Gastrin Gene Expression Is Required for the Proliferation and Tumorigenicity of Human Colon Cancer Cells


Abstract

The majority of human colon cancers express the gastrin gene, and a significant percentage bind gastrin-like peptides. However, it is not known if gastrin gene products are physiologically relevant to the growth and proliferation of human colon cancers. To investigate the functional role of gastrin gene expression, we examined the effect of gastrin antisense (AS) RNA expression on the growth and tumorigenicity of colon cancer cells. The full-length human gastrin cDNA was cloned in the AS direction in a retroviral vector under the transcriptional control of human cytomegalovirus promoter. Three representative human colon cancer cell lines that expressed negligible (Colo-205A) to significant (Colo-320 and HCT-116) levels of gastrin mRNA were transfected with either AS or control vectors and subjected to various growth studies in vitro and in vivo. The proliferative and tumorigenic potential of the AS clones from the gastrin-expressing cell lines was significantly suppressed compared to that of the control clones, whereas the growth of Colo-205A-AS cells (the negative control) was similar to that of the Colo-205A-C-cells, indicating the relative specificity of the antitumorigenic effects of AS gastrin RNA expression. We believe that this is the first evidence that supports a possible critical role of gastrin gene expression in the tumorigenicity of human colon cancers that express the gastrin gene. Because >60-80% of human colon cancers express the gastrin gene, it can be expected that the growth of a significant percentage of these cancers may be critically dependent on the expression of gastrin gene products. Therapeutic measures, such as the AS strategy used in the present study, may therefore prove to be useful in fighting human colon cancers in the future.

Introduction

A possible role of gastrins in the etiology of colon cancers is derived from carcinogenesis studies. Endogenous gastrins and exogenous gastrins (other than tetragastrin) promote the growth of established colon cancers in mice (1–4) and promote carcinogen-induced colon cancers in rats (5–7). Recent studies by Montag et al. (8) further established colon cancers in mice (1–4) and promoted carcinogen-induced colon cancers in rats (5–7). More recent investigations (9–14) have identified a possible role of gastrins in the tumorigenicity of human colon cancers that express the gastrin gene. Because >60-80% of human colon cancers express the gastrin gene, it can be expected that the growth of a significant percentage of these cancers may be critically dependent on the expression of gastrin gene products. Therapeutic measures, such as the AS strategy used in the present study, may therefore prove to be useful in fighting human colon cancers in the future.

Materials and Methods

Construction of the AS3 Gastrin mRNA Expression Vector. A retrovirus vector, LNCX (obtained from Dr. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 23), contains an internal human CMV promoter and was used in construction of the gastrin AS vector (LNC-G-AS), as diagrammatically presented in Fig. 1A. Polyadenylic acid mRNA from a human colon cancer cell line, HCT-116, was reverse-transcribed to cDNA. The full-length gastrin cDNA fragment that contained the entire gastrin open reading frame and 44 bp of the nontranslated 5′ flank sequence was generated by PCR using the 5′ primer HG6 (5′-AGGCCCGAGCTTCGACCAAG-3′) and the AS 3′ primer HG7 (5′-GGTAAGGCCTTGAAGCTTGGTTG-3′), by our published methods (13, 14). The PCR product was subcloned into pSP72 vector that provided unique restriction sites at the proximal ends of the cDNA (5′, Clal; 3′, SmaI). The use of the restriction sites ensured the directional cloning of the gastrin cDNA in the LNCX vector in an AS orientation and placed the cDNA under the transcription control of the CMV promoter (Fig. 1A). The LNC-G-AS DNA was confirmed by restriction and DNA sequence analysis (24).

Transfection of Human Colon Cancer Cell Lines. The human colon cancer cell lines Colo-205A (subcloned in our laboratory from Colo-205; American Type Culture Collection), Colo-320 (American Type Culture Collection), and HCT-116 (obtained from Dr. M. Brattain, Medical College of Ohio, Toledo, OH) were analyzed for relative concentrations of gastrin mRNA by a quantitative competitive RT-PCR method, using gastrin DNA as an internal control as described previously (13, 14). The Colo-205A cells expressed <0.1 copy of gastrin mRNA/cell and were used as a negative control for our studies. The HCT-116 cells expressed ~2.5 copies of gastrin mRNA/cell, whereas the Colo-320 cells expressed ~1–2 copies of gastrin mRNA/cell, in agreement with our previous results (14). Each cell line was transfected with either the control (LNCX) or AS (LNC-G-AS) vector DNA by the Ca2+ precipitation method as described previously (25). NEO-resistant colonies were selected by treating transfected cells with optimal concentrations of G418 (250 ng/ml for HCT-116 cells and 500 ng/ml for Colo-205A and Colo-320 cells) as described previously (25). Drug-resistant clones, selected from separate Petri dishes, were numbered sequentially (Colo-10, AS2, AS3) and expanded in vitro using McCoy’s 5A (HCT-116) and RPMI 1640 (Colo-205A, Colo-320) growth medium (Life Technologies, Inc.) in the presence of 10% FCS (Irvine Scientific).

Analysis of Human Colon Cancer Cell Line Transfectants. The AS clones were analyzed for expression of the AS gastrin mRNA transcript. Total RNA was isolated and analyzed by RT-PCR for the presence of AS gastrin mRNA transcripts using a sense primer derived from the retroviral vector.
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Fig. 1. A, construction of the AS gastrin retroviral vector. Retroviral vector (LNCX) containing the gastrin cDNA in the AS orientation (G-AS) and under the transcription control of the CMV promoter is shown. The long terminal repeat (LTR) sequences, the neomycin phosphotransferase gene (neo), and restriction endonuclease sites used in the analysis in Fig. 1C are indicated. The retroviral vector is propagated in bacteria using a vector DNA sequence (not shown) derived from pBR322. B, BamHI, RV, EcoRV. B, RNA analysis of human colon cancer cell line transfectants. The AS clones were analyzed by electrophoresis on 0.75% agarose gels as described (24). Hybridization with the LNC-G-AS proviral DNA in each of the LNC-G-AS transfectants was confirmed by Southern hybridization and RT-PCR to ascertain the insertion and expression of the LNC-G-AS vector DNA. Genomic DNA (10 μg) was digested with either BamHI (Panel a) or EcoRV (Panel b) using conditions recommended by the manufacturer (New England Biolabs) and analyzed by electrophoresis on 0.75% agarose gels as described (24). Hybridization analysis was performed using a [32P]dCTP-labeled probe representing the entire open reading frame of the gastrin cDNA. Data from representative clones are presented and similar to the data obtained from all the other clones examined. Lane 1, HCT-116-C2; Lane 2, HCT-116-C3; Lane 3, HCT-116 (parent); Lane 4, HCT-116-AS2; Lane 5, HCT-116-AS6; Lane 6, HCT-116-AS5; Lane 7, Colo-320-C3; Lane 8, Colo-320-AS2.

Colo-320 cells transfected with either the gastrin AS vector LNC-G-AS DNA or control vector DNA. Genomic DNA (10 μg) was digested with either BamHI (Panel a) or EcoRV (Panel b) using conditions recommended by the manufacturer (New England Biolabs) and analyzed by electrophoresis on 0.75% agarose gels as described (24). Hybridization analysis was performed using a [32P]dCTP-labeled probe representing the entire open reading frame of the gastrin cDNA, as described in the legend of Fig. 1C.

In Vitro Growth Assays. The in vitro proliferation of AS and control clones was measured in an MTT assay, as described previously (25). Briefly, the cells were plated at equal concentrations (6000/well) in 96-well plates. At the end of 7 days of growth in normal growth medium containing 0—10% FCS, the total number of viable cells was determined in an inverted-light microscope (Nikon, Houston, TX).

In Vivo Growth Assay. The AS and control clones, enumerated in vitro in 1% FCS, were inoculated contralaterally at equal concentrations (0.1—0.5 × 10^6 cells/0.2 ml HBSS) in female nude (athymic) mice, age 2 months (Life Sciences, Inc., Pittsburgh, FL), as per our published procedures (1, 2). Mice were euthanized 22—37 days postinoculation, tumors were harvested, and tumor weights were noted.

RIA Analysis of the Cellular Extracts and Conditioned Media. Concentrations of the processing intermediates of gastrin (progastrin and glygastrin) and amidated gastrins (G17-17NH2) were measured in the CM samples using specific antibodies L-289, L376, and L2, respectively, as described previously (26, 27). Briefly, the cells were expanded in growth medium containing 10% FCS under constant drug selection. Although we were able to expand both the control and AS clones of Colo-205A and HCT-116 cells in vitro, the Colo-320-AS clones demonstrated an almost complete growth arrest within three to five in vitro passages; however, the Colo-320-C clones grew normally (data not shown). We were thus unable to conduct growth studies with the Colo-320-AS clones. Only one of the five Colo-320-AS clones, AS2, produced a sufficient number of cells to permit partial characterization and analysis. We analyzed the Colo-320-AS2 clone using Southern hybridization and RT-PCR to ascertain the insertion and expression of the LNC-G-AS vector (Figs. 1B and C). Southern analysis confirmed the presence of one or more integrated copies of the LNC-G-AS proviral DNA in each of the LNC-G-AS transfectants (representative data from some of the clones is shown in Fig. 1C, Panel a; essentially similar results were obtained from all the other clones). The results from EcoRV digests (Fig. 1C, Panel b) suggest that all of the integrated proviral DNAs in HCT-116-AS clones have not undergone a rearrangement of the transfected retrovirus DNA. The Colo-320-AS2 clone (Fig. 1C, Panel b, Lane 8) contains at least two integrated copies, and one of these has undergone rearrangement. The endogenous gastrin gene is also detected in each of the DNA digests (4.9 kb, BamHI; 8.8 kb, EcoRV). Because the expression of gastrin AS RNA produced such a dramatic effect upon the proliferation of Colo-320 cells, it suggested for the first time that gastrin mRNA expression may indeed be critical to the growth of some colon cancer cell lines. Besides exhibiting an almost complete growth arrest, the size of the Colo-320-AS cells was significantly increased (>10-fold) compared to that of the control clones (data not shown). The Colo-320-AS clones demonstrated distinct morphological differences under electron microscope compared to control clones. The Colo-320-AS

Results and Discussion

Three representative human colon cancer cell lines that express either negligible (Colo-205A) or significant (Colo-320 and HCT-116) levels of gastrin mRNA were transfected with either the control (LNCX) or AS (LNC-G-AS) vector DNA, as described in “Materials and Methods.” The G418-resistant colonies were selected and expanded in growth medium containing 10% FCS under constant drug selection. Although we were able to expand both the control and AS clones of Colo-205A and HCT-116 cells in vitro, the Colo-320-AS clones demonstrated an almost complete growth arrest within three to five in vitro passages; however, the Colo-320-C clones grew normally (data not shown). We were thus unable to conduct growth studies with the Colo-320-AS clones. Only one of the five Colo-320-AS clones, AS2, produced a sufficient number of cells to permit partial characterization and analysis. We analyzed the Colo-320-AS2 clone using Southern hybridization and RT-PCR to ascertain the insertion and expression of the LNC-G-AS vector (Figs. 1B and C). Southern analysis confirmed the presence of one or more integrated copies of the LNC-G-AS proviral DNA in each of the LNC-G-AS transfectants (representative data from some of the clones is shown in Fig. 1C, Panel a; essentially similar results were obtained from all the other clones). The results from EcoRV digests (Fig. 1C, Panel b) suggest that all of the integrated proviral DNAs in HCT-116-AS clones have not undergone a rearrangement of the transfected retrovirus DNA. The Colo-320-AS2 clone (Fig. 1C, Panel b, Lane 8) contains at least two integrated copies, and one of these has undergone rearrangement. The endogenous gastrin gene is also detected in each of the DNA digests (4.9 kb, BamHI; 8.8 kb, EcoRV). Because the expression of gastrin AS RNA produced such a dramatic effect upon the proliferation of Colo-320 cells, it suggested for the first time that gastrin mRNA expression may indeed be critical to the growth of some colon cancer cell lines. Besides exhibiting an almost complete growth arrest, the size of the Colo-320-AS cells was significantly increased (>10-fold) compared to that of the control clones (data not shown). The Colo-320-AS clones demonstrated distinct morphological differences under electron microscope compared to control clones. The Colo-320-AS
cells were multinucleated with euchromatin and a high concentration of mitochondria, whereas the Colo-320-C cells contained the expected heterochromatin and few mitochondria (data not shown). The Colo-320-AS cells, although clearly growth-arrested, were metabolically active and excluded trypan blue dye.

Antiproliferative effects of gastrin AS RNA expression were less dramatic on the growth of HCT-116-AS cells compared to those observed with Colo-320 cells. The in vitro proliferative rate of the HCT-116-C and -AS clones was compared using an MTT assay (as described in "Materials and Methods"). The proliferation of all the HCT-116-AS clones in serum-free medium was only 5% of that measured for the corresponding HCT-116-C clones in serum-free medium (representative data from some of the clones is shown Fig. 2A). The proliferation of all the HCT-116-AS clones increased in response to increasing concentrations of FCS but remained significantly lower than that of HCT-116-C clones at equivalent concentrations of FCS (Fig. 2A). A soft agar clonogenic assay (26) was used to determine the in vitro tumorigenic potential of the cells. The number of colonies formed by HCT-116-AS cells in increasing concentrations of FCS (from all the AS clones) remained only 0–5% compared to that formed by all the HCT-116-C clones (data from representative clones is presented in Figs. 2B and C). Thus, although the proliferative potential of the HCT-116-AS clones (especially in 10% FCS) was not as drastically affected as that of the Colo-320-AS clones, the in vitro clonogenic (tumorigenic) potential of the HCT-116-AS clones (even in 10% FCS) was significantly suppressed compared to that of the HCT-116-C and Colo-320-C clones. Morphologically, the HCT-116-AS cells (from all the AS clones) also seemed to be significantly different compared to the HCT-116-C cells from all the control clones. HCT-116-AS cells were ~2-4-fold larger in size and contained euchromatin, a prominent nucleolus, and signs of microvilli formation, whereas HCT-116-C cells seemed to be normal colon cancer cells with typical heterochromatin (data not shown).

Possible antitumorigenic effects of the expression of gastrin AS RNA were also examined in vivo. Cells from representative HCT-116-AS and -C clones were inoculated into nude mice (as described in "Materials and Methods"). The mice were palpated for tumors from day 10. The mice were euthanized between days 22–37, and tumors, free of host tissues (1, 2), were removed, and tumor weights were noted (Table 1). Tumors were palpable as early as day 12 in all 11 mice inoculated with the HCT-116-C clones. A well-formed tumor was removed from every HCT-116-C inoculation site at the time of euthanasia, and confirmed for vector DNA. On the other hand, a small tumor was palpable only in 1 of 11 mice inoculated with AS cells during the entire course of the study; in all the other mice, no tumor was palpable during the course of the study, and no tumor was observed in the mice at the time of euthanasia. Tumors of equivalent weights were harvested from nude mice inoculated similarly with Colo-205-C and Colo-205-AS cells (data not shown). The significant suppression of tumorigenesis of HCT-116-AS clones in vivo once again suggests that gastrin gene products may play a critical role in the growth and tumorigenesis of human colon cancer cells that express significant concentrations of gastrin mRNA.

The concentrations of amidated gastrin and processing intermediates of gastrin (progastrin and glygastrin; Refs. 27 and 28) in the CMs of the HCT-116-AS and -C cells were measured as described in "Materials and Methods." The concentrations of progastrin and glygastrin in the CM samples of AS clones were <1% compared to those in the CM samples of control clones (Fig. 3). Amidated

<table>
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<th>Mice #</th>
<th>Right side</th>
<th>Weight (mg)</th>
<th>Left side</th>
<th>Weight (mg)</th>
<th>Day of euthanasia (post inoculation)</th>
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<td>1, 2, 3</td>
<td>C3</td>
<td>639 ± 230 (400–850)</td>
<td>AS4</td>
<td>43.3 ± 75 (0.0–130)</td>
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<td>534 ± 127 (397–650)</td>
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<td>48.0 ± 59 (0.0–114)</td>
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<td>7, 8, 9</td>
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<td>217 ± 89 (123–300)</td>
<td>AS10</td>
<td>1.5 ± 2 (0.0–3.0)</td>
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<tr>
<td>10, 11</td>
<td>C7</td>
<td>417 ± 6 (413–422)</td>
<td>AS9</td>
<td>0.0 ± 0.0 (0.0–0.0)</td>
<td>22</td>
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</table>

Table 1 Tumorigenicity of HCT-116-AS and -C clones in vivo in nude mice

The HCT-116-AS and C clones, enumerated in vitro in 1% FCS, were inoculated contralaterally at equal concentrations (0.1-0.5 × 10⁷ cells/0.2 ml HBSS) in female nude (athymic) mice, age 2 months (Life Sciences, St. Petersburg, Fl.), following published procedures (1,2). The weight of tumors harvested from mice on the day of euthanasia was noted. Each data point represents mean ± SD of tumor weights from 2–3 mice inoculated with the indicated AS and control clones growing contralaterally in mice; the range of values measured is given in parentheses.
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**Fig. 3.** Two representative HCT-116-AS (AS2 and AS3) and two representative HCT-116-C (C2 and C3) clones were selected for analysis of gastrin-like peptides by RIA, as described in "Materials and Methods." Each data point represents fmol/10^7 cells and is the mean ± SD of four separate observations from two separate clones. Amidated gastrins were not measured in any of the samples (data not shown).

Gastrins were not measured in the CE and CM samples of either HCT-116-AS or -C clones. The concentrations of progastrins and glygastrins were significantly reduced in the CE samples of AS versus control clones. These results support the conclusion that the antiproliferative effects measured as a result of AS gastrin RNA expression were specific and due to a significant reduction in the concentration of gastrin-like peptides.

The proliferation and tumorigenic potential of the Colo-205A-AS and -C clones was similar (Figs. 2A and B), suggesting that the antiproliferative and antitumorigenic effects of AS expression of gastrin RNA were specific to colon cancer cells expressing significant concentrations of endogenous gastrin mRNA (Colo-320 and HCT-116 cells). No nonspecific effects were measured on either the morphology, tumorigenesis (data not shown), or proliferation (Figs. 2A and B) of Colo-205A cells expressing negligible concentrations of endogenous gastrin mRNA, further confirming the specificity of the effects of AS gastrin RNA expression on only the gastrin-expressing colon cancer cell lines.

**Summary.** Previous studies with anti-gastrin antibodies suggested that gastrins may function as autocrine growth factors for colon cancers (30). Our results confirm that gastrin gene expression may indeed play a physiologically relevant role in the growth of human colon cancer cells that express significant concentrations of gastrin mRNA. In preliminary studies with gastrin AS oligonucleotides (20–23 mer), we observed a significant inhibition in the growth of Colo-320 cells in an MTT assay. This result led to the present study, confirming the specificity of effects with AS gastrin RNA expression. Because AS gastrin RNA expression was effective in suppressing the tumorigenicity of HCT-116 cells (that were primarily expressing progastrin and glygastrin), an important role of processing intermediates of gastrin in the growth and tumorigenicity of colon cancers is suggested. Potent mitogenic effects of glygastrin on several cell types (20–22) supports the novel concept that the processing intermediates of gastrin may play an important autocrine role in the proliferation of colon cancers that needs to be further examined.

An interesting observation was that although the growth suppression was almost complete with Colo-320-AS cells, the proliferative potential of HCT-116-AS cells was reduced but not arrested, suggesting that the expression of gastrin AS RNA was perhaps less effective toward titrating out the effects of endogenous gastrins in cells expressing higher concentrations of gastrin mRNA. Our results further suggest that the expression of gastrin AS RNA via the specific retroviral construct used is likely to be most effective in suppressing the growth of human colon cancers expressing gastrin mRNA; the suppression, however, may be less complete for human colon cancers expressing higher concentrations of gastrin mRNA. Based on our current knowledge that perhaps >60-80% of human colon cancers express gastrin mRNA (9–14, 28), it can be expected that delivery of gastrin AS RNA expression vectors to the site of the tumor may potentially result in significantly suppressing the growth of gastrin-expressing colon cancers. Colon cancers expressing a minimal concentration of gastrin mRNA are not likely to respond to the antitumorigenic effects of gastrin AS RNA expression and may perhaps represent a subset of tumors that have developed autocrine mechanisms independent of gastrin gene products.

**References**


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4 P. Singh and A. Owlia, unpublished observations.
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