

Leptin, Insulin-Like Growth Factor-1, and Insulin-Like Growth Factor-2 Are Mitogens in *Apc*^{Min/+} but not *Apc*^{+/+} Colonic Epithelial Cell Lines

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Abstract

The obese state is associated with elevated circulating levels of insulin, insulin-like growth factors (IGF), and leptin. Research is contradictory regarding the role of these elevated growth factors in colon cancer risk. We hypothesized that colonic epithelial cells that were *Apc* deficient (*Apc*^{Min/+}) but not those expressing wild-type *Apc* (*Apc*^{+/+}) would experience a hyperproliferative and antiapoptotic phenotype when exposed to these growth factors. This hypothesis was addressed using two nontumorigenic murine colonic epithelial cell lines with distinct *Apc* genotypes: *Apc*^{+/+} YAMC cells and *Apc*^{Min/+} IMCE cells. Cells were treated for 48 hours with various concentrations of leptin (0.001-50 ng/mL), IGF-1 (0.1-200 ng/mL), or IGF-2 (0.1-600 ng/mL). In YAMC cells, leptin caused a significant decrease in cell proliferation ($P < 0.01$) compared with controls due to induction of caspase activity and apoptosis. In contrast, in the IMCE cells, leptin

induced a 75% increase in cell proliferation compared with controls ($P < 0.0001$). IGF-1 and IGF-2 also induced 50% greater proliferation in the IMCE cells ($P < 0.001$) compared with controls. Cotreatment of IMCE cells with leptin and either IGF-1 or IGF-2 induced greater proliferation than either growth factor alone ($P < 0.0001$). IMCE cell proliferation caused by leptin only treatment was associated with activation of p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, and nuclear factor- κ B nuclear translocation but not with MAPK kinase or Janus-activated kinase/signal transducers and activators of transcription activation. These data provide the first evidence that leptin may interact with IGFs to promote survival and expansion of colonic epithelial cells that were *Apc* deficient (*Apc*^{Min/+}) but not those expressing wild-type *Apc* (*Apc*^{+/+}). (Cancer Epidemiol Biomarkers Prev 2005;14(7):1646-52)

Introduction

During the past 20 years, obesity has increased at an epidemic rate in the United States. Currently, one in four adults in the United States is considered obese, defined as having a body mass index of ≥ 30 kg/m² (1). Morbidities associated with obesity are second only to smoking in the United States (2). The colon is among several sites, including endometrium, breast, gall bladder, and prostate, that are subject to increased risk of cancer in obese individuals (3). Interestingly, central or visceral obesity greatly increases the risk of a constellation of disease risk factors now called *metabolic syndrome* or syndrome X. The Centers for Disease Control and Prevention estimate that ~47 million Americans have this condition. The clinical presentation includes increased abdominal fat, elevated levels of triglycerides and low-density lipoproteins, low levels of high-density lipoprotein cholesterol, elevated blood pressure, insulin resistance, and prothrombotic and proinflammatory states (4). This insulin-resistant, proinflammatory state is also characterized by elevated blood insulin, insulin-like growth factors (IGF), and leptin. The elevated levels of these biological mediators may be causally related to the development of certain cancers, including colon cancer. Case-control and

cohort studies consistently show a clear positive association between colon cancer and/or colonic polyps with elevated levels of insulin (5-7) and IGF-1 and decreased levels of IGF-binding proteins (8, 9); however, research is lacking regarding an association between elevated leptin and colon cancer.

Colonic epithelial cells possess insulin, IGF-1, IGF-2, and leptin receptors (10, 11). In addition, the insulin and IGF receptors are present at greater levels in tumors compared with normal colonic epithelium (12). Presumably, insulin and IGFs promote tumor progression by increasing proliferation and decreasing the apoptotic potential of abnormal colonic epithelial cells, thereby allowing the cells to survive and acquire additional mutations. In support of this, IGF-1 administration to colon cancer cell lines was shown to protect them from apoptosis by potentiating the tumor necrosis factor- α , mitogen-activated protein kinase (MAPK), and nuclear factor- κ B (NF- κ B) signaling pathways (11). Recently, leptin was shown to be a growth factor for colonic cancer epithelial cells (13) and to promote invasiveness of colon cancer epithelial cells (14).

Although it is relatively clear that these growth factors can promote tumorigenesis, a role for these growth factors early in the progression of normal cells to neoplastic cells is not clear. Using cells with contrasting adenomatous polyposis coli (*Apc*) genotypes, we addressed the role of dose of this gatekeeper tumor suppressor gene in the responsiveness of colonic epithelial cell proliferation to leptin, IGF-1, and IGF-2, growth factors commonly elevated in the obese state. We used a unique model system of conditionally immortalized colonic epithelial cell lines to dissect these early events. These cell lines YAMC (*Apc*^{+/+}) cells and IMCE (*Apc*^{Min/+}) cells are consistent with a normal to preneoplastic transition. Because both cell types are nontumorigenic, the phenotypic changes in the IMCE

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cells (*Apc* deficient) including growth factor induced migration, cell-cell communication, and inducible nitric oxide synthase/cyclooxygenase-2 expression, are consistent with known early phenotypes in human colorectal cancer (15-17). We employed this unique model of normal and preneoplastic colonic epithelial cells to show that leptin and the IGFs have differential, genotype/phenotype-specific effects on the proliferative and apoptotic potential of these cells, two processes that determine cell number homeostasis in the colon. Given the "gatekeeper" status of the *Apc* gene in colon carcinogenesis, it is highly relevant that we have identified a differential effect of leptin on cell proliferation and apoptotic potential in colonic epithelial cells deficient in *Apc* (*Apc*^{Min/+}) compared with wild-type *Apc* (*Apc*^{+/+}).

Materials and Methods

Chemicals. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Growth medium, insulin/transferrin/selenium, murine IFN- γ , and type IV collagen were purchased from Life Technologies (Rockville, MD). Neonatal calf serum and antibiotics were purchased from Gemini Bio-Products (Woodland, CA). All recombinant murine growth factors (IGF-1, IGF-2, and leptin) were purchased from R&D Systems (Minneapolis, MN).

Cells and Cell Culture Conditions. The YAMC (*Apc*^{+/+}) cells were developed from the transgenic SV40 large T antigen mouse. The IMCE (*Apc*^{Min/+}) cells were derived from an F1 hybrid between the SV40 large T antigen transgenic mouse and the *Apc*^{Min/+} mouse (15). Both of these cell lines are non-tumorigenic in nude mice, do not grow in soft agar, and survive in culture only on extracellular matrix proteins such as collagen I (15). Both YAMC (*Apc*^{+/+}) and IMCE (*Apc*^{Min/+}) cells express the heat-labile SV40 large T antigen under the control of an IFN- γ -inducible promoter. At 33°C the temperature-sensitive SV40 large T antigen is active and drives cell proliferation. At 39°C, the temperature-sensitive mutation yields an inactive protein and cells behave as nonproliferating, differentiated colonic epithelial cells (18).

Cells were cultured as previously described (17). Briefly, cells were cultured at 33°C on collagen I until reaching ~70% confluence. Once 70% confluent, cells were transferred to 39°C in serum-free and IFN- γ -free medium for 24 hours before each experiment. This period allows for cessation of SV40 large T antigen-driven cell proliferation, depletion of residual growth factors (serum), and a brief stabilization period. Moving the cells to 39°C at 70% confluence prevents contact inhibition of proliferation while still permitting gap junction formation. These cell lines behave like normal cells in that they are contact inhibited and undergo apoptosis if they achieve maximal confluence. Therefore, conditions are optimized for cells to proliferate slowly for 24 hours at 39°C and then undergo cell death over 5 to 8 days, similar to the life cycle of a normal colonic epithelial cell.

Western Blotting. Cells were grown in T-75 flasks and treated as described above before collection. Briefly, cells were washed twice with cold PBS and harvested by scraping cells into 1 mL of cold lysis buffer (30 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% NP40, and 10% glycerol) per flask. The cell suspension was then sonicated to insure cell lysis and centrifuged for 15 minutes \times 14,000 rpm at 4°C. Nuclear and cytoplasmic fractions were collected using the NE-PER kit according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). Samples were subjected to SDS-PAGE gel electrophoresis and transferred to a polyvinylidene difluoride membrane

(Bio-Rad Laboratories, Hercules, CA). Membranes were probed with primary antibodies against the proteins of interest (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by the appropriate secondary antibody conjugated to horseradish peroxidase (included with femtoLucent kit) and detected by chemiluminescence using the femtoLucent kit (Geno Technology, Inc., St. Louis, MO).

Cell Proliferation. Cell proliferation was measured using a commercially available compound, calcein AM (Molecular Probes, Eugene, OR), that is colorless, nonfluorescent, and cell membrane permeable. The compound fluoresces when cleaved by nonspecific esterases in actively proliferating cells. The cleaved products degrade quickly and are relatively nontoxic to cells. These properties make this compound very useful for proliferation assays in adherent cell lines. We adapted the protocol provided by Molecular Probes to our cells. Briefly, ~1,500 cells per well were seeded in 96-well plates coated with collagen I (BD Biosciences, San Jose, CA) as described above. Cells were left at 33°C overnight to adhere and reach 70% confluence. Plates were then moved to 39°C for 24 hours in serum-free and IFN- γ -free medium to allow for cessation of SV40 large T antigen-driven cell proliferation and a brief stabilization period. The cells were then washed with PBS and treated with 100 μ L of 1 μ mol/L calcein AM in PBS for 30 minutes, and baseline fluorescence was read at an excitation wavelength of 485 nm and emission wavelength of 530 nm in a Cytofluor fluorescent plate reader (Millipore, Bedford, MA). Calcein AM was removed from all wells, and the cells were washed with PBS and treated with growth factors (described below) for 48 hours. After 48 hours, the treatment medium was removed and fluorescence was measured again as described above. Cell proliferation was calculated as the final fluorescent reading minus the first fluorescent reading. This change in fluorescence after 48 hours due to treatment was compared with controls. Unless otherwise indicated, each data point represents the average value over eight wells with SD between the wells used to create error bars. We validated this method using compounds that were known to both stimulate cell proliferation and induce apoptosis in these cell lines (data not shown).

Growth Factor Treatments. After the 24-hour stabilization period at 39°C, the cells were then treated (eight wells per treatment) with various concentrations of leptin (1, 5, 10, and 50 ng/mL), IGF-1, or IGF-2 (1, 10, 50, 100, and 200 ng/mL). Once effective (proliferation different than controls), concentrations of the individual growth factors were established and the cells were cotreated with leptin in combination with IGF-1 or IGF-2.

Caspase Activity Assay. The Caspase-Glo 3/7 and the Caspase-Glo 9 assays (Promega, Madison, WI) were used to detect caspase 3/7 and 9 activity. Cells were cultured in 96-well plates and treated with leptin (1, 12, or 50 ng/mL) as described above. After 48 hours of leptin treatment, 100 μ L of Caspase-Glo reagent was added to each well according to the manufacturer's instructions. Plates were mixed on a plate shaker for 30 seconds and incubated at room temperature for 3 hours. Luminescence was measured using the Synergy HT microplate reader (BIO-TEK, Winooski, VT).

Annexin V/FITC Assay. Apoptosis was assessed in YAMC (*Apc*^{+/+}) cells treated with leptin using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes). Cells were grown in T-75 flasks and treated according to the above description before collection. Cells were detached from flasks using trypsin/EDTA, washed with PBS, centrifuged, and resuspended in 1 \times binding buffer. One hundred microliters of cell suspension was combined with 5 μ L of Annexin V (component A) and 2 μ L of diluted propidium iodide working buffer. The solution was incubated for 15 minutes at room temperature and then 400 μ L of 1 \times binding buffer were added. Finally,

500 μ L of cell solution were diluted with 500 μ L of sheath fluid before analysis by flow cytometry. Bivariate analysis of FITC fluorescence (FL-1) and propidium iodide fluorescence (FL-3) allowed for the distinction of cell stage from viable cells to apoptotic, late apoptotic, or necrotic.

Cell Signal Inhibitor Studies. IMCE (*Apc^{Min/+}*) cells were grown in 96-well plates as described above and treated with 12.5 ng/mL leptin. IMCE (*Apc^{Min/+}*) cells were then cotreated with the following cell signal inhibitors: 1, 50, or 100 nmol/L p38 MAPK inhibitor (SB202190; AG Scientific, San Diego, CA); 1, 10, or 100 μ mol/L Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) inhibitor (AG490; AG Scientific); 1, 10, or 100 μ mol/L MAPK kinase (MEK) inhibitor (UO126; Promega); and 1, 100, or 1,000 nmol/L NF- κ B inhibitor (pyrrolidine dithiocarbamate; Tocris, Ellisville, MO). Results were compared with leptin-treated (proliferation) control and an untreated control.

Statistical Analysis. Cell proliferation and cell proliferation inhibition data were assessed statistically by comparing treated cell proliferation with control cell proliferation within each cell type. Differences in proliferation were compared using ANOVA in combination with Tukey's multiple comparisons test. Caspase data were assessed statistically by comparing treated cell caspase activity to control cell caspase activity in YAMC (*Apc^{+/+}*) cells. Pairwise differences in caspase activity were compared using ANOVA in combination with Tukey's multiple comparisons test. Fluorescence-activated cell sorting data were assessed statistically by comparing the percent of treated cells versus control cells in each gated quadrant using ANOVA in combination with Tukey's multiple comparisons test. The Prism software package (Graph Pad, San Diego, CA) was used for this analysis.

Results

Western Blotting for Receptor Status. We employed Western blot analysis to confirm the presence of the leptin receptor (Ob-R) and IGF-1 and IGF-2 receptors in YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) cells. It was critical to confirm the presence of the receptors for these growth factors and to rule out the possibility of no effect due to lack of receptor presence. These receptors were found in relatively comparable quantities in these two cell lines (Fig. 1B and Fig. 2B-D).

Cell Proliferation. Cell proliferation was determined using calcein AM, a commercially available chemical that fluoresces when cleaved by nonspecific esterases in proliferating cells. YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) cells were treated with concentrations of leptin ranging from 1 pg/mL to 50 ng/mL. These concentrations were chosen to represent the physiologic range of low to high circulating concentrations of leptin. In YAMC (*Apc^{+/+}*) cells, leptin significantly decreased cell proliferation at concentrations as low as 390 pg/mL (Fig. 1A). An opposite effect was observed in IMCE (*Apc^{Min/+}*) cells. Leptin induced cell proliferation in IMCE (*Apc^{Min/+}*) cells in a concentration-dependent manner from 10 to 100 pg/mL ($P < 0.001$), with maximal cell proliferation observed at 390 pg/mL and higher ($P < 0.0001$). YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) cells were treated with IGF-1 (0.1-200 ng/mL) or IGF-2 (0.1-600 ng/mL) at various concentrations representative of physiologic low to high concentrations. Neither IGF-1 (Fig. 2A) nor IGF-2 (Fig. 2C) significantly altered cell proliferation in YAMC (*Apc^{+/+}*) cells. However, IGF-1 and IGF-2 significantly increased cell proliferation in IMCE (*Apc^{Min/+}*) cells at 1 ng/mL and higher concentrations ($P < 0.01$). We wanted to assess the effect of cotreatment of high levels of leptin and IGF-1 on cell proliferation to mimic the physiologic state in which multiple growth factors are elevated, as in obese

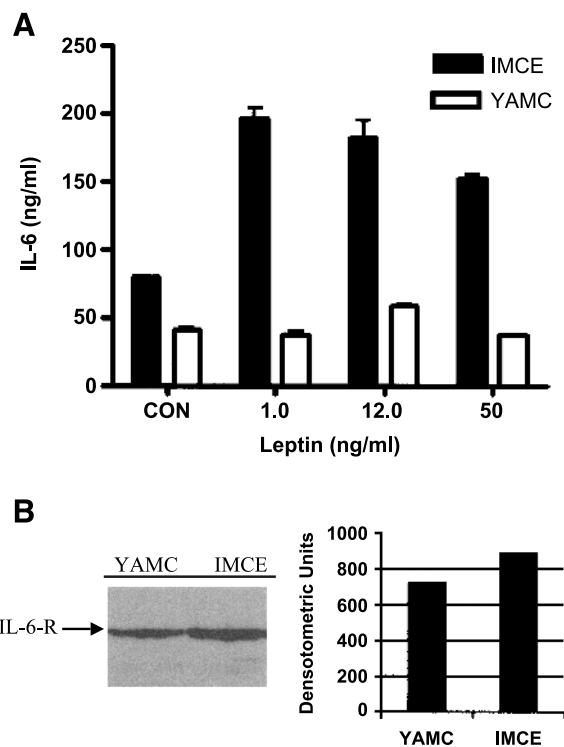


Figure 1. A. Effect of leptin on the proliferation of YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) colonic epithelial cells. Cells were treated with leptin from 0.001 to 50 ng/mL for 48 hours. Representative of three separate experiments. \blacklozenge , $P < 0.01$ (compared with untreated control); *, $P < 0.001$ (compared with untreated control); **, $P < 0.0001$ (compared with untreated control). B. Western blot analysis using an antibody against Ob-R and the densitometric analysis comparing Ob-R protein from untreated YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) control cells.

individuals. Therefore, IMCE (*Apc^{Min/+}*) cells were treated with 50 ng/mL leptin, 100 ng/mL IGF-1, or a cotreatment of 50 ng/mL leptin and 100 ng/mL IGF-1. The cotreatment resulted in a significant increase in cell proliferation compared with leptin or IGF-1 alone ($P < 0.01$; Fig. 3A). In a separate experiment, IMCE (*Apc^{Min/+}*) cells were treated with 50 ng/mL leptin, 200 ng/mL IGF-2, or a cotreatment of 50 ng/mL leptin and 200 ng/mL IGF-2 (Fig. 3B). Again, the cotreatment resulted in a significant increase in cell proliferation compared with leptin or IGF-2 alone ($P < 0.01$).

Caspase Activity. To investigate whether increased apoptosis was involved in the antiproliferative effect of leptin on YAMC (*Apc^{+/+}*) cells, these cells were treated with 1, 12.5, or 50 ng/mL leptin and caspase activity was measured. Irrespective of the concentration tested, leptin induced significantly greater caspase 3/7 activation compared with untreated controls ($P < 0.001$ for each concentration; Fig. 4A). Similarly, leptin significantly increased caspase 9 activity ($P < 0.01$ at 1 and 50 ng/mL leptin versus control), with maximal activity induced with 12.5 ng/mL leptin ($P < 0.001$; Fig. 4B).

Annexin V/Propidium Iodide Staining. To assess further the effect of leptin on apoptosis on YAMC (*Apc^{+/+}*) cells, we chose the optimal leptin concentration based on caspase activity (12.5 ng/mL) to use for flow cytometric analysis of apoptotic stage. Significantly fewer leptin-treated YAMC (*Apc^{+/+}*) cells were viable compared with controls ($68 \pm 4.0\%$ versus $80 \pm 5.0\%$; $P < 0.01$) when treated with this dose of leptin. More leptin-treated YAMC (*Apc^{+/+}*) cells were observed

staining for early ($13 \pm 2.0\%$ versus $2 \pm 1.0\%$) and late ($19 \pm 2.0\%$ versus $13 \pm 3.0\%$) apoptotic stages compared with controls ($P < 0.05$ for each comparison; Fig. 5).

Cell Signal Pathway Activation. Because increased proliferation was observed in IMCE cells in response to leptin, IGF-1, and IGF-2, we investigated whether changes in the phosphorylation status of MAPK and NF- κ B signaling molecules were associated with this phenomenon. Leptin treatment of IMCE (*Apc^{Min/+}*) cells caused rapid phosphorylation of p42/44 MAPK and p38 MAPK (Fig. 6A). In addition, leptin also caused a time-dependent increase in nuclear accumulation of NF- κ B (Fig. 6B). However, leptin treatment did not result in phosphorylation of MEK (data not shown).

Cell Signaling Inhibitor Experiments. We assessed the effect of several inhibitors of intracellular signaling pathways associated with cell proliferation in IMCE (*Apc^{Min/+}*) cells to gain insight into which ones might be involved with the leptin-associated proliferation we observed in these cells. Cotreatment of IMCE (*Apc^{Min/+}*) cells with leptin (12.5 ng/mL) and the MEK (U0126) and JAK/STAT (AG490) inhibitors did not inhibit the leptin-induced proliferation at any tested doses (data not shown). In fact, the highest doses of these inhibitors caused cell death. However, cotreatment of IMCE (*Apc^{Min/+}*) cells with leptin (12.5 ng/mL) and the p38 MAPK inhibitor (SB202190) reduced leptin-induced proliferation by $\sim 75\%$ ($P < 0.001$; Fig. 6C) at each dose tested. Cotreatment with the NF- κ B inhibitor (pyrrolidine dithiocarbamate) maximally inhibited proliferation by 62% at the 100 nmol/L dose ($P < 0.001$; Fig. 6C).

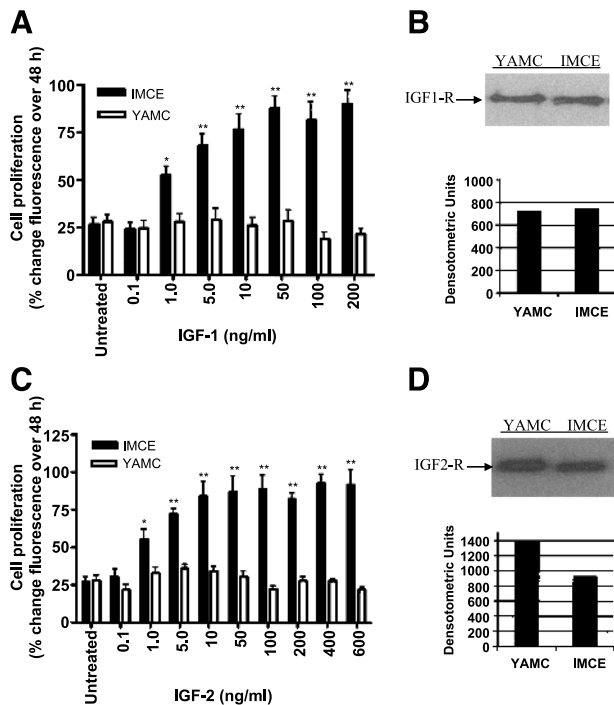


Figure 2. The effect of IGF-1 (A) and IGF-2 (C) on the proliferation of YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) colonic epithelial cells. YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) cells were treated with (A) IGF-1 (0.1–200 ng/mL) or (B) IGF-2 (0.1–600 ng/mL) for 48 hours. Representative of three separate experiments. *, $P < 0.01$ (compared with untreated control); **, $P < 0.001$ (compared with untreated control). Western blot analysis using an antibody against IGF1-R (B) and IGF2-R (D) and the densitometric analysis comparing the protein from untreated YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) control cells.

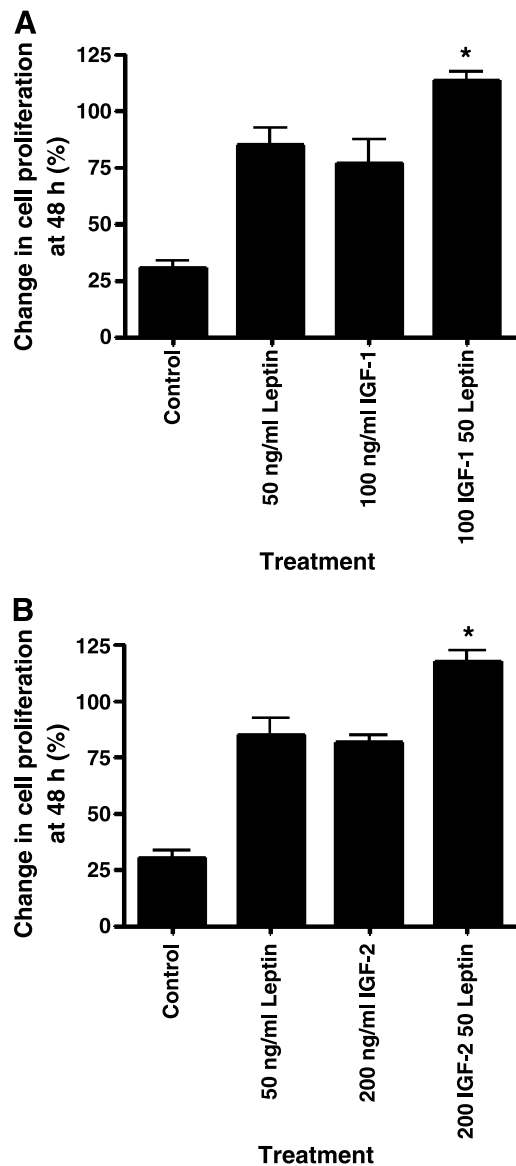


Figure 3. Effect of cotreatment of leptin and IGF-1 or IGF-2 on the proliferation of IMCE (*Apc^{Min/+}*) colonic epithelial cells. IMCE (*Apc^{Min/+}*) cells were treated with (A) 50 ng/mL leptin, 100 ng/mL IGF-1, or a cotreatment of 50 ng/mL leptin and 100 ng/mL IGF-1 or (B) with 50 ng/mL leptin, 200 ng/mL IGF-2, or a cotreatment of 50 ng/mL leptin and 200 ng/mL IGF-2 for 48 hours. *, $P < 0.01$ (compared with leptin or IGF alone). Representative of three separate experiments.

Discussion

Cancer is thought to result from the successive acquisition of distinct phenotypes that facilitate independent cell survival and growth. These phenotypes may include, in a cell type-specific fashion, the evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, and ultimately tissue invasion and metastasis (19). The sequence and consequences of the acquisition of these phenotypes is of great interest, and models to study this process would be of value. Cell culture systems are lacking for the study of the factors that potentially influence the early stages of colon cancer. Most research published to date uses cell lines isolated from colon tumors capable of invasion and metastasis. Because the biology of cancer is known to be stage specific, metastatic

cells are not relevant for the study of early events in colon carcinogenesis. As such, the cell model system employed must reflect the cancer stage under investigation.

Because colorectal cancer arises from the interaction of dietary, hormonal, and genetic susceptibility factors, it is important to use relevant reductionist model systems to evaluate the interaction of these factors on cell number homeostasis in colonic epithelial cells. The effect of leptin on processes related to cell number homeostasis in normal, preneoplastic, or metastatic colonic epithelial cells is controversial. Leptin causes cell proliferation in metastatic cell lines (13, 20), whereas it decreases cell proliferation in mouse colon (21) and reduces formation of aberrant crypt foci in azoxymethane-treated rat colon (22).

We assessed the effects of leptin, IGF-1 and IGF-2 on colonic epithelial cell homeostasis using a range of physiologically relevant concentrations of these growth factors associated with human metabolic syndrome. We identified a differential effect of leptin on cell proliferation and apoptotic potential between two nontumorigenic epithelial cells with distinct *Apc* genotypes. Given the "gatekeeper" role of this important protein in colon carcinogenesis, it is highly relevant that we identified a differential effect of leptin on cell proliferation and apoptotic potential in YAMC (*Apc*^{+/+}) colonic epithelial cells and IMCE (*Apc*^{Min/+}) colonic epithelial cell lines, both of which are nontumorigenic. We showed that leptin reduced cell proliferation in YAMC (*Apc*^{+/+}) cells

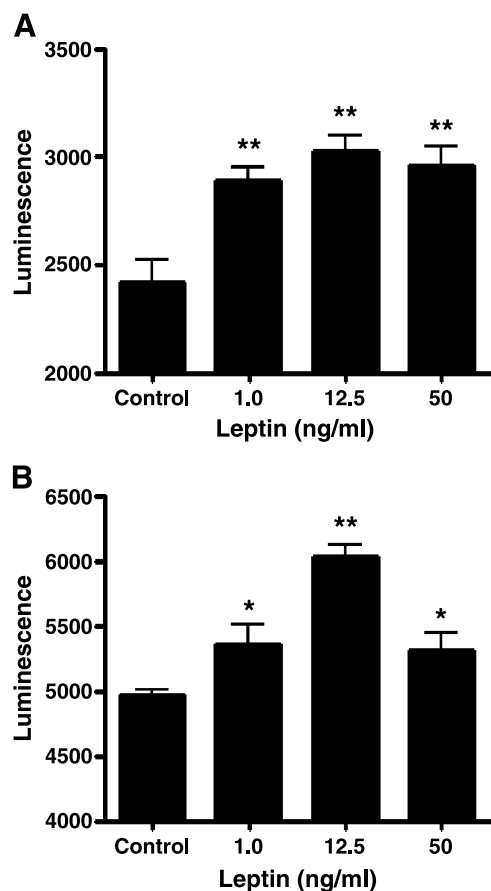


Figure 4. The effect of leptin on caspase activity in YAMC (*Apc*^{+/+}) colonic epithelial cells. YAMC (*Apc*^{+/+}) cells were treated with 1, 12.5, or 50 ng/mL leptin for 48 hours and induction of (A) caspase 3/7 activity or (B) caspase 9 activity was measured. *, $P < 0.01$ (compared with control); **, $P < 0.001$ (compared with control). Representative of three separate experiments.

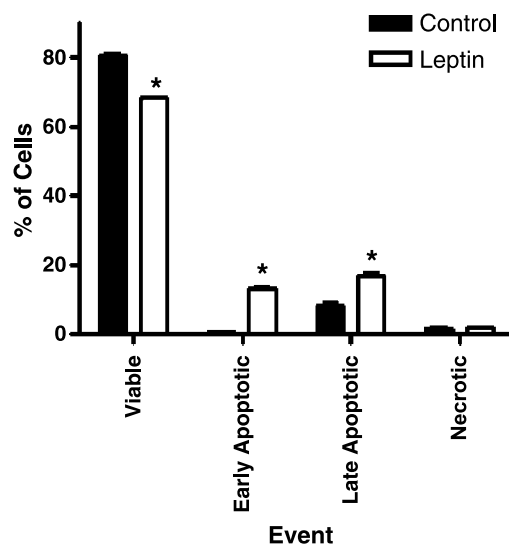


Figure 5. The effect of leptin on apoptosis in YAMC (*Apc*^{+/+}) colonic epithelial cells. YAMC (*Apc*^{+/+}) cells were treated with 12.5 ng/mL leptin and stained with Annexin V/propidium iodide, and apoptotic stage was assessed using flow cytometric analysis. *, $P < 0.05$ (compared with control for each quadrant). Representative of three separate experiments.

(Fig. 1A) and that this effect was associated with an induction of caspase activity (Fig. 4) and apoptosis (Fig. 5). In contrast, leptin, IGF-1, and IGF-2 induced cell proliferation in colonic epithelial cells possessing one truncated copy of *Apc* [IMCE (*Apc*^{Min/+}) cells; Figs. 1A and Fig. 2A and C, respectively]. IMCE cell proliferation was augmented (Fig. 3) when IMCE (*Apc*^{Min/+}) cells were treated with IGF-1 or IGF-2 in combination with leptin.

The dramatically different proliferative responses to leptin in IMCE (*Apc*^{Min/+}) and YAMC (*Apc*^{+/+}) cells cannot be explained by significant differences in relative abundance of the leptin receptor, Ob-R (Fig. 1B). It is rational to speculate that signaling events downstream of these receptors may be responsible for the observed phenotypic differences. To investigate this possibility, we tested whether the MEK, JAK/STAT, p-42/44 MAPK, p38 MAPK, or NF- κ B proteins showed changes associated with leptin treatment in IMCE (*Apc*^{Min/+}) cells. We showed that leptin treatment of these cells induced phosphorylation of p42/44 MAPK and p38 MAPK (Fig. 6A) and activation of NF- κ B as evidenced by NF- κ B nuclear accumulation (Fig. 6B). Therefore, the survival and proliferation of preneoplastic IMCE (*Apc*^{Min/+}) cells caused by leptin treatment is associated with MAPK and NF- κ B activation. Although others have shown that the proliferative effect of leptin is dependent on MEK and JAK/STAT activation (23), inhibition of MEK and JAK/STAT pathways was not found to be critical for the proliferative effect of leptin/IGF-1 treatments in our experiments (data not shown).

The majority of previous studies of intracellular signaling events initiated by leptin and the insulin-like growth factors was done in cell lines isolated from metastatic tumors (24-26). In tumor cell lines, the vast differences in ploidy, the presence of activated oncogenes, and the inactivation of tumor suppressor genes can create aberrant signal transduction compared with quiescent and nontumorigenic cell lines, like YAMC (*Apc*^{+/+}) and IMCE (*Apc*^{Min/+}) cells, which more closely model the normal epithelial cell phenotype.

The proliferative response of IMCE (*Apc*^{Min/+}) cells to leptin treatment was inhibited by a specific enzymatic inhibitor of p38 MAPK activation (SB202190; Fig. 6) lending further

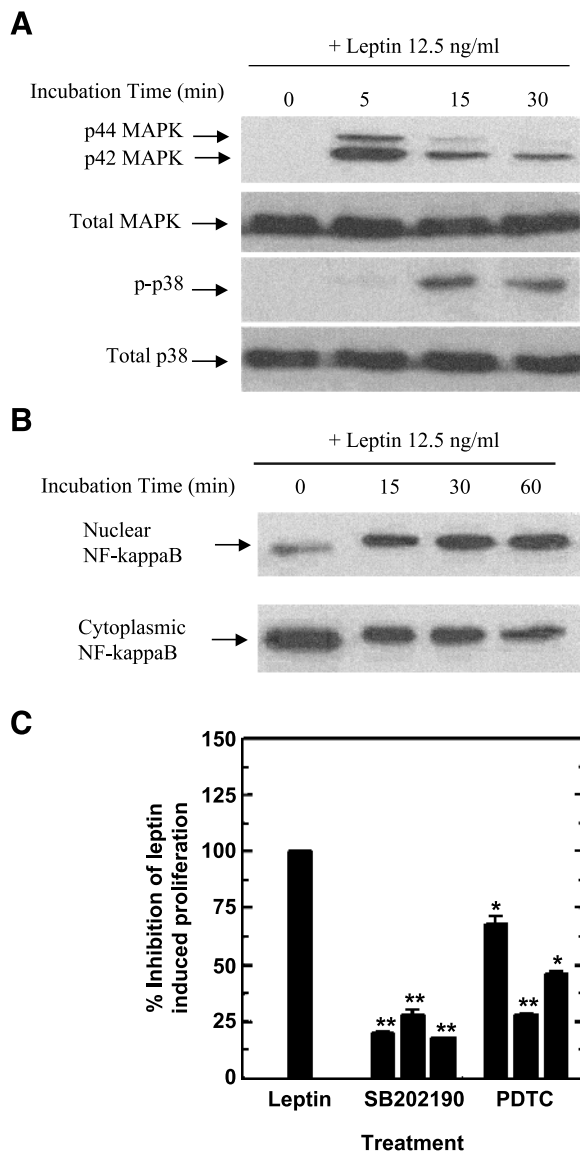


Figure 6. Activation by leptin of p42/44 and p38 MAPK (A) or nuclear translocation of NF- κ B (B) in IMCE (*Apc^{Min/+}*) cells. Cells were treated overnight in serum-free medium before exposure to leptin (12.5 ng/mL). At the indicated times, cellular extracts were prepared and Western blot analysis was done. Blots are one representative experiment of three. C. Effect of cotreatment of leptin and cell signaling inhibitors on the proliferation of IMCE (*Apc^{Min/+}*) cells. Cells were cotreated with 12.5 ng/mL leptin and the following cell signal inhibitors for 48 hours: 1, 50, or 100 nmol/L p-38 MAPK inhibitor (SB202190) and 1, 100, or 1,000 nmol/L NF- κ B inhibitor (PDTC). *, $P < 0.01$ (compared with leptin control); **, $P < 0.001$ (compared with leptin control). Representative of three separate experiments.

support that this pathway is important in proliferation of these nontransformed cells. Leptin binding of the long form of the leptin receptor activates extracellular signal-regulated kinase (p42/44 MAPK) which in turn activates p38 MAPK (27). This leptin-induced activation of p38 via p42/44 activation may be associated with the nuclear translocation of NF- κ B we observed in leptin-treated IMCE (*Apc^{Min/+}*) cells. In support of this possibility, Jijon et al. showed that NF- κ B-inducing kinase activates NF- κ B transcriptional activity through a p38 MAPK-dependent phosphorylation pathway (28). Furthermore, as seen in Fig. 6C, in IMCE (*Apc^{Min/+}*) cells,

p38 inhibition led to a greater reduction in the leptin-induced cell proliferation than NF- κ B inhibition, and this may be due to the fact that enzymatic inhibitors of p38 block both p38 phosphorylation and p38 activation-induced NF- κ B activation seen in thymic epithelial cells (29).

Recent evidence has shown a critical role for NF- κ B activation in epithelial cells associated with decreased apoptosis and hence increased cancer risk (30, 31). Enhanced epithelial cell survival resulting from NF- κ B activation could allow for the accumulation of additional mutations in cell growth and apoptosis control genes associated with colon carcinogenesis (32). Colon cancer risk is increased in individuals with inflammatory bowel conditions; leptin is associated with these processes (22, 33, 34). Our cell signaling data in IMCE (*Apc^{Min/+}*) cells are consistent with the hypothesis that NF- κ B activation enhances cell survival and proliferation and hence neoplastic potential in preneoplastic cells. This observation is consistent with data from rodent studies that show that leptin prevents the formation of aberrant crypt foci in azoxymethane-induced colon cancer (22). These data, in addition to our current findings, argue that leptin has stage-dependent effects on cell number homeostasis in colonic epithelial cells.

In summary, our data provide the first evidence that the obesity-associated and adipose-derived hormone, leptin, may interact with IGFs to promote survival and expansion in a model of preneoplastic [IMCE (*Apc^{Min/+}*)] but not normal [YAMC (*Apc^{+/+}*)], colonic epithelial cells. The findings presented here provide evidence to support our view that the hormonal profile of obesity may preferentially promote the survival and proliferation of preneoplastic colonic epithelial cells [IMCE (*Apc^{Min/+}*)] and that leptin is not a growth factor for normal colonic epithelial cells [YAMC (*Apc^{+/+}*)]. The findings from these studies, if further confirmed relevant in human model systems, have broad implications for the prevention and treatment of obesity-associated cancers such as colorectal cancer.

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Correction

In an article in the July 2005 issue of *Cancer Epidemiology, Biomarkers, & Prevention* (1), Figure 1 contained two errors. Figure 1A was not the correct figure, and in Figure 1B the label on the western is IL-6 and should be Ob-R. The corrected version of Figure 1 appears below.

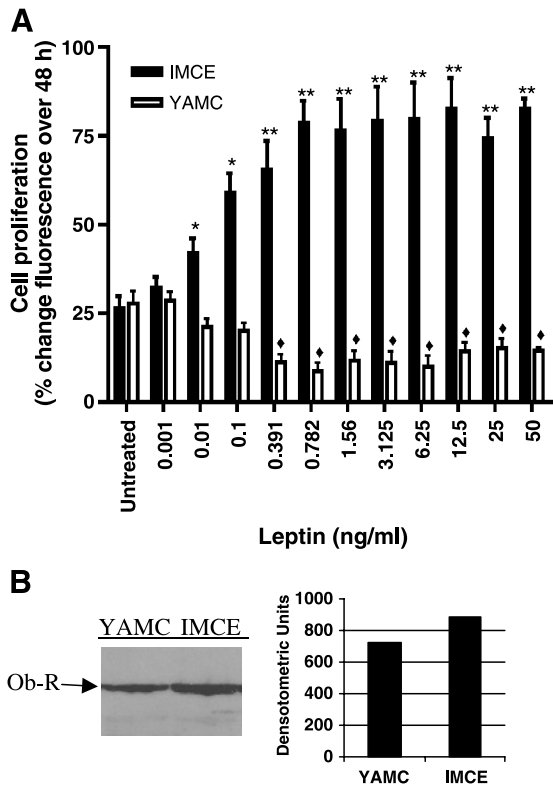


Figure 1. A. Effect of leptin on the proliferation of YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) colonic epithelial cells. Cells were treated with leptin from 0.001 to 50 ng/mL for 48 hours. Representative of three separate experiments. ♦, *P* < 0.01 (compared with untreated control); *, *P* < 0.001 (compared with untreated control); **, *P* < 0.0001 (compared with untreated control). **B.** Western blot analysis using an antibody against Ob-R and the densitometric analysis comparing Ob-R protein from untreated YAMC (*Apc^{+/+}*) and IMCE (*Apc^{Min/+}*) control cells.

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1. Fenton JI, Hord NG, Lavigne JA, et al. Leptin, Insulin-Like Growth Factor-1, and Insulin-Like Growth Factor-2 Are Mitogens in *Apc^{Min/+}* but not *Apc^{+/+}* Colonic Epithelial Cell Lines. *Cancer Epidemiol Biomarkers Prev* 2005;14: 1646-52.

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