Progastrin-Induced Secretion of Insulin-Like Growth Factor 2 From Colonic Myofibroblasts Stimulates Colonic Epithelial Proliferation in Mice

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BACKGROUND & AIMS: Many colon cancers produce the hormone progastrin, which signals via autocrine and paracrine pathways to promote tumor growth. Transgenic mice that produce high circulating levels of progastrin (hGAS) have increased proliferation of colonic epithelial cells and are more susceptible to colon carcinogenesis than control mice. We investigated whether progastrin affects signaling between colonic epithelial and myofibroblast compartments to regulate tissue homeostasis and cancer susceptibility. **METHODS:** Colonic myofibroblast numbers were assessed in hGAS and C57BL/6 mice by immunohistochemistry. Human CCD18Co myofibroblasts were incubated with recombinant human progastrin (rhPG)(1–80) for 18 hours, and proliferation was assessed in the presence of pharmacologic inhibitors. The proliferation of human HT29 colonic epithelial cells was assessed after addition of conditioned media from CCD18Co cells incubated with progastrin. The effects of the insulin-like growth factor (IGF)-I receptor antagonist AG1024 were investigated in cultured HT29 cells and on the colonic epithelium of hGAS mice compared with mice that did not express transgenic progastrin (controls). **RESULTS:** The colonic mucosa of hGAS mice contained greater numbers of myofibroblasts that expressed α–smooth muscle actin and vimentin than controls. Incubation of CCD18Co myofibroblasts with 0.1 nmol/L rhPG(1–80) increased their proliferation, which required activation of protein kinase C and phosphatidylinositol-3 kinase. CCD18Co cells secreted IGF-II in response to rhPG(1–80), and conditioned media from CCD18Co cells that had been incubated with rhPG(1–80) increased the proliferation of HT29 cells. The colonic epithelial phenotype of hGAS mice (crypt hyperplasia, increased proliferation, and altered proportions of goblet and enteroendocrine cells) was inhibited by AG1024. **CONCLUSIONS: Progastrin stimulates colonic myofibroblasts to release IGF-II, which increases proliferation of colonic epithelial cells. Progastrin might therefore alter colonic epithelial cells via indirect mechanisms to promote neoplasia.**

Keywords: Mouse Model; Colon Cancer; PKC; PI3K.

The colonic epithelium is organized into crypts that rapidly self-renew via stem cells, which are believed to be located at or near the crypt base.[1] Tissue self-renewal is precisely regulated to maintain normal homeostasis and avoid malignant transformation. Under normal conditions, colonic epithelial stem cells self-renew asymmetrically and daughter cells divide further in the transit-amplifying region in the bottom two-thirds of the crypt. Cells then differentiate into secretory (goblet, enteroendocrine) or absorptive (colonocyte) lineages as they migrate up the crypt axis until they are shed into the lumen. Surrounding the colonic crypts are sheaths of mesenchymal cells, including myofibroblasts, which participate in regulating homeostasis through reciprocal signaling with the epithelial compartment. Myofibroblasts produce Wnt signals[2] and several growth factors, such as hepatocyte growth factor and insulin-like growth factors (IGFs),[3] which increase colonic epithelial cell proliferation. Factors that disrupt homeostasis in this niche may therefore render the colon more susceptible to development of cancer.

Progastrin is a precursor of the fully processed hormone gastrin and is produced by cotranslational cleavage of the precursor molecule preprogastrin (reviewed in Dockray et al[4]). Amidated forms of gastrin are produced mainly by G cells in the gastric antrum, where they function as gastric acid secretagogues. Gastrin precursors are also biologically active; in particular, they disrupt colonic mucosal homeostasis and are involved in development of colon cancer. Preprogastrin is expressed by many colon cancers,[5] but because these tumors lack the enzymes required to process progastrin to amidated forms,[6–8] immature gastrins such as progastrin and glycine-extended gastrin (G-Gly) are secreted, leading to high local and circulating concentrations of these hormones.[9] Progastrin is mitogenic to colonic cell lines[10,11] and increases colonic epithelial proliferation in progastrin-overexpressing (hGAS) mice.[12–14] hGAS mice have colonic crypt hyperplasia, increased numbers of colonic goblet cells, and increased

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Abbreviations used in this paper: CCK-2R, cholecystokinin-2 receptor; CgA, chromogranin A; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; G-Gly, glycine-extended gastrin; G-17, gastrin-17; hGAS, high circulating levels of progastrin; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; rhPG, recombinant human progastrin; RLU, relative luminescence unit; RT-PCR, reverse-transcription polymerase chain reaction; SMA, smooth muscle actin.

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susceptibility to colonic tumor development after treatment with the carcinogen azoxymethane,[15,16] possibly as a result of persistent epithelial mitosis following DNA-damaging stimuli.[13]

We therefore hypothesized that progastrin regulates colonic homeostasis and carcinogenesis susceptibility by exerting effects on the myofibroblast compartment of the colonic mucosa in addition to its well-documented effects on epithelial cells. We have assessed colonic myofibroblast numbers in hGAS mice and have used a human colonic myofibroblast cell line (CCD18Co) to investigate the effect of exogenous recombinant human progastrin (rhPG) (1–80) on myofibroblasts in vitro. Various pharmacologic agents were used to investigate the signaling pathways involved.

Materials and Methods

Animals

Ten- to 12-week-old C57BL/6 mice (Charles River Laboratories, Margate, Kent, England) and hGAS mice[12] on the C57BL/6 genetic background were maintained at the University of Liverpool in England. Animals were fed a commercially prepared diet, given water ad libitum, and maintained on a 12:12-hour light/dark cycle. All experiments were performed with UK Home Office (Animals Scientific Procedures Act 1986) and local ethical committee approval.

Treatment With AG1024

Groups of 5 sex-matched hGAS and C57BL/6 mice aged 10 weeks were treated with 3 injections of 30 μg AG1024 (Merck Chemicals Ltd, Nottingham, England) in 15% dimethyl sulfoxide (DMSO) or vehicle control intraperitoneally at 0, 24, and 48 hours and were killed 52 hours after the first injection.

Tissue Preparation and Scoring

Colons were fixed in 4% formalin and embedded in paraffin. Four-micrometer sections of distal colon were stained with H&E for morphological assessment or Alcian blue/periodic acid–Schiff for goblet cell identification or underwent immunohistochemistry for chromogranin A (CgA), Ki67, or DCAMKL-1. Fifty hemi-crypts per mouse were assessed for the number of mitotic, goblet, Ki67-positive, CgA-positive, and DCAMKL-1–positive cells. Data are presented as positive cells per hemi-crypt or on a cell positional basis.[13]

Immunohistochemistry

CgA and DCAMKL-1 immunohistochemistry was performed using an EnVision Plus Kit (Dako UK Ltd, Cambridgeshire, England). Sections were subjected to heat-mediated antigen retrieval (10 mmol/L citric acid buffer), incubated for 1 hour at room temperature with rabbit anti-CgA (1/1000; Abcam, Cambridge, England) or rabbit anti–DCAMKL-1 (1/50; Cambridge Bioscience Ltd, Cambridge, England), washed and incubated with biotin-labeled anti-rabbit polymer and 3,3′-diaminobenzidine chromogen, and counterstained with hematoxylin. Ki67 immunohistochemistry was also performed following heat-mediated antigen retrieval, but after overnight incubation with rat anti-Ki67 primary antibody (1/20; Dako) at 4°C, the signal was detected with a biotin-conjugated rabbit anti-rat secondary antibody (1/200; Dako), Vectastain ABC (Vector Laboratories, Peterborough, England), and 3,3′-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Gillingham, England).

For evaluation of myofibroblast cell numbers, sections were incubated with rabbit anti—α–smooth muscle actin (SMA) antibody (1/100; Abcam) in 10% normal goat serum and/or guinea pig anti-vimentin antibody (1/50; Fitzgerald Industries International, North Acton, MA) in 1% bovine serum albumin overnight at 4°C. Sections were then incubated with Texas Red–conjugated anti-rabbit and/or fluorescein isothiocyanate–conjugated anti–guinea pig immunoglobulins (Jackson ImmunoResearch Laboratories, Cambridgeshire, England) (1/200 or 1/600 dilutions, respectively) and mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector Laboratories Ltd). Three fields of view (40× objective lens) per mouse were scored for total number of mucosal cells (by DAPI-stained nuclei) and for positively stained mucosal cells using a fluorescence microscope (BX51; Olympus Microscopy, Essex, England). Data are presented as the mean percentage of α-SMA– or vimentin-positive cells per total number of mucosal cells.

CCD18Co Cell Culture

The human colonic myofibroblast cell line CCD18Co (American Type Culture Collection, Manassas, VA) was cultured at 37°C with 5% CO_2 in Eagle's modified essential media (LGC Standards, Teddington, England) containing 10% fetal calf serum (FCS) (Gibco Invitrogen Ltd, Paisley, Scotland), 0.5% 2 mmol/L L-glutamine (Sigma-Aldrich), and penicillin/streptomycin (Sigma-Aldrich). Cells were detached using trypsin/EDTA (Sigma-Aldrich) and seeded at 1000 cells/well in 96-well plates. Media was changed to serum-free media at 24 and 48 hours to synchronize the cell cycle. At 72 hours, cells were treated with rhPG(1–80),[17] gastrin-17 (G-17), G-Gly, or 50 mmol/L phorbol myristate acetate (PMA), a known inducer of protein kinase C (PKC), for 18 hours. Cells were also pretreated for 20 minutes before addition of 0.1 nmol/L rhPG(1–80) with the cholecystokinin-2 receptor (CCK-2R) inhibitor YM022 (10 nmol/L; Tocris Bioscience, Bristol, England), PKC inhibitor RO-32-0432 (1 μmol/L; Calbiochem, Nottingham, England), mitogen-activated protein kinase (MAPK) inhibitors PD98059 (20 μmol/L; Calbiochem) and U0126 (10 μmol/L; Cell Signaling Technology, Beverly, MA), or the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (20 μmol/L; Calbiochem). Serum-free media and media containing 10% FCS were used as negative and positive controls.

Generation of Conditioned Media From CCD18Co Cells

CCD18Co cells were grown for 24 hours and media was replaced with serum-free media. This process was repeated 24 hours later and 72 hours after initial plating (48 hours after being in serum-free media). Cells were then treated with 0.1 nmol/L rhPG(1–80) in fresh serum-free media or serum-free media alone (negative control). Conditioned media was collected 18 hours posttreatment and centrifuged at 1000 rpm for 5 minutes to remove cells.

Protein Extraction and Western Blotting

Conditioned media was harvested from CCD18Co cells on 3 separate occasions and proteins were concentrated and desalted using Amicon Ultra-15 centrifugal filter units, 3kDa