Uroguanylin Treatment Suppresses Polyp Formation in the \textit{Apc}^{Min/+} Mouse and Induces Apoptosis in Human Colon Adenocarcinoma Cells via Cyclic GMP


ABSTRACT

The enteric peptides, guanylin and uroguanylin, are local regulators of intestinal secretion by activation of receptor-guanylate cyclase (R-GC) signaling molecules that produce cyclic GMP (cGMP) and stimulate the cystic fibrosis transmembrane conductance regulator-dependent secretion of Cl\(^-\) and HCO\(_3\)\(^-\). Our experiments demonstrate that mRNA transcripts for guanylin and uroguanylin are markedly reduced in colon polyps and adenocarcinomas. In contrast, a specific uroguanylin-R-GC, R-GCC, is expressed in polyps and adenocarcinomas at levels comparable with normal colon mucosa. Activation of R-GCC by uroguanylin in vitro inhibits the proliferation of T84 colon cells and elicits profound apoptosis in human colon cancer cells, T84. Therefore, down-regulation of gene expression and loss of the peptides may interfere with renewal and/or removal of the epithelial cells resulting in the formation of polyps, which can progress to malignant cancers of the colon and rectum. Oral replacement therapy with human uroguanylin was used to evaluate its effects on the formation of intestinal polyps in the \textit{Min/+} mouse model for colorectal cancer. Uroguanylin significantly reduces the number of polyposis found in the intestine of \textit{Min/+} mice by \textit{ca.} 50\% of control. Our findings suggest that uroguanylin and guanylin regulate the turnover of epithelial cells within the intestinal mucosa via activation of a cGMP signaling mechanism that elicits apoptosis of target enterocytes. The intestinal R-GC signaling molecules for guanylin regulatory peptides are promising targets for prevention and/or therapeutic treatment of intestinal polyps and cancers by oral administration of human uroguanylin.

INTRODUCTION

Chemoprevention evolved during the last decade as a viable strategy for cancer prevention, with the aim of controlling the development of cancer through pharmacological and/or dietary intervention prior to the appearance of clinically detectable tumors with malignant properties (1). The pathogenesis of colorectal cancer is characterized as a multistep process that begins with increased proliferation and/or decreased apoptosis of epithelial cells in the mucosa resulting in the generation of polyposis, followed by progression to adenomas and then to adenocarcinomas (2, 3). One of the major mechanisms for this transformation is an imbalance between cell proliferation and apoptosis, leading to irregularities in the biochemical processes governing renewal of the intestinal epithelium (4). Therefore, restoration of normal mechanisms that maintain homeostasis of the intestinal mucosa is an attractive strategy for development of prophylactic intervention measures to prevent and/or treat colorectal cancers.

Uroguanylin and guanylin are small peptides that are related both in primary structures and in biological activities (5–7). Both of these regulatory peptides are produced at high concentrations throughout the intestinal mucosa. A cell-surface receptor in the intestine that has been identified at the molecular level is R-GCC, which is specifically activated by the guanylin peptides (7, 8). R-GCC proteins are localized on the luminal surface of enterocytes in the intestinal tract (7, 8). Binding of uroguanylin or guanylin to the extracellular domain of R-GCC stimulates production of the intracellular second messenger, cGMP. cGMP activates the CFTR protein that serves as an apical membrane channel governing Cl\(^-\) and HCO\(_3\)\(^-\) efflux from enterocytes lining the intestinal tract (9, 10). Activation of CFTR channel proteins and the subsequent enhancement of transepithelial secretion of Cl\(^-\) and HCO\(_3\)\(^-\) is the main driving force for secretion of Na\(^+\) and water into the intestinal lumen (7). Therefore, a growing body of evidence strongly suggests that one of the major physiological functions of the guanylin hormones is to regulate fluid and electrolyte transport in the intestinal tract by serving as local regulators of CFTR activity.

In addition to a role for uroguanylin and guanylin as hormonal modulators of intestinal fluid and electrolyte secretion, there may be other physiological functions for the guanylin family of cGMP-regulating peptides. Recent experiments demonstrate that expression of the R-GCC form of receptors for uroguanylin and guanylin is maintained at normal levels in both primary and metastatic cancers of the colon and rectum (11). This finding resulted in the suggestion that R-GCC may serve as a specific marker for colon tumors in the body (11). In contrast, expression of guanylin mRNA is markedly down-regulated in adenocarcinomas of the colon (12). Loss of guanylin production in the colon may have deleterious effects on tumor growth, but uroguanylin is also produced in the colon mucosa and regulates the activity of R-GCC and intestinal anion and fluid secretion. Thus, we investigated the expression of both uroguanylin and guanylin mRNAs in colon polypos and adenocarcinomas and tested the efficacy of uroguanylin as a potential therapeutic agent in the treatment of colon tumors.

We found that mRNA transcripts for guanylin and uroguanylin are severely decreased in both polypos and adenocarcinomas of human colon relative to the mRNA levels found in the surrounding normal colon mucosa. The mRNA expression of R-GCC was essentially normal in both polypos and cancers of the colon. In addition, we demonstrate for the first time that treatment with uroguanylin leads to induction of apoptosis in human colon carcinoma cells \textit{in vitro}, and that oral administration of human uroguanylin suppresses the formation and apparent progression of polypos in the \textit{Min/+} mouse animal model of colorectal cancer (13).

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2 The abbreviations used are: cGMP, cyclic GMP; CFTR, cystic fibrosis transmembrane conductance regulator; R-GC, receptor-guanylate cyclase; R-GCC, uroguanylin-R-GC; BrdUrd, 5-bromo-2’-deoxyuridine; IIBMX, isobutylmethylxanthine; KRB, Krebs-Ringer bicarbonate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; GL, gastrointestinal; RT-PCR, reverse transcription-PCR; ANP, atrial natriuretic peptide; ST, heat-stable toxin.
UROGUANYLIN, cGMP, AND COLON CANCER

MATERIALS AND METHODS

Cell Culture. Human T84 colon carcinoma cells were obtained from the American Type Culture Collection at passage 52. Cells were grown in a 1:1 mixture of Ham’s F-12 medium and DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg/ml streptomycin. The cells were fed fresh medium every third day and split at a confluence of ~80%.

Tissue Collection. Samples of normal colon and tumors were obtained following colon resections for adenocarcinoma under a human experimentation protocol that was approved by the Institutional Review Board for Missouri University School of Medicine/Truman VA Hospital. Mucosa samples from normal colon tissues adjacent to the colon adenocarcinomas were isolated from the submucosal tissue by scraping the luminal surface with a microscope slide to separate mucosa from the underlying tissue. Portions of the tumors were collected and processed as an intact tissue. Tissues from 11 subjects between the ages of 48 and 82 years representing 2 female and 9 male patients were used in this study.

Isolation of RNA. RNA was extracted from tissue using a combination of the TRI reagent method (Molecular Research Center, Inc., Cincinnati, OH) and the RNAeasy kit (Qiagen, Valencia, CA). The tissue was homogenized in TRI reagent following the manufacturer’s protocol. After phase separation with chloroform, the aqueous supernatant phase containing total RNA was removed and mixed with an equal volume of 70% ethanol and lysis buffer without β-mercaptoethanol. The resulting mixture was loaded onto the RNAeasy columns and then processed following the protocol provided by the manufacturer.

Northern Assays. Total RNA (20 μg) isolated from colon tissues was subjected to electrophoresis in formaldehyde-agarose gels and then transferred to nylon membranes (Zeta-Probe; Bio-Rad Laboratories, Inc., Hercules, CA). The membranes were prehybridized for 2 h at 65°C in ExpressHyb solution (Clontech, Palo Alto, CA) and then hybridized with human guanylin, uroguanylin, and GC-C cDNAs overnight at 65°C. All cDNA probes were labeled with 32P by random priming (Boehringer Mannheim, Indianapolis, IN). RNA blots were then washed twice with 2× SSC-0.1% SDS for 3 min at room temperature, followed by a 15-min wash at 60°C with 0.2× SSC-0.1% SDS. Exposure to X-ray film was performed at ~80°C with intensifying screens.

RT-PCR-Southern Assays. Oligo(dexothyimidine)18 primed cDNAs were synthesized from 3 μg of total RNA using reverse transcriptase (SuperScript II; Life Technologies, Gaithersburg, MD). Two PCR primers, 5’ primer (5’-GAACCCAGGGAGCGGCTAG-3’) and 3’ primer (5’-CTGGTGGCT-CAGGGTACC-3’), were designed from regions flanking the open reading frame of human pre-pro-uroguanylin cDNA (14). A PCR product of the expected size of 384 bp was amplified from colon cDNAs after 25 cycles at 93°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min using Taq DNA polymerase (United States Biochemical Corp., Cleveland, OH). The pair of primers for RT-PCR of pre-pro-uroguanylin were 5’ primer (5’-AATCTCAG-GAAGTCTGC3’) and 3’ primer (5’-GTGAGCCAGATTTAC-3’). These primers produced 174-bp cDNAs for human guanylin using the PCR conditions of 25 cycles at 93°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min. The primers for amplification of GC-C cDNAs were 5’ primer (5’-CAATTCGACAAAAAAGCAGAGAG) and 3’ primer (5’-GAAATGTCGCCATT-CAGGTAG). These primers produced a 235-bp GC-C product using the PCR conditions of 25 cycles at 93°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min. The PCR-generated cDNA products were subjected to electrophoresis on 1% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5) containing ethidium bromide and then transferred to nylon membranes. Southern hybridization was carried out using uroguanylin and guanylin cDNA probes. Prehybridization was for 1 h at 65°C with either ExpressHyb or PerfectHyb Plus (Sigma Chemical Corp., St. Louis, MO) solutions, and then hybridization was for 3 h at 65°C. Blots were washed as described above and exposed to X-ray films at ~80°C with intensifying screens.

Cell Proliferation Assays. Approximately 10,000 cells (T-84; CaCo-2) were inoculated in each well of 96-well plates. After an incubation period of 3 days, the various concentrations of human uroguanylin were added to the media, and cells were allowed to grow until they formed monolayer monolayers. Subsequently, BrdUrd labeling agent (BrdUrd in PBS) was added (final concentration, 100 μM), and cells were reincubated for an additional 24 h. Monolayers of cells were washed, and the incorporation of BrdUrd was measured following the manufacturer’s instructions (Boehringer Mannheim Corp.).

cGMP Accumulation Bioassays with T84 Cells. The human uroguanylin peptide (NDDCFLCNVACTGCL) was custom synthesized by Multiple Peptide Systems (San Diego, CA). Biological activity of the synthetic peptide was assayed as reported previously (6). Briefly, the confluent monolayers of T84 cells in 24-well plates were washed twice with 250 μl of DMEM containing 50 mM HEPES (pH 7.4), preincubated at 37°C for 10 min with 250 μl of DMEM containing 50 mM HEPES (pH 7.4) and 1 mM IBMX, followed by incubation with uroguanylin (0.1 mM to 10 μM) for 30 min. The medium was aspirated, and the reaction was terminated by the addition of 3% perchloric acid. After centrifugation and neutralization with 0.1 N NaOH, the supernatant was used directly for measurements of cGMP by using the ELISA kit (Caymen Chemical, Ann Arbor, MI).

Using Chamber Assays. The seromuscular layer of human colon mucosa was removed by blunt dissection, and one to four mucosal sheets from each specimen (~1 cm2) were used. To isolate intestinal mucosa from mice, animals were sacrificed by CO2, and the proximal portion of the colon distal to the cecum was dissected from the intestinal tract. Intestinal tissue was placed in ice-cold, oxygenated KRB solution, opened along the mesenteric border and then pinned with the luminal-side down on a pliable silicone surface. The outer muscle layers were stripped by shallow dissection with a scalpel and fine forceps. Mouse proximal colon and human colon tissues, consisting of only mucosa and submucosa, were mounted between two Ussing half-chambers and bathed on both sides with KRB solutions in a manner similar to that reported previously (9). Electrometric measurements were monitored with an automated voltage clamp, and direct-connecting voltage and Isc were recorded. Tissues were equilibrated under short-circuit conditions until Isc had stabilized, and the potential difference across the epithelium was measured intermittently.

Apoptosis Assays. T84 cells were grown in 35-mm dishes for 7 days. The cells were washed once with serum-free DMEM and incubated with the same media containing different concentrations of human uroguanylin for the indicated times. After this incubation, cells were quickly collected by trypsinization, and the cell pellet was washed twice with PBS. Cells were resuspended in PBS at a concentration of ~106 cells/ml. For demonstration of nucleosomal ladders, the apoptotic DNA was isolated from these cells by following the instructions of the DNA fragmentation analysis kit (Boehringer Mannheim Corp.). The apoptotic DNA was separated on a 1.8% agarose gel electrophoresis, followed by staining with ethidium bromide. Induction of apoptosis by uroguanylin was further demonstrated by using the TUNEL assay with human CaCo-2 colon adenocarcinoma cells as per the instructions of the In Situ Cell Death Detection kit (Boehringer Mannheim Corp., Indianapolis, IN).

Mini/+ Mouse Model. Male C57BL/6-J-ApcMin+/+ mice, a strain containing a fully penetrant dominant mutation in the Apc gene, were obtained at 4–5 weeks of age from The Jackson Laboratory (Bar Harbor, ME). All mice were fed a high-fat AIN-93G diet (Research Diets, Inc., New Brunswick, NJ), tap water to drink ad libitum, and housed in a humidity- and temperature-controlled room with a 12-h light-dark cycle. After 1 week of quarantine period, the animals were randomly divided into three groups of 10 animals each and ear tagged. Animals were fed the same diet containing different concentrations of human uroguanylin (0, 10, and 20 μg of uroguanylin/10 g of diet). The mice were also given additional amounts of either 10 or 20 μg of human uroguanylin or vehicle (0.2 ml of PBS containing 20% polyethylene glycol) by oral gavage twice a week. Food consumption and body weight of these animals were monitored weekly. At the end of 17 weeks, animals were sacrificed by CO2 asphyxiation, and the GI tracts were removed. After flushing with PBS, the GI tract was divided as sections of duodenum (~5 cm from the stomach), jejunum (middle portion, ~10–13 cm), ileum (~5 cm proximal to the cecum), and the entire colon. Tissues were opened longitudinally, washed with the Streck fixative (Streck Laboratories, Inc., Omaha, NE), and placed between two layers of blotting paper in a tray containing the tissue fixative. Polyps and other tumors were counted independently by four different observers. The results are expressed as a mean of the total number of polyps for each individual animal as recorded by the four observers. Analysis of the data obtained from all observers revealed a statistically nonsignificant, interobserver variance. Sections of these tissues were viewed under constant magnification (~×10) to measure the diameter of intestinal polyps in situ.
RESULTS

Synthetic Human Uroguanylin Is Biologically Active. Human uroguanylin was chemically synthesized, and the relative potencies of the synthetic peptides were evaluated using a cGMP accumulation bioassay with intact T84 cells (5, 6). We observed biological activity in several isoforms of this peptide that exhibited similar physicochemical properties. The major peptide peak, exhibiting a potent biological activity, was further purified by reverse phase-HPLC to ~99% purity and used for this study. To ensure that the synthetic form of human uroguanylin had efficacy in both mouse and human colon mucosa, its biological activity was examined using mouse and human colonic mucosa mounted in Ussing chambers. Fig. 1 compares the short circuit current (Isc) responses of colon mucosa to 1 μM uroguanylin after pretreatment with tetrodotoxin and amiloride, which reduces and stabilizes the Isc in both human and mouse colon (10). Addition of uroguanylin to the luminal reservoir stimulates a rapid increase in Isc, which was sustained for the course of the experiments. Isc is a measurement of the electrogenic secretion of Cl⁻ and HCO₃⁻ across the colon mucosa when amiloride is used to inhibit the electrogenic absorption of Na⁺. These results confirm that synthetic human uroguanylin is effective in stimulating the transepithelial secretion of Cl⁻ and HCO₃⁻ in the mucosa of both human and mouse intestine. Therefore, we used the synthetic human uroguanylin for all of the subsequent experiments that are presented in this communication.

Uroguanylin Induces Apoptosis. To test the efficacy of human uroguanylin on cell growth in vitro, the effects of uroguanylin on proliferation of T84 cells were measured. Uroguanylin treatment caused a concentration-dependent inhibition of cell growth, reaching an ~70% inhibition of growth at 10 μM peptide (Fig. 2). In contrast, a biologically inactive variant of this peptide neither inhibits cell growth nor stimulates the production of cGMP (data not shown), suggesting that the growth inhibition by active uroguanylin is mediated by intracellular cGMP.

To evaluate the effect of uroguanylin on DNA fragmentation, a hallmark response of cells undergoing programmed cell death, preconfluent monolayers of T84 cells were incubated for 16 h with serum-free DMEM medium. After this incubation, cells were treated with different concentrations of uroguanylin for 16 h, and DNA was isolated to evaluate DNA fragmentation patterns using agarose gel electrophoresis. DNA isolated from control cells as well as from the cells treated with a biologically inactive form of uroguanylin exhibited little if any detectable DNA fragmentation, consistent with a low basal incidence of apoptosis under the serum-free conditions (Fig. 3A). On the other hand, DNA from uroguanylin-treated cells exhibited relatively extensive DNA fragmentation. The action of uroguanylin to elicit DNA fragmentation in T84 cells was also detected by 2 h after treatment, and this action of uroguanylin was greatly augmented by the addition of an inhibitor of cGMP hydrolysis, IBMX (Fig. 3B).

Induction of apoptosis by uroguanylin treatment of human colon cancer cells was further investigated using CaCo-2 cells and the TUNEL assay for apoptosis. Uroguanylin treatment significantly increased the generation of apoptotic cells as compared with vehicle treated CaCo-2 cells (Fig. 4). About 25% of the CaCo-2 cells exhibited positive reaction for apoptosis induced by treatment with uroguanylin, as detected using the TUNEL method. These results confirm and extend the finding that uroguanylin induces apoptosis in human colon cancer cells in vitro and suggest that uroguanylin induces apoptosis in human colon cancer cells by a cGMP-dependent signaling mechanism.
T84 cells were treated with either vehicle ( ), 1mM IBMX ( o r 1mM IBMX plus 10 ), and normal mucosa tissues. These findings raise the possibility that (Fig. 6). The levels of R-GCC mRNAs were similar in both polyps when compared with transcript levels in the normal colon mucosa uroguanylin and guanylin mRNAs is also markedly reduced in polyps polyps isolated from the colon. Interestingly, the expression of expression is reduced in colon cancers (12, 15). Thus, we examined the expression of uroguanylin, guanylin, and R-GCC mRNAs in adenocarcinomas and in the adjacent normal colon mucosa of human subjects. Northern blot analyses showed that mRNA transcripts for both uroguanylin and guanylin were undetectable in all specimens of colon cancers examined, whereas the adjacent normal mucosa from the same patients exhibited robust expression of guanylin and uroguanylin mRNAs (Fig. 5). The levels of R-GCC mRNAs were similar in both the adenocarcinomas and normal mucosa. Similar expression patterns for uroguanylin and guanylin mRNAs were found when RNA preparations from 11 additional specimens of colon mucosae and tumors were analyzed by highly sensitive RT-PCR-Southern assays. Guanylin and uroguanylin transcripts were markedly reduced in 10 of 11 specimens of colon adenocarcinomas as compared with the levels of mRNA in the adjacent normal mucosa (data not shown). These finding suggest a possible link between colon cancer and expression of these cGMP-regulating peptides in the intestinal epithelium.

Because the formation of adenomatous polyps is an early stage in the development of adenocarcinomas, we also examined the expression of guanylin, uroguanylin, and R-GCC in samples of benign polyps isolated from the colon. Interestingly, the expression of uroguanylin and guanylin mRNAs is also markedly reduced in polyps when compared with transcript levels in the normal colon mucosa (Fig. 6). The levels of R-GCC mRNAs were similar in both polyps and normal mucosa tissues. These findings raise the possibility that

Human Uroguanylin Inhibits the Formation of Polyps in the Min/+ Mouse. Because the biological activity of synthetic uroguanylin was not affected by digestion with trypsin, chymotrypsin, V-8 protease, and human gastric and intestinal juices (data not shown), we expected that uroguanylin should be bioavailable when administered p.o. This view is consistent with the previous findings that intragastric administration of uroguanylin to suckling mice elicits increases in fluid secretion into the intestinal lumen, whereas treatment with guanylin was ineffective (16). Therefore, we evaluated the effect of oral administration of human uroguanylin on the formation of polyps in the Min/+ mouse model of colorectal cancer, which is the most widely used animal model to assess the chemopreventive properties of dietary nutrients and therapeutic agents (13). These animals carry a dominant mutation in one allele of the Apc genes. When the mice are raised on a high-fat diet, polyps begin developing throughout the intestine at about 55 days of age. Development of polyps in the intestinal lumen reduces the transit of intestinal contents, which decreases food consumption and reduces growth as the disease progresses. Administration of uroguanylin in the diet together with

Fig. 3. Uroguanylin induces DNA fragmentation in T84 cells. A, approximately 2 x 10⁶ cells were seeded into 35-mm dishes and cultured for 7 days. Preconfluent monolayers were washed with serum-free DMEM and further incubated with the same media containing the indicated concentrations of human uroguanylin. After the cells were collected by trypsinization and washed twice with PBS, the cells were immediately used for DNA isolation according to the instructions contained in the DNA fragmentation analysis kit (Boehringer Mannheim Corp.). The fragmentation of DNA was analyzed by electrophoresis using 1.8% agarose gels with ethidium bromide. Apoptotic DNA provided with the kit was used as positive control, M (Lane 1), and a functionally inactive variant of human uroguanylin (V) was used as negative control (Lane 6). Different concentrations of uroguanylin, as indicated, were examined (Lanes 2-5). B, preconfluent monolayers of T84 cells were treated with either vehicle (C), 1 mM IBMX (D), 10 μM uroguanylin (U), or 1 mM IBMX plus 10 μM uroguanylin (I+U). After 2 h of incubation, the cellular DNA was isolated and subjected to electrophoresis as described above. Camptothecin (Ca) is the positive control DNA preparation supplied with the assay reagents to demonstrate DNA ladders.

Fig. 4. Uroguanylin induces apoptosis in CaCo-2 cells. Cells were cultured on microscope slides until semiconfluent monolayers were formed. This was followed by treatment with human uroguanylin (1 μM) for 48 h. Induction of apoptosis was detected by fluorescence microscopy directly after the TUNEL reaction according to the instructions contained in the In Situ Cell Death Detection kit (Boehringer Mannheim Corp.). A, vehicle-treated cells; B, uroguanylin-treated cells.
oral doses of uroguanylin on a twice-weekly protocol of administration caused a significant increase in both food consumption and body weight gain over the course of this experiment (data not shown). In addition, the Min/+ mice treated with uroguanylin were clearly healthier by external examination and more active than control animals.

At the end of the study, all animals were sacrificed, and the number and distribution of polyps in the small intestine and colon tissues were measured (Table 1). Intestinal tracts of the vehicle-treated control group contained 48.3 ± 7.7 (mean ± SE) polyps/animal. Most of the polyps were located throughout the small intestine, and only a few polyps were found in the colon. We did not measure the precise size of each polyp. However, the sizes of polyps in control Min/+ mice were in the range of approximately 3–5 mm in diameter and were clearly visible. In addition, three animals in the control group also developed globular tumors in the duodenum that were similar in appearance to that of carcinomas. Oral administration of uroguanylin reduced the total number of polyps by ~50% from 48.3 ± 7.7 per animal to 23.3 ± 3.1 ($P < 0.05$). Furthermore, this peptide also appeared to inhibit the progression of polyps because the majority of polyps in these animals were not clearly visible and were very small (e.g., <2.0 mm in diameter). In addition, uroguanylin-treated Min/+ mice did not develop globular tumors in the duodenum and had no polyps in the colon. The appearance of polyps in the colon and carcinoma-like tumors in the small intestine of Min/+ mice usually occurs at the later and more severe stages of disease. The absence of carcinomas in the small intestine and polyps in the colon of uroguanylin-treated mice suggests that this peptide may inhibit both the formation of tumors and the progression of polyps into more advanced tumors. Taken together, our results strongly suggest that oral administration of uroguanylin substantially reduces the formation and/or growth of polyps in the Min/+ mouse model of colon cancer.

**DISCUSSION**

Uroguanylin and guanylin are potent physiological regulators of intestinal fluid and electrolyte transport (7). Their structural homologies and profiles of biological activities suggest that uroguanylin and guanylin have similar physiological functions. We have reported recently that uroguanylin and guanylin cooperatively regulate a signaling pathway that modulates intestinal salt and fluid transport via a cellular mechanism involving cGMP-mediated activation of CFTR, which serves as a channel for the passage of Cl$^-$ and HCO$_3^-$ through the apical membranes of target cells (10, 17, 18). Both uroguanylin and guanylin participate cooperatively in the regulation of intestinal secretion that is influenced by variable mucosal pH values at the apical surface of this epithelium (10, 17). Moreover, both peptides are produced at remarkably high levels in the human colon and are secreted into the intestinal lumen (7). It is likely that both guanylin and uroguanylin act to regulate intestinal secretion during digestion as a major physiological action of these peptide hormones in the body.

Another potential physiological role for guanylin and uroguanylin that was revealed in the present study concerns the activation of a cGMP signal transduction pathway that may help to regulate the turnover of epithelial cells and maintain homeostasis of the intestinal mucosa. The biochemical processes that influence the longevity of cells in the intestinal mucosa coupled with those processes involved in the regulation of cell proliferation and differentiation function together in an integrated fashion to maintain homeostasis of a normal intestinal epithelium. Abnormalities in the processes of programmed cell death and/or renewal of the epithelium as characterized by increased cell proliferation and/or suppressed apoptosis may lead to the formation of tumors within the intestinal tract (19). Our findings clearly demonstrate that the expression of mRNAs encoding uroga-

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**Fig. 5. Expression of uroguanylin and guanylin is suppressed in adenocarcinomas.** Total RNA (20 μg) isolated from normal colon mucosa (M) and tumors (T) of nine patients was subjected to Northern hybridization assays using $^{32}$P-labeled cDNA probes that are specific for: A, guanylin (GN) and R-GCC; and B, uroguanylin (UGN) and R-GGCC. Loading estimates for RNA in each lane can be derived from the ethidium bromide-stained 28S rRNA shown at the bottom panel of each blot.

**Fig. 6. Expression of uroguanylin and guanylin is suppressed in polyps.** cDNAs were prepared by reverse transcription of RNA isolated from normal colon mucosa (M) and polyps (T-P) from four patients and then were amplified by PCR using guanylin-specific, uroguanylin-specific, and GCC-specific oligonucleotide primers for 25 cycles of PCR. Southern hybridization assays used $^{32}$P-labeled guanylin-, uroguanylin- and GCC-specific cDNA probes. Ethidium bromide staining was performed to examine the cDNA products in gels before transfer and Southern hybridization.
Table 1  Oral administration of uroguanylin inhibits polyp formation in the Min/+ mouse

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
<th>Total</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.4 ± 0.8</td>
<td>20.9 ± 5.4</td>
<td>14.3 ± 2.6</td>
<td>0.7 ± 0.2</td>
<td>48.3 ± 7.7</td>
<td>Three animals showed globular tumors in their duodenum. The diameter of polyps was in the range of 3–5 mm.</td>
</tr>
<tr>
<td>B</td>
<td>2.0 ± 0.5</td>
<td>21.2 ± 4.2</td>
<td>8.7 ± 1.6</td>
<td>0.2 ± 0.1</td>
<td>32.1 ± 5.2</td>
<td>The diameter of polyps was in the range of 3–4 mm.</td>
</tr>
<tr>
<td>C</td>
<td>2.2 ± 0.6</td>
<td>12.5 ± 1.6</td>
<td>8.7 ± 1.8</td>
<td>0.0</td>
<td>23.3 ± 3.1</td>
<td>Polyps were not clearly visible and were very small (&lt;2.0 mm in diameter).</td>
</tr>
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</table>

* A: control group, n = 10; B: uroguanylin, oral gavage 10 µg/day, n = 10; C: uroguanylin, oral gavage 20 µg/day, n = 10.
* Results are expressed as mean ± SE, n = 10.
* Compared with control at P < 0.05.

Uroguanylin and guanylin are markedly suppressed in both adenocarcinomas and polyps of human colorectal cancer, and that treatment with uroguanylin to activate R-GCC receptors induces apoptosis in human T84 and CaCo-2 colon carcinoma cells by a cGMP-dependent mechanism. Furthermore, oral administration of uroguanylin to the Min/+ mouse model of colorectal cancer not only inhibited the formation of polyps, but this cGMP-regulating peptide apparently delayed the progression of intestinal polyps during the course of treatment. Taken together, these findings suggest that down-regulation of uroguanylin and guanylin gene expression may be one mechanism underlying the derangement of programmed cell death in the intestinal mucosa that is associated with the formation of polyps in the intestine.

It is important to note that expression of uroguanylin and guanylin mRNAs is virtually eliminated in the specimens of adenocarcinomas and polyps that were examined in this study. It is noteworthy that even in the ultrasonographic RT-PCR-Southern assays detected only very low levels of transcripts for uroguanylin and guanylin in some of the polyps and adenocarcinomas. Our results are in agreement with a recent report suggesting that expression of guanylin mRNA is markedly reduced in human colorectal adenocarcinoma as revealed by in situ hybridization histochemistry (12). Both guanylin and uroguanylin genes have recently been mapped to chromosome 4 of mice and to a syntenic position on human chromosome 1p34–35 (20, 21). This chromosomal region is frequently associated with loss of heterozygosity in human colon carcinoma (22). In the Min/+ mouse tumor model, adenozae multiplicity and growth rates are regulated by tumor suppressor Apc genes (23). This gene is mutated with loss of function of the gene product in the vast majority of colorectal cancers in the human (24). The principal function of Apc genes is to regulate growth and apoptosis of epithelial cells via the Wnt signal transduction cascade (25). Because uroguanylin and guanylin are essentially lost in both adenomatous polyps and adenocarcinomas of human colon, it is tempting to speculate that these enteric hormones may be involved mechanistically at early stages of colon carcinogenesis. This possibility is consistent with our results in the Min/+ mouse model of colorectal cancer, where oral administration of uroguanylin inhibits both the formation and apparent progression of polyps. Although the biochemical mechanism(s) for this chemopreventive property of uroguanylin is not known, it is plausible that uroguanylin and guanylin both participate in a cGMP-mediated signaling mechanism in target enterocytes that regulates mucosal cell turnover by initiating programmed cell death. Therefore, diminished expression of uroguanylin and guanylin genes in cells within the intestinal mucosa that have inactivating mutations of Apc genes may lead to an inhibition of programmed cell death and contribute to the formation of tumors in the colon and rectum.

Several lines of evidence have implicated a potential role of K\(^+\) efflux in the induction of apoptosis (26–32). The question that naturally arises concerns the existence of a putative relationship between uroguanylin/cGMP-induced apoptosis on the one hand and K\(^+\) and CI\(^-\) transport in colon carcinoma cells on the other. Uroguanylin and guanylin stimulate transepithelial CI\(^-\) secretion (10) and K\(^+\) efflux (7) by activation of receptors with intrinsic guanylate cyclase activity. Other cGMP-regulating peptides, such as ANP, have also been shown to activate K\(^+\) conductance in rat mesangial cells and induce apoptosis in cardiac myocytes by a cGMP-dependent mechanism (33, 34). Furthermore, pretreatment of rat endothelial cells with either ANP or 8-bromo-cGMP causes a marked accumulation of the nuclear phosphoprotein, p53, a tumor suppressor protein known to induce apoptosis in many cell types (35). Therefore, it seems possible that uroguanylin and guanylin produced in the intestinal mucosa and released locally may initiate apoptosis of epithelial cells lining the GI tract mucosa by activating R-GCC and/or other intestinal receptors that produce cGMP upon activation by the peptides.

Hormones and neurotransmitters that activate cGMP signal cascades, such as uroguanylin, ANP, and nitric oxide, are all capable of producing apoptosis in target cells (33–35). Apoptotic cells are distinguishable from living cells by a number of biochemical and morphological features. Although cell shrinkage has been invariably observed in cells undergoing programmed cell death, the underlying mechanism by which apoptotic cells achieve this feat is poorly understood. The inhibition of K\(^+\) loss by raising external K\(^+\) concentrations (36, 37) or by exposing cells to K\(^+\) channel blockers (38) results in an abrogation of apoptosis. Our proposed model suggests that uroguanylin may stimulate intestinal fluid secretion by activation of an intracellular cGMP signaling pathway, which promotes K\(^+\) recycling across the basolateral plasma membranes of enterocytes and a concomitant activation of anion channels at the apical surface of the uroguanylin target cells (10). Thus, it seems plausible that the same cellular signaling machinery that is activated by cGMP to regulate intestinal fluid secretion may also be used for the induction of programmed cell death within the intestinal epithelium.

It should be emphasized that uroguanylin and guanylin were discovered because these hormonal peptides are the endogenous agonists for intestinal R-GCC that is also activated by heat-stable toxin (ST) peptides, which are secreted by enteric bacteria that cause diarrhea (7). The ST peptides are uroguanylin-like in both primary structures and profiles of biological activities. Thus, it is possible that chronic and/or periodic infections with ST-secreting bacteria in the intestine elicit beneficial therapeutic actions for people living in the developing nations, where a relatively low incidence of colorectal cancer is found (39). Regular exposure and enteric infection with enterotoxigenic Escherichia coli or other intestinal microbes that secrete ST peptides into the colon could provide a uroguanylin-like agonist that stimulates...
cGMP production in epithelial cells containing mutations in ApoC or other genes involved in tumor formation. This action of the ST peptides could elicit apoptosis in cells at relatively early stages of tumor formation. Stimulation of cGMP production by ST in benign tumors may also inhibit the progression of polyps to adenocarcinomas of the colon. Although the hypothesis is speculative, we offer this suggestion to reinforce the historical importance of E. coli ST peptides in the discovery of guanylin and uroguanylin and once again connect the pathophysiology of bacterial ST peptides to the physiology of guanylin regulatory peptides in the intestinal tract (5–7).

To summarize, this study demonstrates for the first time that uroguanylin induces apoptosis in human colon cancer cells in vitro, and oral uroguanylin inhibits the formation of polyps in the Min/+ mouse animal model of colorectal cancer in vivo. We conclude with the suggestion that uroguanylin and other members of the guanylin peptide family may have therapeutic efficacy for prevention and/or treatment of colon cancer.

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Uroguanylin Treatment Suppresses Polyp Formation in the Apc\textsuperscript{Min/+} Mouse and Induces Apoptosis in Human Colon Adenocarcinoma Cells via Cyclic GMP

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