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A gastrin precursor, gastrin-gly, upregulates VEGF expression in colonic epithelial cells through an HIF-1-independent mechanism

Claudine Bertrand¹, Aline Kowalski-Chauvel¹, Catherine Do¹, Cécile Résa¹, Souad Najib¹, Laurence Daulhac², Timothy C. Wang³, Audrey Ferrand¹, and Catherine Seva¹

¹Cancer Department, INSERM U.858, (I2MR) Institute of Molecular Medicine, Toulouse Cedex 4, France

²Laboratoire de Pharmacologie, INSERM U.766, Université Clermont 1, Faculté de Pharmacie, Clermont-Ferrand, France

³Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY

Abstract

One of the major angiogenic factor released by tumor cells is VEGF. Its high expression is correlated with poor prognosis in colorectal tumors. In colon cancer, gastrin gene expression is also upregulated. In these tumors, gastrin precursors are mainly produced and act as growth factors. Recently, a study has also shown that the gastrin precursor, G-gly induced *in vitro* tubules formation by vascular endothelial cells suggesting a potential proangiogenic role. Here, we demonstrate that stimulation of human colorectal cancer cell lines with G-gly increases the expression of the proangiogenic factor VEGF at the mRNA and protein levels. In addition, blocking the progastrin autocrine loop leads to a downregulation of VEGF. Although HIF-1 is a major transcriptional activator for VEGF our results suggest an alternative mechanism for VEGF regulation in normoxic conditions, independent of HIF-1 that involves the PI3K/AKT pathway. Indeed we show that G-gly does not lead to HIF-1 accumulation in colon cancer cells. Moreover, we found that G-gly activates the PI3K/AKT pathway and inhibition of this pathway reverses the effects of G-gly observed on VEGF mRNA and protein levels. In correlation with these results, we observed *in vivo*, on colon tissue sections from transgenic mice overexpressing G-gly, an increase in VEGF expression in absence of HIF-1 accumulation. In conclusion, our study demonstrates that gastrin precursors, known to promote colon epithelial cells proliferation and survival can also contribute to the angiogenesis process by stimulating the expression of the proangiogenic factor VEGF *via* the PI3K pathway and independently of hypoxia conditions.

Keywords

colon cancer; gastrin; intracellular signaling; VEGF

Angiogenesis is essential for tumor growth and metastasis. It is initiated by tumor cells releasing angiogenic factors that induce growth of new blood vessels. Indeed recruitment of new blood vessels by the tumor provides nutrients and oxygen and facilitates dissemination

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Correspondence to: Catherine Seva, Cancer Department, INSERM U.858, (I2MR) Institut de Medecine Moleculaire de Rangueil, 1 avenue J. Poulhes, BP 84225, 31432 Toulouse Cedex 4, France, Tel.: 33-5-61-32-24-08, Fax: 33-5-61-32-24-03, Cathy.seva@inserm.fr.

Claudine Bertrand and Aline Kowalski-Chauvel contributed equally to this work.

of cancer cells that will give rise to metastasis. This complex process involves basement membrane degradation, endothelial cell migration, proliferation and tubule formation. One of the major angiogenic factor released by tumor cell is VEGF. Its high expression levels have been correlated with poor prognosis in colorectal tumors.¹

Colorectal cancer (CRC) remains the second most common cause of cancer-related death worldwide. Once metastasis has occurred in CRC, a complete cure of the cancer is unlikely. In patients with advanced colon cancer, wherein the tumor has penetrated beyond the bowel wall and there is evidence of metastasis to distant organs, the 5-year survival rate is less than 10%. As a consequence, it is important to identify the factors involved in the development, the survival but also the spreading of colon cancer cells toward the body through the blood vessels.

The hormone gastrin (Gamide) is known as a potent stimulant of gastric acid secretion.² In gastrointestinal cancers and more particularly in colon cancer, gastrin gene expression is upregulated. Indeed this gene is a target of oncogenic pathways frequently activated in these cancers such as APC/beta-catenin or Ras pathways.^{3–5} However, in these tumors, gastrin is incompletely matured and gastrin precursors, such as G-gly, are mainly produced. High concentrations of gastrin precursors, including G-Gly, have been observed in colon tumors and in blood of patients with colorectal cancer. These precursors represent 90 to 100% of the gastrin peptides produced by colon tumor while they are absent from healthy tissue.⁶ Interestingly, the resection of the colorectal tumor induces a decrease in the concentration of the precursors in the blood suggesting that the tumor is the source of the peptides. In addition, clinical studies have reported that hypergastrinemia is associated with an increased risk of colorectal cancer.

G-gly has been reported to promote the proliferation of human gastrointestinal tumor cells.^{7–10} The trophic effects of this peptide have also been confirmed *in vivo*. MTI/G-gly transgenic mice overexpressing G-gly display hyperproliferation of the colonic mucosa likely *via* the upregulation of several signalling pathways, including Src, PI3-K/Akt, JAK2/STAT3, ERKs.^{11,12} Furthermore, perfusion of G-gly into rats or gastrin-deficient mice results in proliferation of colonic mucosal cells, the formation of aberrant crypt foci and increases the sensitivity to azoxymethane, a colon carcinogen.¹¹ G-gly is also known to inhibit apoptosis and promote the migration of human colon cancer cells.^{13,14} More recently, a study has also shown that G-gly induced *in vitro* the tubule formation by human vascular endothelial cells in a manner similar to what is observed with VEGF suggesting a potential proangiogenic role for this peptide.¹⁵

The aim of the current study is to investigate whether glycine-extended gastrin regulates the expression of proangiogenic factors such as VEGF in colon cancer cells and to analyze the cellular mechanisms responsible. Here we report in 3 different human colon cancer cell lines that G-gly increases VEGF expression in normoxic conditions. Although numerous factors, including growth factors and hormones, have been shown to regulate VEGF expression in normoxic conditions *via* HIF-1,^{16–18} a transcription factor which binds a consensus hypoxia response element on the VEGF promoter, our results suggest an alternative mechanism for VEGF regulation by G-gly that is independent of this transcription factor but requires the PI3-Kinase/AKT pathway.

Material and Methods

Cell culture

The human colon cancer cell lines DLD1, HT29 and Lovo were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in RPMI (DLD1) or

DMEM (HT29, Lovo) supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO₂. In all experiments, cells were serum-starved for 18 hr prior G-gly stimulation. Hypoxic conditions were achieved by culturing cells in a sealed hypoxic chamber (1% O₂, 5% CO₂). For proliferation assays, cells were counted by using a Coulter electronic counter.

Animals

MTI/G-Gly (in a FVB/N background) and control FVB/N mice used in this study have been previously described.¹² At least 4 MTI/G-gly mice and 4 corresponding control littermates mice (22–24 weeks) were used. All procedures were approved by animal facility care committee.

RNA extraction, reverse transcription, real-time PCR

Total RNA was isolated from colon cancer cells by using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA). After pre-treating RNA with DNase (Invitrogen, Carlsbad, CA), cDNA was produced from 1 µg of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). VEGF or gastrin mRNA expression was determined *via* real-time PCR, using fluorescent SYBR green dye (Applied Biosystems, Framingham, MA) to allow semi quantitative analysis of gene expression levels. Amplification was conducted using ABI-Stepone + Detection System (Applied Biosystems, Framingham, MA). Relative fold changes were determined using the $2^{-\Delta\Delta CT}$ method, in which *ACTB* gene was used for normalization. Forward and reverse primers used: gastrin, forward-TCCATCCATCCATAGGCTTC reverse-CCACACCTCGTGGCAGAC; ACTB, forward-GCGCGGCTACAGCTTCA reverse-CTTAATGTACGCACGATTTC; VEGF, forward-CGAGGGCCTGGAGTGTGT

Gastrin SiRNA and ShRNA

Lovo cells (1×10^5 cells ml⁻¹ in 6-well plates) were transiently transfected with 60 nM of Silencer Negative control siRNA or Gastrin silencer pre-designed siRNA (from Ambion) using the transfection agent siPORT NeoFX according to the manufacturer protocol. Gastrin mRNA expression was controlled 48, 72 and 96 hr after transfection using real time PCR as described above.

HT29 cells were stably transfected using a shRNA plasmid for Human gastrin (SuperArray Bioscience Corporation) according to manufacturer's instructions. After 3 weeks of selection in Neomycin, stably transfected cell pools were used for the experiments. Gastrin mRNA expression was controlled using real time PCR as described above.

Western-blot analysis

Western-blot analyses were performed on cell lysates. Fractions, containing identical levels of proteins, were separated by SDS-PAGE and analyzed by western-blot with the indicated antibodies as described previously.^{19,20} The immunore-activity was visualized with an enhanced chemiluminescence system. Primary antibodies used for western blot were: VEGF (Santa cruz Biotechnology), HIF-1alpha (BD Transduction Laboratories), beta-Tubuline (SIGMA), phospho-AKT (Cell Signaling), GAPDH (Chemicon International).

Detection of VEGF by ELISA

Cells were plated in 35-mm dishes. After 24 hr, the supernates were collected and analyzed for VEGF using the Duo set ELISA development system from R&D system according to manufacturer's instructions. Results were normalized by counting the cells in each well.

Immunofluorescent staining of cells

Cells were grown on 12-well plates containing cover slides. Cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% triton X-100, blocked in 1% FCS-PBS and incubated with primary antibodies according to standard immunofluorescence methods.

Primary antibodies used for immunofluorescent staining of cells were: VEGF (Santa cruz Biotechnology), HIF-1alpha (BD Transduction Laboratories).

Secondary antibodies coupled to Alexa-488 were purchased from Molecular Probe (Invitrogen, France). Slides were mounted in fluorescent mounting medium (DAKO) and analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. Images were assembled using Adobe Photoshop software. For semi-quantitative comparisons, identical volumes of antibody mix were used for all samples and identical exposure times taken. Immunofluorescent intensity was analyzed in at least 3 independent experiments using the image analyzer ImageQuant.

Immunofluorescent staining of tissues

For immunofluorescent staining of tissues we used formaldehyde-fixed, paraffin-embedded tissues. Antigen retrieval was performed on dewaxed sections by microwaving slides in 10 mM citrate buffer (pH 6). After Serum blocking and application of primary antibodies, the detection was done using Alexa Fluor 488 goat anti-rabbit antibody. Control slides, where the primary antibody was replaced by diluted nonimmune rabbit IgG, were checked for nonspecific reactivity before assessment of the staining. Slides were analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. For comparisons, identical volumes of antibody mix were used for all samples. Immunofluorescence intensity was analyzed using the image analyzer ImageJ.

Blood vessels counting

Blood vessels were visualized by immunofluorescent staining as described above using a CD31 specific antibody (PECAM-1 from Santa-Cruz). Immunostained blood vessels were counted using the microscope software Axio Vision from Zeiss. The total section was screened and the results were normalized to tissue area.

Immunohistochemistry

For staining of tissues by immunohistochemistry we used formaldehyde-fixed, paraffin embedded tissues. Antigen retrieval was performed on dewaxed sections by microwaving slides in 10 mM citrate buffer (pH 6). After Serum blocking and application of primary antibodies we used the DakoCytomation Envision + System-HRP according to the manufacturer protocol.

Statistical analysis

Means $\pm$ SE and Student *t* tests were performed using “GraphPad Prism.” *** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$.

Results

G-gly regulates VEGF expression in human colon cancer cells

We first analyze whether G-gly regulates the expression levels of VEGF mRNA in HT29 and DLD1, 2 human colon cancer cell lines known to proliferate in presence of exogenous G-gly. Using real time RT-PCR we compared the levels of VEGF mRNA from G-gly treated (1 nM) or control cells. In response to G-gly, we observed a 2-fold increase in the

expression of *VEGF* gene after G-gly treatment (Fig. 1*a*). A similar increase in VEGF mRNA was also observed under hypoxia, a recognized potent stimulant of this proangiogenic factor.

Using 2 different approaches, we next analyzed whether induction of VEGF mRNA levels in response to G-gly resulted in a parallel increase of the protein.

By immunofluorescence microscopy using antibodies specific for VEGF we observed a higher level of staining in colon cancer cells treated with G-gly (Fig. 1*b*). In addition, we also confirmed by western blot analysis (Fig. 1*c*) and ELISA (Fig. 1*d*) the increase in VEGF protein levels in G-gly-stimulated cells. Similarly, VEGF protein levels were increased by CoCl₂ known to mimic hypoxic conditions (Figs. 1*b* and 1*c*).

Immuno-staining of VEGF and blood vessels number in colonic mucosa of FVB/N and MTI/G-Gly transgenic mice

To analyze *in vivo* the relevance of VEGF expression in response to G-gly under normoxic conditions, we studied, by immunohistochemistry methods, VEGF expression on colon tissue sections from control mice (FVB/N) or MTI/G-gly mice which overexpress glycine-extended gastrin, resulting in colonic mucosa hyperplasia, hyperproliferation and an increased susceptibility to intestinal neoplasia.²¹ As shown in Figure 2*a*, tissues derived from MTI/G-gly mice showed an upregulation of VEGF (right panel) as compared to control mice (left panel). In correlation with these results we also observed a significant increase in blood vessels number, visualized by CD31 immunostaining, in MTI/G-gly mice as compared to control FVB/N mice (Fig. 2*b*).

G-gly does not increase HIF-1 α protein levels

As mentioned in the introduction, VEGF expression is highly regulated by hypoxia *via* the transcription factor, HIF-1. HIF-1 is a dimer composed of HIF-1 α and HIF-1 β that bind to the hypoxia response element on the VEGF promoter. Although HIF-1 β is constitutively expressed, HIF-1 α is undetectable in normoxic conditions but strongly induced under hypoxia. Recently several growth factors have been shown to regulate VEGF expression in normoxic conditions through the induction of HIF-1 α .^{16–18} Therefore we tested the hypothesis that G-gly upregulates VEGF *via* HIF-1 α protein. By immunofluorescence analysis using an antibody that detects specifically HIF-1 α , we observed, as expected, an accumulation of HIF-1 α protein in the nucleus of HT29 and DLD1 cells under hypoxic conditions (Fig. 3*a*). These results were confirmed by western blot analysis on both cell lines (Fig. 3*b*). In contrast, using these 2 approaches we found that HIF-1 α protein is not induced by G-gly, suggesting an alternative mechanism for the regulation of VEGF by this hormone (Figs. 3*a* and 3*b*).

We also analyzed, by immunohistochemistry methods, the expression of HIF-1 α on colon tissue sections from MTI/G-gly mice or control mice (FVB/N). In contrast to what we observed for VEGF expression, the regulation of HIF-1 α was not affected in transgenic mice compared to control animals (Fig. 3*c*). These results are consistent with an upregulation of VEGF expression by G-Gly independently of HIF-1.

Signaling pathways involved in VEGF expression stimulated by G-gly

Although HIF-1 is a key regulator of VEGF expression, other signaling pathways, including the PI3-kinase/AKT pathway, have been shown to induce VEGF independently of HIF-1 under normoxic conditions.^{18,22–26} We previously demonstrated the involvement of this signaling pathway in the proliferative effects of G-gly.^{9,12} In addition, we recently reported the upregulation of the PI3-kinase/AKT pathway in the colon of MTI/G-gly mice. Here we

first verified, in the HT29 and DLD1 cell lines, the activation of this pathway in response to G-gly.

Using antibodies specific for the activated, phosphorylated forms of AKT, a downstream effector of the PI 3-Kinase we performed western-blot analysis on lysates from colon cancer cells incubated in the presence or in absence of G-gly for the indicated times. In response to G-gly, we detected an increased phosphorylation of AKT indicating that the PI3-Kinase/AKT pathway was activated (Fig. 4).

To determine the cellular mechanism by which G-gly could increase *VEGF* gene expression, we examined G-gly-regulated *VEGF* gene expression in HT29 and DLD1 cells by quantitative real-time PCR in the presence of LY294002, a specific inhibitor which blocks the PI 3-Kinase pathway. When cells were preincubated with LY294002, the response to G-gly was abolished (Fig. 5a), indicating that the PI 3-Kinase pathway mediates G-gly-increased VEGF mRNA expression in colon cancer cells. These results were confirmed at the protein level by immunofluorescence staining using antibodies specific for VEGF. The increase in VEGF protein levels in response to G-gly was also reversed in the presence of the PI3-Kinase inhibitor in both cell lines (Fig. 5b).

Blocking autocrine gastrins decreases VEGF production by colon cancer cells

As mentioned in the introduction, gastrin precursors are produced by colorectal tumors. In addition, they can play an important role in maintaining cell proliferation by acting as autocrine growth factors.⁶ We analyzed, by quantitative real-time PCR, the expression levels of gastrin mRNA in 3 different human colon cancer cell lines relative to normal human colon. As expected, we observed high levels of gastrin mRNA in all cell lines compared to normal colon, with the highest expression in Lovo cells (Fig. 6a). Therefore we transfected this cell line with either a small interfering RNA (siRNA) directed against the gastrin gene or a scrambled control. gastrin mRNA expression was measured 48–96 hr after transfection. Down regulation of the gastrin gene was significant after 48 hr (40%) however, a maximal decrease of 80% was achieved at 72 hr with the gastrin siRNA. This downregulation was not maintained after 96 hr (Fig. 6b). The condition of 72 hr was used to analyze the ability of the gastrin siRNA to block cell proliferation as well as AKT phosphorylation, 2 events known to be activated by gastrin precursors. Both cell growth and AKT phosphorylation were significantly inhibited in Lovo cells transfected with the gastrin siRNA as compared to scrambled control (Figs. 6c and 6d). These results confirm the autocrine role of gastrin peptides on colon cancer cells. However, the partial inhibition suggests the involvement of other growth factors secreted by the tumor cells. Similarly 72 hr after transfection with the gastrin siRNA we observed a decrease in VEGF expression at the protein level indicating that blocking autocrine gastrins decreases VEGF production by colon cancer cells (Fig. 6e).

We also used another human colon cancer cell line, HT29 stably transfected with a shRNA directed against the gastrin gene or a scrambled control. A decrease of more than 70% in the gastrin mRNA expression was observed in cell pools transfected with the gastrin shRNA as compared to the scramble control (Fig. 7a). Similarly to what we observed in the Lovo cells, blocking autocrine gastrins in HT29 leads to a decrease in VEGF expression at the mRNA and protein level (Figs. 7b and 7c). Cell growth was also significantly inhibited in HT29 cells stably transfected with the gastrin shRNA as compared to scrambled control and addition of exogenous VEGF can restore cellular proliferation (Fig. 7d).

Discussion

Gastrin precursors are highly expressed by colon cancer cells and play an important role in colon carcinogenesis by stimulating cell proliferation and decreasing apoptosis. However, they are also increased in early stages of colon carcinogenesis.⁶

Tumor growth and metastasis are also regulated by proangiogenic factors released from tumor cells into the microenvironment. One of the most potent angiogenic factors is VEGF, which is also commonly overexpressed in colorectal cancers and associated with a poor clinical outcome.¹

In the present study, we hypothesized that gastrin precursors might also contribute to tumor progression by regulating the expression of proangiogenic factors such as VEGF in colon cancer cells. Our results demonstrate that gastrin precursors play a role in the regulation of VEGF independently of hypoxia. Treatment of human colorectal cell lines with exogenous G-gly increases VEGF at the mRNA and protein levels. In addition, blocking the progastrin autocrine loop leads to a downregulation of VEGF.

HIF-1 is a major transcriptional activator of VEGF known to be regulated by environmental factors such as hypoxia but also by certain growth factors, cytokines or hormones produced by the tumors. Whereas hypoxia upregulates VEGF by decreasing HIF-1 α protein degradation, numerous growth factors have been shown to stimulate VEGF expression by increasing HIF-1 α synthesis through the ERK and/or PI3-Kinase/AKT pathways.^{16,27–29} In both cases, HIF-1, accumulated in the nucleus, binds to an HIF response element (HRE) present on the VEGF promoter. Here our results suggest an alternative mechanism for VEGF regulation in normoxic conditions, independent of HIF-1 that involves the PI3K/AKT pathway. Indeed we show that G-gly, does not lead to HIF-1 α accumulation in DLD1 and HT29 cells in contrast to what is observed under hypoxia in these cell lines. In addition we found that G-gly activates the PI3K/AKT pathway and that the PI3K inhibitor LY294002 reverses the effects of G-gly observed on VEGF mRNA and protein level. In correlation with these results, we observed *in vivo*, on colon tissue sections from transgenic mice overexpressing G-gly, an increase in VEGF expression in absence of HIF-1 accumulation. Previously we reported in this model the up-regulation of the PI3-Kinase/AKT pathway. VEGF over-expression in the colon of these transgenic mice was associated with an increase in the number of blood vessels as measured by CD31 expression, an adhesion molecule expressed by vascular endothelial cells.

These results are in accordance with other reports that show dissociation between PI3K/AKT-dependent and HIF-1-dependent VEGF upregulation. In particular, expression of constitutively active AKT leads to VEGF upregulation independently of hypoxia and HIF-1,³⁰ likely *via* the activation of the transcription factor SP1.²⁶ Activation of the PI3K pathway by either Ki-Ras mutations or loss of PTEN, a negative regulator of the PI3K pathway gave similar results.^{26,31} Maity *et al.* also suggest that a growth factor such as EGF could regulate VEGF expression *via* a pathway involving PI3K but independent of HIF-1.²² Indeed, in this study, VEGF promoter in which the Hypoxic Response Element was deleted was still upregulated by EGF and downregulated by inhibition of PI3K. In addition, the VEGF promoter contains several β -catenin/Tcf binding sites that could be involved in the expression of this angiogenic factor in colon cancer.^{24,25} Inactivation of the tumor suppressor APC occurs early during colon carcinogenesis and leads to accumulation of β -catenin, a mediator of the Wnt pathway. Translocation of β -catenin into the nucleus and its interaction with transcription factors of the TCF/Lef family leads to the expression of target genes. Transfection of colon epithelial cells with activated β -catenin has been shown to upregulate VEGF expression. It is interesting to notice that gastrin precursors produced by

colon cancer cells are capable to increase the activity of the transcription factor β -catenin/Tcf-4 likely *via* the PI3K pathway.³²

In conclusion, our study demonstrates that gastrin precursors, known to promote colon epithelial cells proliferation and survival and to be expressed along the colon adenoma-carcinoma sequence, can also contribute to the angiogenesis process by stimulating the expression of the potent proangiogenic factor VEGF *via* the PI3K pathway and independently of hypoxia conditions.

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Figure 1.

G-gly regulates VEGF expression in human colon cancer cells. DLD1 and HT29 cells were treated or not with 1 nM of G-gly, 250 μ M CoCl₂ or exposed to hypoxia (1%) for 6 hr as indicated. (a) Total RNA was isolated and VEGF mRNA expression was determined by real time PCR as described in methods. Quantifications of 3 experiments are presented as means $\pm$ SE. (b) Cells were grown for 24 hr on 12-well plates containing cover slides. Following treatments, cells were then similarly fixed and stained with anti-VEGF antibodies using standard immunofluorescence techniques as described in "Methods." Representative micrographs from 3 independent experiments are shown. (c) Expression of VEGF protein was also examined by Western analysis following treatment of the cells with G-gly or CoCl₂. Blots were also probed with an antibody against tubulin to ensure equal loading of proteins. (d) VEGF expression in cell supernates was also measured by ELISA. Results were normalized by cell counting. Representative data from 3 experiments are shown. Quantifications of 3 experiments are presented as means $\pm$ SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2.

Immuno-staining of VEGF and blood vessels number in colonic mucosa of FVB/N and MTI/G-Gly transgenic mice. (a) Sections of paraffin-embedded colonic mucosa were immunostained with VEGF antibodies using standard immunofluorescence techniques as described in "Methods." Micrographs from representative fields of the stained sections were taken (original magnification $\times 40$). Immunofluorescent intensity was analyzed in at least 4 independent experiments using the image analyzer ImageJ. Results of immunofluorescence quantification are expressed as percentages of the control values (FVB/N). (b) Blood vessels were visualized by immunofluorescent staining as described in "Methods," using a CD31 specific antibody. Immunostained blood vessels were counted using the microscope software Axio Vision from Zeiss. The total sections were screened and the results were normalized to tissue area. Quantifications of 3 experiments are presented as means $\pm$ SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 3.

G-gly does not increase HIF-1α protein levels. DLD1 and HT29 cells were treated or not (control) with 1 nM of G-gly or exposed to hypoxia (1%) for the time indicated. (a) Cells were grown for 24 hr on 12-well plates containing cover slides. Following treatments, cells were then similarly fixed and stained with anti-HIF1α antibodies using standard immunofluorescence techniques as described in "Methods." Representative micrographs from 3 independent experiments are shown. (b) Expression of HIF1α protein was also examined by Western analysis. Blots were also probed with an antibody against tubuline to ensure equal loading of proteins. Representative data from 3 experiments are shown. (c) Immunohistochemistry was performed on sections of paraffin-embedded colonic mucosa with anti-HIF1α antibodies. Micrographs from representative fields were taken (original magnification $\times 40$). Results are representative of 3 experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4.

G-gly induces AKT activation in human colon cancer cells. DLD1 and HT29 cells were treated or not with 1 nM of G-gly for the time indicated. AKT activation was determined on cell lysates by immunoblot using antibodies directed against the activated phosphorylated forms or AKT. Blots were also probed with tubuline to ensure equal loading of protein. Representative data from 3 experiments are shown. Quantifications of 3 experiments are presented as means $\pm$ SE.

Figure 5.

Role of the PI3-kinase/AKT pathway in VEGF expression stimulated by G-gly. Cells were pretreated for 30 min. with a specific PI3-kinase inhibitor (LY294002, 20 μ M) prior to G-gly stimulation. (a) Total RNA was isolated and VEGF mRNA expression was determined by real time PCR as described in methods. Quantifications of 3 experiments are presented as means $\pm$ SE. (b) Cells were grown for 24 hr on 12-well plates containing cover slides. Following treatments, cells were then similarly fixed and stained with anti-VEGF antibodies using standard immunofluorescence techniques as described in "Methods." Representative micrographs from 3 independent experiments are shown. Immunofluorescent intensity was analyzed in at least 3 independent experiments using the image analyzer ImageQuant. Quantifications of 3 experiments are presented as means $\pm$ SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6.

Blocking autocrine gastrins decreases VEGF production in the human colon cancer cell line Lovo. (a) Gastrin mRNA expression from different human colon cancer cell lines (HT29, DLD1, Lovo) or normal epithelium was determined by real time PCR as described in methods. (b) Lovo cells were transiently transfected with Silencer Negative control siRNA or Gastrin silencer siRNA as described in "Methods." Gastrin mRNA expression was controlled 48, 72 and 96 hr after transfection using real time PCR. (c–e) After 72 hr of transfection with Silencer Negative control siRNA or Gastrin silencer siRNA, proliferation rates in Lovo cells (c) were determined by MTT assay, AKT phosphorylation (d) and VEGF protein expression (e) were determined on cell lysates by immunoblot. Blots were also probed with GAPDH or tubuline (as indicated) to ensure equal loading of protein. Representative data from 3 experiments are shown. (a–e) Quantifications of 3 experiments are presented as means $\pm$ SE.

Figure 7.

Blocking autocrine gastrins decreases VEGF production in the human colon cancer cell line HT29. HT29 cells were stably transfected with a shRNA directed against the gastrin gene or a scrambled control. Stably transfected cell pools were used for the experiments. (a, b) Gastrin or VEGF mRNA expression was measured using real time PCR. (c) VEGF expression in cell supernates was measured by ELISA. Results were normalized by cells counting. (d) Proliferation rates of HT29 in absence or presence of VEGF (10 ng ml^{-1}) were determined by cell counting. Representative data from 3 to 4 experiments are shown. Quantifications of 3 to 4 experiments are presented as means $\pm$ SE.