV-empty vector (T84-V) or the MSCV-MMP-9 vector (T84-MMP-9). Cells were treated *in vitro* as in *A* and correspondent *in vivo* tumor formation in mouse diaphragms was quantified by RT-PCR for human GCC, a specific T84 cell marker (24,25). Levels of GCC mRNA were calculated as $2^{[(\text{blank Ct}) - (\text{sample Ct})]}/\mu\text{g}$ of mouse β -actin mRNA, where Ct is the sample threshold cycle number of mice not injected (blank) or injected (sample) with cancer cells. Numbers of animals were: PBS, 13 (T84), 11 (T84-V) and 14 (T84-MMP-9); ST, 10 (T84), 12 (T84-V) and 11 (T84-MMP-9). *, $P < 0.05$ versus respective control. *C*, Proposed antimetastatic actions of GCC signaling in colorectal cancer (see Discussion for description).