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Leptin induces an Apc genotype-associated colon epithelial cell chemokine production pattern associated with macrophage chemotaxis and activation

Jenifer I.Fenton[1,2,*], Stephen D.Hursting[1,3,4], Susan N.Perkins[1,3] and Norman G.Hord[2]

[1]Cancer Prevention Fellowship Program, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD, USA, [2]Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI, USA, [3]Laboratory of Biosystems and Cancer, National Cancer Institute, Bethesda, MD, USA and [4]Division of Nutritional Sciences, University of Texas, Austin, TX, USA

*To whom correspondence should be addressed at: Jenifer I Fenton, 210 G.M. Trout Bldg, Michigan State University, East Lansing, MI 48824
Email: imigjeni@msu.edu

Leptin is an adipocyte-derived cytokine associated with obesity and inflammation recently shown to influence colon epithelial cell fate and colon inflammation. Thus, the purpose of this study is to investigate the influences of leptin exposure on the production of proinflammatory signals by a model of normal [YAMC ($Apc^{+/+}$)] and preneoplastic [IMCE ($Apc^{Min/+}$)] colon epithelial cells. Here, we characterize the production of specific CC and CXC chemokines by IMCE and YAMC cells using an antibody-based cytokine array. Further, since epithelial cells are hypothesized to be accessory to the inflammatory response, we assessed the ability of supernants from leptin-exposed colon epithelial cells to activate macrophage chemotaxis and nitric oxide production. Both YAMC and IMCE cells produced the following chemokines from the CC family; MCP-1, MIP-3α, TCA-3, CTACK and RANTES. These cell lines also produced the following CXC chemokines; MIP-2, CXCL18, KC and LIX. Conditioned media from leptin-treated YAMC and IMCE cells induced nitric oxide production by macrophages ($P < 0.05$). However, only conditioned media from leptin-treated IMCE cells induced macrophage chemotaxis ($P < 0.05$). These data imply that preneoplastic but not normal cells may selectively attract immune cells that promote their survival and transformation. Taken together with our previous data, we conclude that leptin promotes the proliferation of a model of preneoplastic cells (IMCE) and induces the production of chemokines which may activate macrophages and promote macrophage cell chemotaxis. These data provide a rational basis for leptin-induced cross-talk between preneoplastic epithelial cells and immune cells that may influence the promotional phase of carcinogenesis.

Introduction

Obesity is directly associated with an increased risk of cancer at several organ sites, including colon, breast (in postmenopausal women), endometrium, esophagus and kidney (1,2). However, the underlying pathophysiological mechanisms regarding cancer risk is ambiguous. Intra-abdominal fat is immunologically active and excess amounts are associated with the proinflammatory milieu promoted by obesity that may underlie many disease associations (3). This insulin-resistant, proinflammatory state is also characterized by elevated blood insulin, insulin-like growth factors and leptin. The elevated levels of these biological mediators may be related to the development of certain cancers, including colon cancer (4). Therefore, adipose tissue secretes hormones and cytokines that may play a role in the systemic inflammatory state that is linked to obesity and subsequent cancer risk. One such adipocyte-derived hormone leptin, which plays a crucial role in regulating energy balance, is elevated in obese individuals (5). In addition, hyperleptinemia is associated with inflammatory bowel disease (IBD) and colon cancer risk (6,7). Leptin is a cytokine and possesses immunomodulatory activities such as modulation of T cell phenotypes such as phagocytic function (8–10). A wide variety of immune, normal epithelial and epithelial-derived tumor cell types including colon epithelial cells express the leptin receptor which mediates leptin's biological effects (11).

It is hypothesized that leptin may act locally within the gastrointestinal tract to influence intestinal functions, such as nutrient absorption (12). However, it is unclear whether elevated systemic leptin levels may have pathophysiological implications for colon epithelial cells and the development of colon cancer, or if it may indirectly mediate the process via a wide variety of other cell types located in the gut. To date, leptin's effect on intestinal inflammation has primarily been studied in the context of immune cells. Siegmund *et al.* identified leptin as a critical mediator of intestinal inflammation using a mouse model lacking the leptin receptor (Ob-R) (13). More recently, leptin was shown to affect gut immune response, partly by acting on the long isoform of its receptor expressed on T lymphocytes (14).

A growing body of evidence has associated the activation of components of the innate and adaptive immune response with epithelial cell transformation and the promotion of epithelial carcinogenesis (15–17). Various models describing the process leading to colon cancer begin with the initiating event such as inherited or somatic mutations in the adenomatous polyposis coli (*Apc*) tumor suppressor gene. The normal cellular functions of APC, including proliferation, migration, differentiation and apoptosis are disrupted by these truncating mutations (18). APC participates in these processes via regulation of Wnt signaling in association with axin and glycogen synthase 3β to target the adhesion protein and transcription factor β-catenin for ubiquitin-mediated proteosomal degradation. Excess cytosolic β-catenin resulting from dysregulated degradation results in nuclear translocation, dimerization with Tcf/LEF (T cell factor/lymphoid enhancer factor) and transcriptional activation of target genes

Abbreviations: IBD, inflammatory bowel disease; IFN-γ, interferon-γ; PTX, pertussis toxin.

such as cyclin D1 which enhances survival and proliferation of initiated cells (19). If these initiated precancerous cells ($Apc^{+/-}$) survive, they proliferate during the promotional phase of carcinogenesis. During this phase further genetic damage can be incurred such as mutations in other genes, for example, p53 or deleted in colon cancer (DCC) (20). In active IBD there is an increase in the mucosal macrophage population which secrete cytokines and reactive oxygen species (21). These non-genetic stimuli can encourage the survival and proliferation of initiated cells in the case of inflammation-associated colorectal cancer, like cases associated with colitis or IBD (16).

Activation of epithelial cells by bacterial pathogens or apposing immune cells to produce high concentrations of chemokines, cytokines and growth factors can cause them to become 'accessory' cells in the inflammatory process. Epithelial cell production of immunomodulatory mediators can modulate neoplastic phenotypes such as angiogenesis and cell growth and survival (22). *In vitro* models, animal studies and clinical evidence lends support to the hypothesis that cancer development largely depends on the ability of survival-advantaged mutant cells (such as $Apc^{Min/+}$ colonic epithelial cells) to hijack and exploit the normal physiological processes of the host (23). It is now recognized that each stage of cancer development is exquisitely susceptible to regulation by innate and adaptive immune cells (16). Whereas fully activated antitumor immunity by adaptive immune cells might result in eradication of malignant cells, chronic activation of various types of innate immune cells in or around premalignant tissues may actually promote tumor development (20).

Cross-talk between immune and epithelial is a characteristic of tumor promotion even at the earliest stages of carcinogenesis (24). Macrophages are important innate immune cells that play an important role in tumor development. Evidence supporting a role for macrophages in tumor progression can be inferred from studies examining the link between tumor-associated macrophage (TAM) levels and prognosis. Upon histologic analysis, high TAM numbers are an independent prognostic factor in many forms of cancer (25). These observations correlate with animal studies using macrophage-depleted mice to investigate the role of macrophages in tumor progression *in vivo* (26,27).

Our previous data shows that leptin preferentially promotes the survival and proliferation of a preneoplastic colon epithelial cell line [IMCE ($Apc^{Min/+}$)], and that leptin is not a growth factor for a normal colon epithelial cell line [YAMC ($Apc^{+/+}$)] (28). Since leptin is an adipocyte-derived cytokine and influences colon epithelial cell fate, it is important to address the interaction between leptin exposure and inflammation promoting factors by colon epithelial cells. The purpose of this study is to investigate the role of leptin exposure on the production of proinflammatory signals by colon epithelial cells and subsequent associated migration and activation of macrophages.

Using cells with contrasting *adenomatous polyposis coli* (*Apc*) genotypes, we hypothesized that leptin treatment can influence the production of proinflammatory signals by colon epithelial cells differing in *Apc* genotype. We utilized a unique model system of conditionally immortalized non-tumorigenic colon epithelial cell lines to dissect these early events (29). These cell lines [YAMC ($Apc^{+/+}$) cells and IMCE ($Apc^{Min/+}$) cells] have proven to be excellent models of normal and preneoplastic cells. As such, the phenotypic changes in the IMCE ($Apc^{Min/+}$) cells, including growth factor-induced migration, cell–cell communication and iNOS/COX-2 expression, are consistent with known early phenotypes in human colorectal cancer (30–32). Indeed, unstimulated IMCE cells showed an enhanced total cytosolic β-catenin, and a markedly greater amount of nuclear beta-catenin/LEF-1 DNA binding complex in response to nitric oxide than YAMC cells (31,33). These preneoplastic phenotypes are consistent with known early phenotypes in human colorectal cancer. In $Apc^{Min/+}$ mice, a heterozygous mutation in *Apc* has been demonstrated to be sufficient to reduce microtubule polymerization and Cx43 expression in intestinal epithelial cell lines (34). These data suggest that the progressive loss of wild-type *Apc* in colorectal carcinogenesis is associated with the acquisition of growth promoting and apoptosis inhibitory capabilities that promote neoplasia.

We employed these unique model cells to demonstrate that leptin induced an *Apc*-genotype-dependent specific differential production of specific CC and CXC chemokine family members. Further, we show that supernants from leptin-treated IMCE ($Apc^{Min/+}$) cells induced macrophage chemotaxis and nitric oxide production. Given the 'gatekeeper' status of the *Apc* gene in colon carcinogenesis, it is highly relevant that normal and preneoplastic cells produce different levels of potentially immunomodulatory chemokines.

Materials and methods

Chemicals

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

YAMC and IMCE cells and cell culture conditions

The YAMC ($Apc^{+/+}$) cells were developed from the transgenic SV40 large T antigen mouse (35). The IMCE ($Apc^{Min/+}$) cells were derived from an F1 hybrid between the SV 40 large T antigen transgenic mouse and the $Apc^{Min/+}$ mouse (30). 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 (30,35). 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 non-proliferating, differentiated colon epithelial cells (36). Cells were cultured as described previously (32). 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 h 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 by allowing enough room for a low level 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 h at 39°C and then undergo cell death over 5–8 days, similar to the life cycle of a normal colon epithelial cell.

Proliferation assay

IMCE ($Apc^{Min/+}$) cells were grown in 96-well plates as described above. Briefly, ~1500 cells/well were seeded in 96-well plates coated with collagen I (BD Biosciences; San Jose, CA, USA) 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 h in serum-free and IFN-γ-free medium to allow for cessation of SV40 large T antigen-driven cell proliferation and to achieve

stability. After the 24 h stabilization period at 39°C the cells were treated (8 wells per treatment) with leptin (1 or 50 ng/ml) or co-treatment combinations with pertussis toxin (PTX) (Biomol; Plymouth Meeting, PA, USA), a drug that uncouples G proteins from receptors by ADP ribosylating a cysteine near the carboxy terminus of the A subunit (37). Since chemokine receptors are G-protein receptors, PTX thus serves as a global inhibitor of chemokine receptors. Briefly, cells were pretreated with 100 ng/ml PTX and then co-treatments were carried out (38).

Cell proliferation was measured after 48 h of treatment as described previously (28). Briefly, cell proliferation was measured using a commercially available compound, calcein AM (Molecular Probes, Eugene, OR, USA), that is colorless, non-fluorescent and cell membrane permeable. The compound fluoresces when cleaved by non-specific esterases in actively proliferating cells. After 48 h of treatment, the cells were treated with 100 µl of 1 µM calcein AM in PBS for 30 min, and 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, USA). We previously confirmed that this technique measures cell proliferation via flow cytometric analysis in a previous article (28).

Leptin treatment of colon epithelial cells

After the 24 h stabilization period at 39°C, the IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) cells were treated with leptin (1 or 50 ng/ml). Conditioned medium was collected 48 h after treatment and stored at −80°C. Total protein was measured using the BCA assay (Pierce Biotechnology, Rockford, IL, USA) in the conditioned media samples to serve as an adjustment factor if necessary. However, total protein was not different between the conditioned media samples (data not shown). The dose of leptin and collection period was based on our previously published data with leptin and these cell lines (28). Leptin was measured by commercial ELISA (R&D Systems, Minneapolis, MN, USA) in the conditioned media after the 48 h treatment period to verify that leptin was no longer present in the media to rule out a direct effect of leptin on macrophage activation and migration.

Cytokine antibody array

After incubation for 48 h at 39°C, the conditioned medium was harvested from the IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) cells treated with leptin (1 or 50 ng/ml) and centrifuged at 1500× *g* for 10 min to remove cell debris and stored at −80°C until cytokine analysis. Total protein was not significantly different across the conditioned media samples (data not shown). Therefore, conditioned medium (undiluted) was probed for cytokine profile using the RayBio® Mouse Cytokine Antibody Array 3.1 kit according to the manufacturer's instructions (RayBiotech®; Norcross, GA, USA). Briefly, membranes were blocked with a blocking buffer, and then 2 ml of medium from each culture of treated cells was individually added and incubated at 4°C overnight. Membranes were washed; 1 ml of primary biotin-conjugated antibody was added and incubated at room temperature for 2 h. The membranes were then incubated with 2 ml of horseradish peroxidase-conjugated streptavidin at room temperature for 30 min and cytokine presence was detected by chemiluminescence. Films of array dots were scanned with a densitometer and converted to densitometric units using Quantity One® software (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's instruction. Data were generated according to recommendations from RayBiotech. Briefly, autoradiography films were digitized and circles were measured using QuantityOne® software. Data were imported into an Excel spreadsheet, normalized against a control across membranes and final values were calculated using the RayBio® Murine Cytokine 3.1 Analysis Tool.

Macrophage chemotaxis

RAW 264.7 murine macrophage cells (kindly provided by Dr J Pestka, Michigan State University) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 Unit/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2-humidified incubator at 37°C. Macrophage cell number was assessed by trypan blue dye exclusion using a hematocytometer. Cells were then collected and prepared as per manufacturer instructions for the QCM™ chemotaxis (8 µM) cell migration assay (Chemicon, Temecula, CA, USA). Briefly, 30 000 RAW cells were seeded in the upper chamber of the provided 96-well plates. The lower chambers were filled with conditioned medium from the IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) control or leptin-treated cells. The plates were incubated overnight to allow for RAW 264.7 cell migration through the pores and into the lower chamber or to the outside bottom of the chamber. Any cells attached to the outside of the chamber were detached using the provided detachment buffer and collected according to manufacturer instructions. Any present cells were detected using a compound that fluoresces when exposed to non-specific enzymes in live cells (provided with the kit). The plate was read at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a Cytofluor fluorescent plate reader (Millipore, Bedford, MA, USA) and data were analyzed.

Nitric oxide assay—macrophage activation

Macrophages were cultured in 24 well plates as described, medium was removed and macrophages were treated with previously collected conditioned medium from control and leptin-treated IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$). Macrophages were treated for 24 h with conditioned medium from IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) and NO was measured in the medium. Macrophage co-treatment experiments with the IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) conditioned medium and neutralizing antibodies were carried out using anti-murine MCP-1, MIP-3α or MIP-2 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or isotype control at concentrations of 1, 0.1 or 0.01 µg/ml for 24 h.

That medium, exposed to macrophages for 24 h, was collected and assayed for nitric oxide as described previously. Briefly, nitrite, a stable end product of nitric oxide metabolism, was measured in conditioned medium using the Greiss reaction and sodium nitrite as a standard. Aliquots of 75 µl of conditioned medium were added to an equal volume of a freshly prepared mixture of equal proportions of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride. The absorbance of the solution was read at 540 nm after 10 min incubation and NO concentrations were determined using a standard curve of sodium nitrite.

Statistical analysis

Data were assessed statistically by comparing supernants isolated from control cells with supernants from leptin-treated cells. Differences in proliferation, cytokine production, NO production and cell migration were compared using ANOVA in combination with Tukey's multiple comparisons test. Nitric oxide values are reported as mean ± SEM µM of NO. The Prism® software package (Graph Pad, San Diego, CA, USA) was utilized for this analysis.

Results

Leptin analysis of conditioned media

Leptin was measured by commercial ELISA in the conditioned media after the 48 h treatment period to verify that leptin was no longer present in the media. The purpose of this experiment was to rule out a direct effect of any residual (added) leptin on macrophage activation and migration (data not shown).

Cell proliferation

YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells were treated with concentrations of leptin ranging from 0.01 to 50 ng/ml. These concentrations were chosen to represent a similar physiological range of low to high circulating concentrations of leptin. As observed previously, in YAMC ($Apc^{+/+}$) cells leptin significantly decreased cell proliferation. An opposite effect was observed in IMCE ($Apc^{Min/+}$) cells as seen in Figure 1A (28). Using PTX as a global inhibitor of G-protein chemokine receptors, we co-treated with leptin and PTX to determine whether blocking chemokines could explain the proliferative response induced by leptin in IMCE cells. PTX alone had no effect on cell survival (Figure 1B).

CC chemokine production

Both YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells produced the following chemokines from the CC family; monocyte chemoattractant protein-1 (MCP-1; CCL2), macrophage inflammatory protein-3α (MIP-3α; CCL20), T cell activation-3 (TCA-3; CCL1), cutaneous T cell-attracting chemokine (CTACK; CCL27) and CC-chemokine-5 (RANTES; CCL5) (Figure 2). Leptin treatment (1 or 50 ng/ml) of IMCE ($Apc^{Min/+}$) cells induced at least a 3-fold increase in RANTES ($P < 0.05$) and a >10-fold increase in MIP-3α and TCA-3 ($P < 0.05$) with the highest production at 1 ng/ml leptin ($P < 0.001$). Leptin

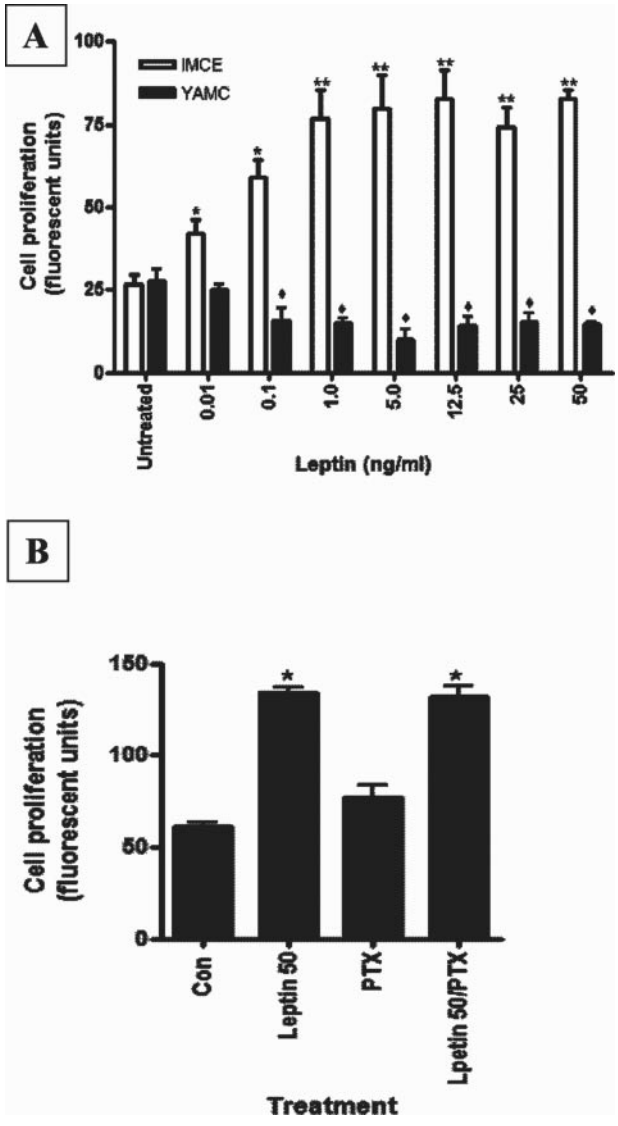

Fig. 1. (**A**) The effect of leptin on the proliferation of IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colon epithelial cells. Cells were treated with leptin from 0.01 to 50 ng/ml for 48 h. Con, Control. Results are representative of three separate experiments. *$P < 0.01$ (compared with untreated YAMC control); *$P < 0.001$ (compared with untreated IMCE control); **$P < 0.0001$ (compared with untreated IMCE control). (**B**) The effect of leptin and pertussis toxin (PTX) on proliferation of IMCE ($Apc^{Min/+}$) colon epithelial cells. Cells were pretreated with 100 ng/ml PTX for 1 h and then leptin (50 ng/ml) was added for 48 h. Con, Control. Results are representative of three separate experiments. *$P < 0.01$ (compared with untreated control).

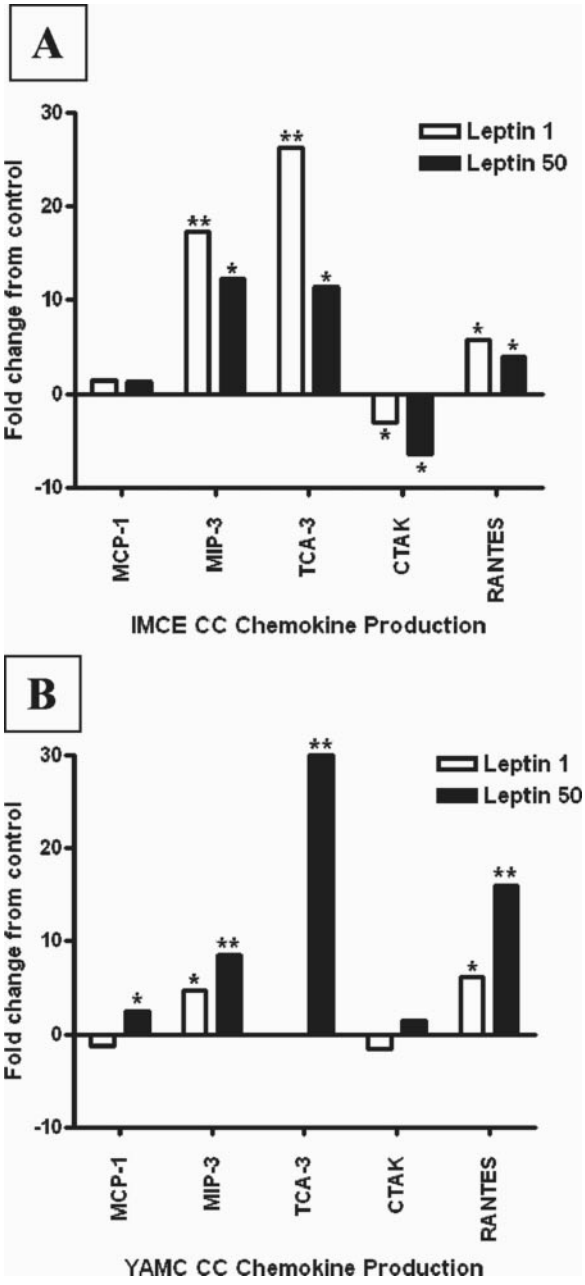

Fig. 2. (**A**) The effect of leptin on IMCE ($Apc^{Min/+}$) cell production of CC chemokines. (**B**) The effect of leptin on YAMC ($Apc^{+/+}$) cell production of CC chemokines. Cells were treated with leptin (1.0 or 50 ng/ml) for 48 h and conditioned media was collected and exposed to RayBio® Mouse Cytokine Antibody Array 3.1 kit according to instructions. Results are representative of two separate experiments. *$P < 0.05$ (compared with untreated control); **$P < 0.001$ (compared with untreated control).

treatment of IMCE ($Apc^{Min/+}$) cells induced a significant decrease in CTAK ($P < 0.05$) and did not induce any change in MCP-1 (Figure 2A). Leptin treatment (1 or 50 ng/ml) of YAMC ($Apc^{+/+}$) cells induced a 3-fold increase in MCP-1 and a 30-fold increase in TCA-3 at 50 ng/ml ($P < 0.05$), a 5- and 8-fold increase in MIP-3α at 1 and 50 ng/ml ($P < 0.05$) and a 7- and 18-fold increase in RANTES ($P < 0.05$) with no change in CTACK production (Figure 2B). See Table I for those CC chemokines present on the protein array but were not detectable in our conditioned media samples.

CXC chemokines production

Both YAMC ($Apc^{+/+}$) and IMCE ($Apc^{Min/+}$) cells produced the following chemokines from the CXC family; macrophage inflammatory protein-2 (MIP-2; CXCL2), CXCL16, KC (CXCL1) and LPS induced CXC chemokine (LIX; CXCL6). Leptin treatment (1 or 50 ng/ml) of IMCE ($Apc^{Min/+}$) cells induced a 30- and 10-fold increase in MIP-2, respectively ($P < 0.001$), a 30-fold increase in CXCL18 ($P < 0.001$) and at least a 3-fold increase in KC and LIX ($P < 0.05$) at 1 and 50 ng/ml (Figure 3A). Leptin treatment (1 or 50 ng/ml) of YAMC ($Apc^{+/+}$) cells induced a 5- and 7-fold increase in MIP-2 ($P < 0.05$), a 7- and 25-fold increase in CXCL16 ($P < 0.001$). At 50 ng/ml leptin treatment of YAMC ($Apc^{+/+}$) cells, KC and LIX increased by at least 3-fold ($P < 0.05$; Figure 3B). See Table I for those CXC chemokines present on the protein

Table I. Chemokines on protein array not produced by IMCE or YAMC cells

CC chemokines	CXC chemokines	Other chemokines
Eotaxin	MIG	Fractalkine
Eotaxin-2	PF-4	
TARC		
TECK		
MIP-1 alpha		
MIP-3 beta		
MIP-1 gamma		
MCP-5		

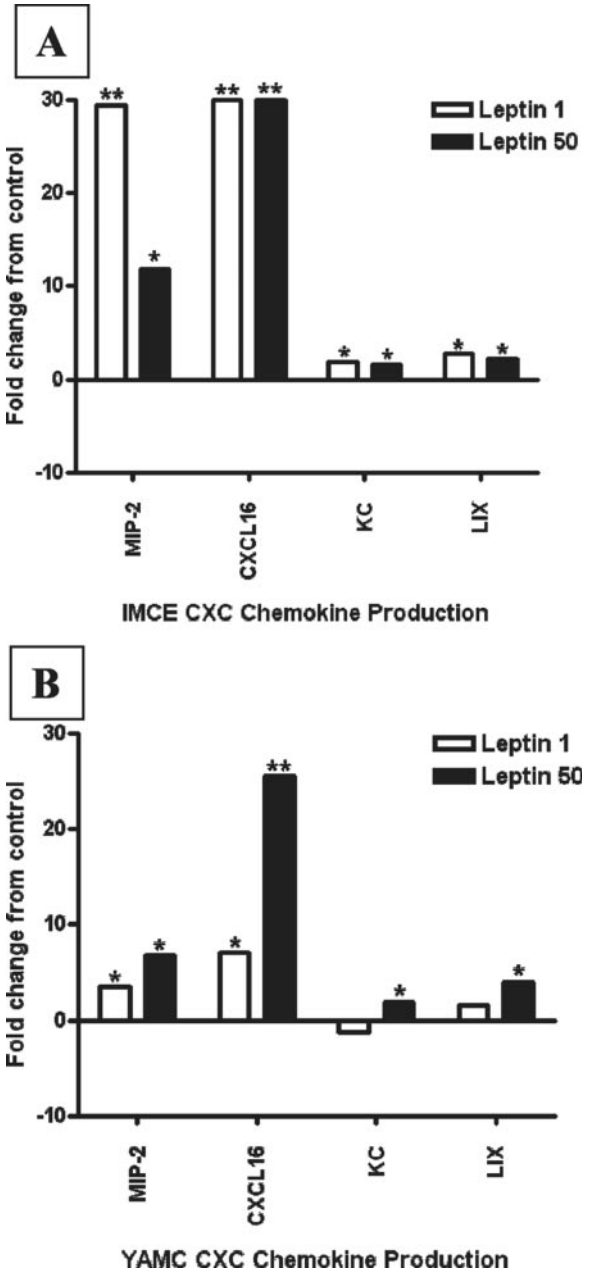

Fig. 3. (**A**) The effect of leptin on IMCE ($Apc^{Min/+}$) cell production of CXC chemokines. (**B**) The effect of leptin on YAMC ($Apc^{+/+}$) cell production of CXC chemokines. Cells were treated with leptin (1.0 or 50 ng/ml) for 48 h and conditioned media was collected and exposed to RayBio® Mouse Cytokine Antibody Array 3.1 kit according to instructions. Results are representative of two separate experiments. $^{*}P < 0.05$ (compared with untreated control); $^{**}P < 0.001$ (compared with untreated control).

array but were not detectable in our conditioned media samples.

Macrophage nitric oxide production

RAW 264.7 cells were exposed to conditioned medium from IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) or leptin-treated cells and macrophage nitric oxide production was measured. Prior to macrophage treatment, NO was undetectable in the conditioned medium from IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) cells (data not shown).

Conditioned medium from IMCE ($Apc^{Min/+}$) cells treated with 1 or 50 ng/ml leptin induced macrophage nitric oxide production (23.7 ± 3.4 versus 6.6 ± 0.04; $P < 0.05$) with maximal production at 50 ng/ml (46.0 ± 5.7 versus 6.6 ± 0.4; $P < 0.001$) (Figure 4A). Conditioned medium from YAMC ($Apc^{+/+}$) cells treated with 1 or 50 ng/ml leptin induced macrophage nitric oxide production (9.5 ± 0.6 versus 1.7 ± 0.2; $P < 0.05$) with maximal production at 50 ng/ml compared with control (40.8 ± 4.0 versus 1.7 ± 0.2; $P < 0.001$) (Figure 4A). In addition, macrophages were also treated with leptin alone (as a macrophage leptin control) at 1 and 50 ng/ml and nitric oxide was undetectable (data not shown).

Macrophage chemotaxis

RAW 264.7 cells were exposed to conditioned media from IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) control or leptin-treated cells and cell migration through pores was measured. Conditioned medium from IMCE ($Apc^{Min/+}$) cells treated with 1 or 50 ng/ml leptin induced macrophage cell migration ($P < 0.05$; Figure 4B). Conditioned medium from YAMC ($Apc^{+/+}$) cells treated with leptin did not induce macrophage cell migration different from control (Figure 4B).

Antibody neutralization experiments—macrophage nitric oxide production

Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media was partially blocked by co-treatment with anti-MCP-1 antibody at only the highest concentration (1 μg/ml) ($P < 0.01$; Figure 5A). Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media was blocked in a concentration-dependent fashion by co-treatment with anti-MIP-3α antibody (1 and 0.1 μg/ml) ($P < 0.05$; Figure 5B). Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media was partially blocked by co-treatment with an anti-MIP-2 neutralizing antibody at 0.01 and 0.1 μg/ml ($P < 0.01$). The anti-MIP-2 neutralizing completely blocked NO production antibody at the highest concentration tested ($P < 0.001$; Figure 5C).

Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media was partially blocked by co-treatment with anti-MCP-1 antibody at the highest antibody concentration [1 μg/ml ($P < 0.01$; Figure 6A)]. Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media was also partially blocked by co-treatment with anti-MIP-3α antibody at the highest antibody concentration (1 μg/ml) ($P < 0.01$; Figure 6B). Macrophage nitric oxide production induced by leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media was completely blocked by co-treatment with an anti-MIP-2 neutralizing antibody at all concentrations tested ($P < 0.001$; Figure 6C). The addition of a non-specific isotype

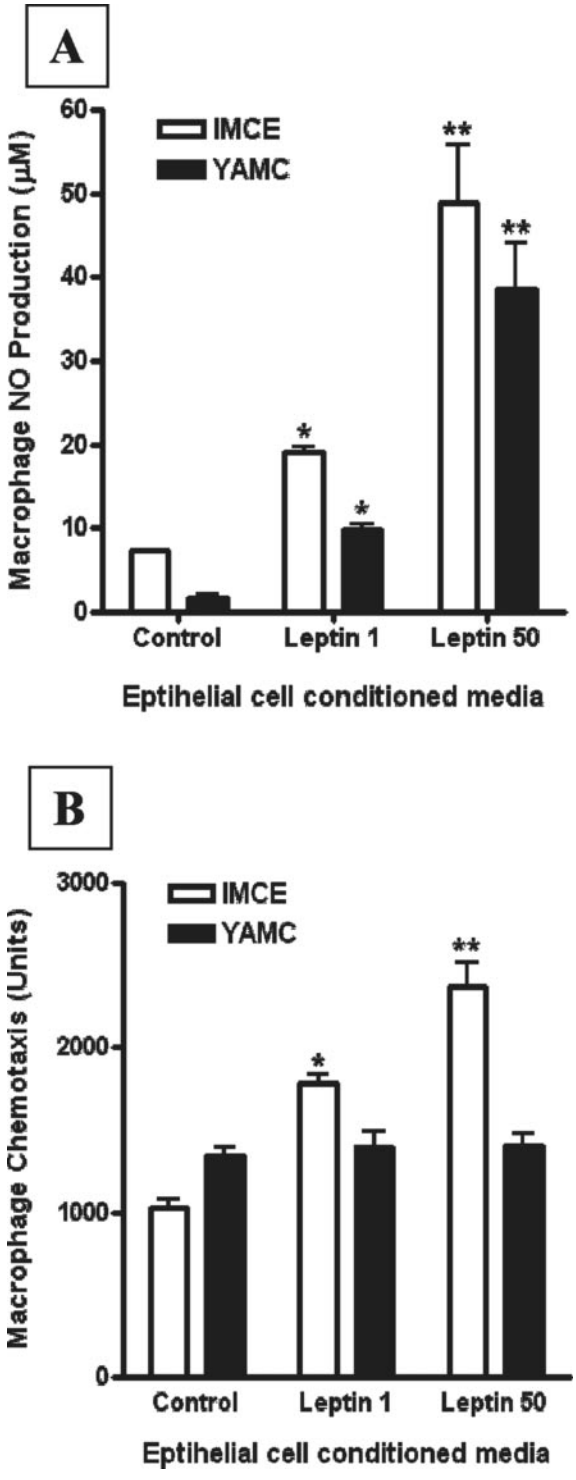

Fig. 4. (**A**) The effect of conditioned media from IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) control or leptin (1 or 50 ng/ml) treated cells on RAW 264.7 macrophage cell nitric oxide production at 24 h. (**B**) The effect of conditioned media from IMCE ($Apc^{Min/+}$) or YAMC ($Apc^{+/+}$) control or leptin (1 or 50 ng/ml) treated cells on RAW 264.7 macrophage cell chemotaxis. RAW 264.7 cells were exposed to conditioned media for 24 h and chemotaxis was measured. Results are representative of two separate experiments. $^{*}P < 0.05$ (compared with untreated control); $^{**}P < 0.001$ (compared with untreated control).

control antibody did not inhibit macrophage nitric oxide production induced by leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) or IMCE ($Apc^{Min/+}$) conditioned medium (data not shown).

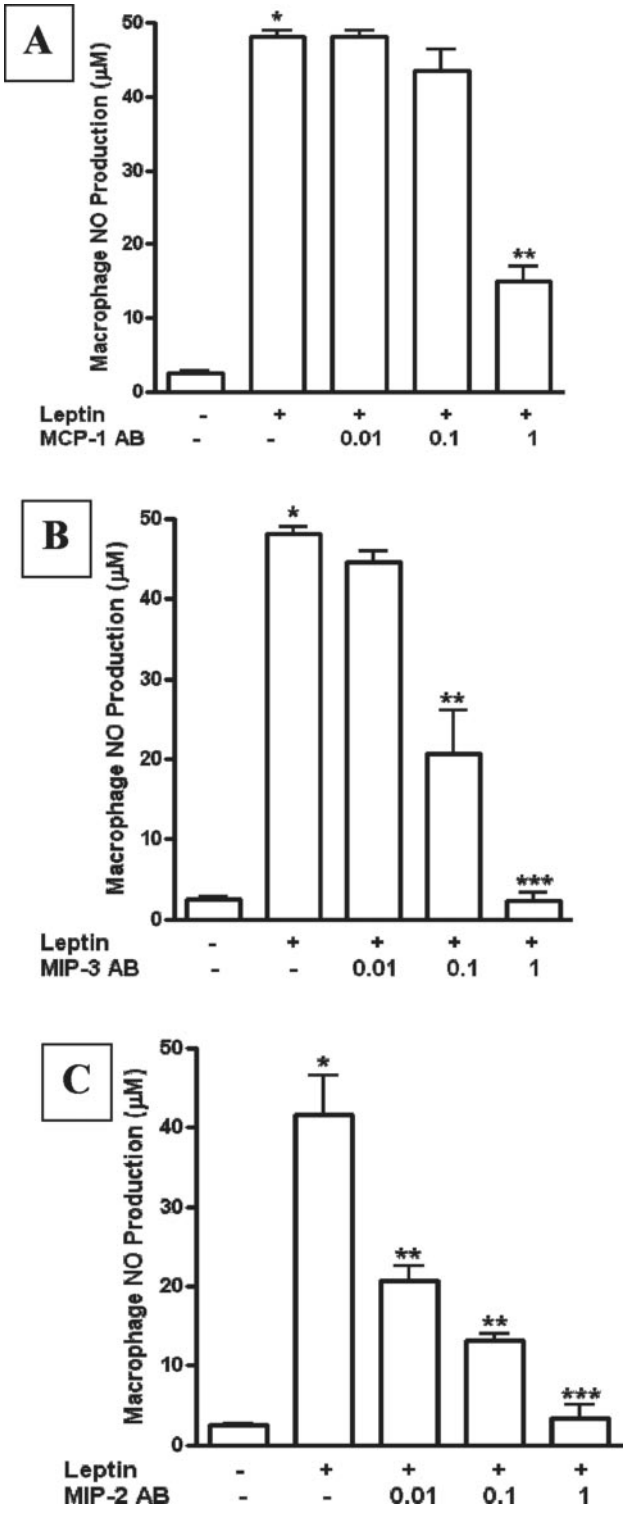

Fig. 5. (**A**) The effect of leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media co-treated with anti-MCP-1 antibody on RAW 264.7 macrophage cell nitric oxide production. (**B**) The effect of leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media co-treated with anti-MIP-3α antibody on RAW 264.7 macrophage cell nitric oxide production at 24 h. (**C**) The effect of leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned media co-treated with anti-MIP-2 antibody on RAW 264.7 macrophage cell nitric oxide production at 24 h. RAW 264.7 cells were exposed to conditioned media and the specified neutralizing antibody (0.01, 0.1 or 1.0 μg/ml) for 24 h and nitric oxide production was measured. Results are representative of three separate experiments. $^{*}P < 0.05$ (compared with untreated control); $^{**}P < 0.01$ (compared with I-L50); $^{***}P < 0.001$ (compared with I-L50).

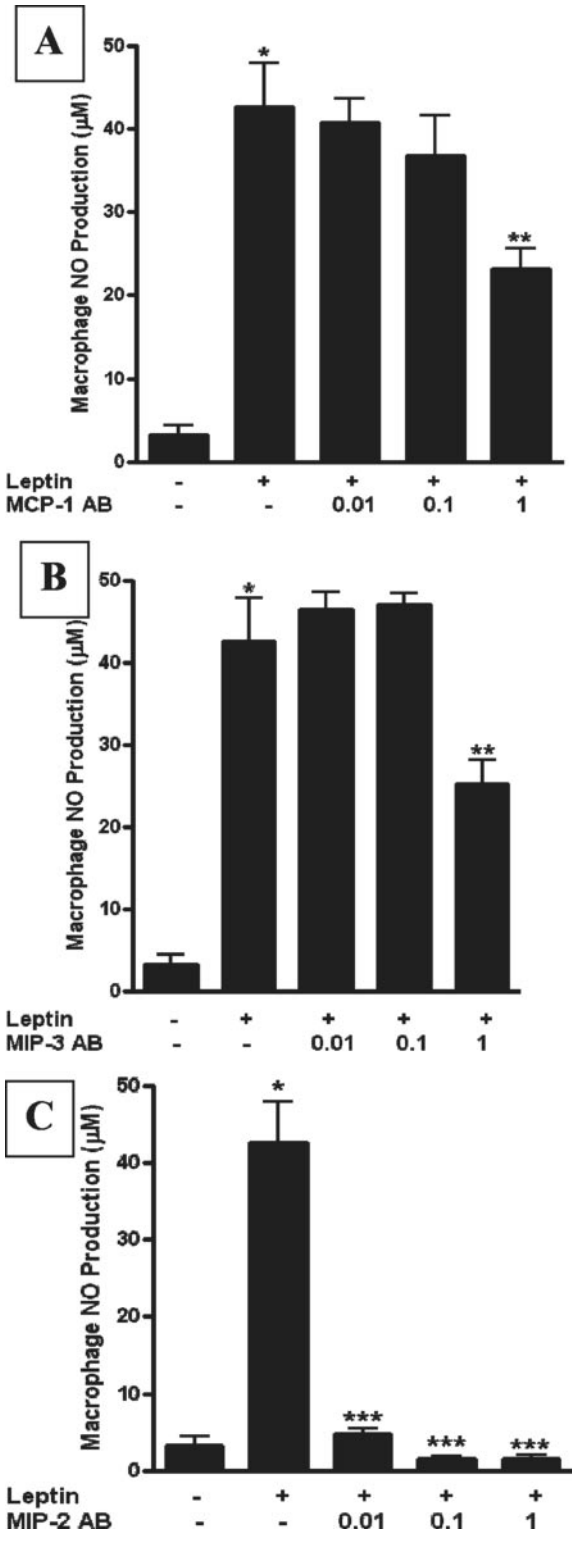

Fig. 6. (**A**) The effect of leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media co-treated with anti-MCP-1 antibody on RAW 264.7 macrophage cell nitric oxide production. (**B**) The effect of leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media co-treated with anti-MIP-3α antibody on RAW 264.7 macrophage cell nitric oxide production at 24 h. (**C**) The effect of leptin-treated (50 ng/ml) YAMC ($Apc^{+/+}$) conditioned media co-treated with anti-MIP-2 antibody on RAW 264.7 macrophage cell nitric oxide production at 24 h. RAW 264.7 cells were exposed to conditioned media and the specified neutralizing antibody (0.01, 0.1 or 1.0 μg/ml) for 24 h and nitric oxide production was measured. Results are representative of three separate experiments. $^{*}P < 0.05$ (compared with untreated control); $^{**}P < 0.01$ (compared with Y-L50), $^{***}P < 0.001$ (compared with Y-L50).

Antibody neutralization experiments—macrophage chemotaxis

Macrophage chemotaxis co-treatment experiments with the IMCE ($Apc^{Min/+}$) conditioned medium and neutralizing antibodies were carried out using anti-murine MCP-1, MIP-3α or MIP-2 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). None of these co-treatments was able to block IMCE ($Apc^{Min/+}$) cell conditioned medium induced macrophage chemotaxis. The addition of a non-specific isotype control antibody did not inhibit macrophage chemotaxis induced by leptin-treated (50 ng/ml) IMCE ($Apc^{Min/+}$) conditioned medium (data not shown).

Discussion

Leptin treatment of colon epithelial cells causes the elaboration of a variety of biological signals that can induce a range of phenotypes consistent with cell survival, proliferation and engagement of innate and adaptive immune cells. Our previous data has shown that leptin, a systemic mediator of inflammation associated with obesity, may act on the colon epithelial mucosal microenvironment to promote the survival and proliferation of initiated colon epithelial cells [IMCE ($Apc^{Min/+}$)] but not normal colon epithelial cells [YAMC ($Apc^{+/+}$)] (28). This difference was not due to protein abundance of the leptin receptor. As shown previously the long form of the leptin receptor was relatively similar between the two cell types by western blot analysis (28). In fact, we show that the proliferative difference is likely due to a trans-IL-6 signaling mechanism activated in IMCE but not YAMC cells (39). Yet, we observed that leptin was able to induce chemokine production in both YAMC and IMCE cells. Using PTX as a global inhibitor of G-protein chemokine receptors, we co-treated with leptin and PTX to determine whether blocking G-protein-mediated chemokine signaling inhibited the proliferative response induced by leptin in IMCE cells. PTX did not inhibit IMCE cell proliferation due to leptin indicating that chemokines signaling via PTX-sensitive chemokine receptors were not responsible for the observed proliferative effect. Therefore, we were compelled to understand what other role(s) relevant to colon carcinogenesis these chemokines might play.

We speculated that IMCE cells, a model of an initiated or preneoplastic epithelial cell, may possess the ability to facilitate the promotional influence of immune cells by promoting their activation and chemotaxis though the production of proinflammatory mediators induced by leptin treatment. To test this hypothesis, we identified chemokines produced by IMCE ($Apc^{Min/+}$) and YAMC ($Apc^{+/+}$) colon epithelial cells treated with leptin and chose to evaluate macrophage activation and chemotaxis as outcomes of leptin-induced phenotypes by colon epithelial cells. Epithelial cells possess the ability to make signals that can chemoattract macrophages (40). While the specific role of macrophages and their infiltration into tissue in cancer progression and metastasis is controversial, it is clear that macrophage-epithelial apposition is associated with cancer (41). A recent study provides compelling evidence that macrophages and the dynamic microenvironment in which they live facilitate malignant outgrowth and eventual metastatic spread of evolving neoplastic cells (26).

Infiltration of inflammatory cells can promote epithelial carcinogenesis, perhaps by directing promotional influences

on epithelial cells (e.g. activation of oncogenes, etc.), by activation of specific signaling pathways in initiated cell, or by altering epithelial–stromal interactions (42). We utilized cell lines which represent the earliest event in colon carcinogenesis; one cell line is normal and the other possesses a truncated copy of *Apc*, a protein involved in targeting β-catenin for proteosomal degradation and considered key susceptibility protein in colon cancer. Using these cell lines, we demonstrate the potential for epithelial cells to shape the innate immune response to leptin exposure depending on their *Apc* genotype. The data presented here characterizes the CC and CXC chemokine production by colon epithelial cells contrasting in *Apc* genotype induced by leptin treatment (1 and 50 ng/ml). We describe the differential leptin-induced production of five CC chemokines and four CXC chemokines in colon epithelial cells contrasting in *Apc* genotype using a novel protein array method. Based on these results, it was logical to speculate on the specific phenotypes of immune cells activated by the pattern of chemokines produced.

We surveyed two phenotypes of macrophage function: macrophage chemotaxis and macrophage nitric oxide production. We show that conditioned medium from leptin-treated IMCE ($Apc^{Min/+}$) cells induces macrophage chemotaxis and nitric oxide production at 1 and 50 ng/ml leptin treatment. Both cell types, when exposed to leptin, release products that induce NO production by macrophages. However, the IMCE ($Apc^{Min/+}$) cells appear to secrete factors in response to leptin at a lower dose than YAMC ($Apc^{+/+}$) cells. Further, IMCE ($Apc^{Min/+}$) cells produced leptin-induced factors able to induce macrophage chemotaxis. This observation was consistent with our hypothesis that, in response to a systemic signal, preneoplastic cells are capable of elaborating signals that results in the activation of immune cells. While normal cells [YAMC ($Apc^{+/+}$)] were able to induce macrophage NO production in response to leptin, this response is hypothesized to act only on local resident macrophages as our data indicate that they do not make a chemoattractant signals in response to leptin. In contrast, preneoplastic [IMCE ($Apc^{Min/+}$)] cells display the ability to both activate and chemoattract macrophages in response to leptin, which occurs at lower concentrations of leptin than normal cells. We cannot rule out that part of the observed effect could be due to increasing cell numbers over the course of the leptin treatment. However, one would expect that total protein levels would increase in the media. As total protein concentrations were similar across all conditioned media samples (data not shown) we do not believe the elevated chemokine/cytokine effect is due to changes in cell number. We believe the effect to be specific. Given the fact that leptin-induced differences in chemokine secretion between YAMC and IMCE cells can vary up to 20-fold, it is unlikely that differences in cell number of 2- to 3-fold account for the differences in chemokine secretion.

Among the candidate macrophage activation and chemoattractant signals elaborated by leptin treatment, we identified MIP-2 and to a lesser extent MIP-3α and MCP-1 as signals involved in the elaborated macrophage phenotypes. MIP-2 is an excellent candidate for involvement in intestinal epithelial-immune cross-talk. MIP-2 is expressed in intestinal epithelial cells after stimulation with LPS or proinflammatory cytokines and is not expressed in epithelial cells isolated from healthy intestine *in vivo* (43). MIP-2 expression is increased in IBD and dextran sulfate sodium-induced inflammation (30,31). In addition, MIP-2 secretion by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine (44). To a lesser extent MIP-3α and MCP-1 are associated with intestinal inflammation. MIP-3 is induced in response to inflammation (45) and is involved in maturation of intestinal macrophages (46). In intestinal epithelial cells, MCP-1 production was enhanced in response to an intracellular pathogen (47). However, we acknowledge that other measured and unmeasured epithelial factors may also play a role in macrophage activation. This is evidenced by the lack of concordance in the concentration dependence of MIP-2 change across leptin concentration (Figure 1) and macrophage NO production induced by conditioned medium from leptin-treated colon epithelial cells. Research is ongoing to elucidate the role of leptin-induced colon epithelial cell production of MIP-2. It is possible that expression of the MIP-2 receptor CXCR-2 or other chemokine receptors may explain the observations. However, the chemokine network has built-in redundancies; chemokines are known to bind to more than one receptor and most receptors can respond to more than one chemokine. As such, our data identify only a few of the potential immunomodulatory factors elaborated by IMCE cells; these data do not rule out the existence of other factors secreted in response to leptin that may be responsible for these measured effects. In addition, we are also examining the response of other innate immune cells to see if they respond similarly.

Basal production of protective factors by intestinal epithelial cells, including chemokines, cytokines and heat shock proteins, plays a critical role in intestinal homeostasis and repair (48). In contrast, the inducible production of chemokines and cytokines by endocrine factors and hormones, or in response to tissue damage or bacterial pathogens, are critical to innate and adaptive immune responses (49). Importantly, inducible chemokines are becoming recognized as important components in the promotional phase of carcinogenesis (42). Most tumors engage in a complex chemokine network which can influence immune responses to the tumor, direct the cellular composition of leukocyte infiltrate and also play a role in angiogenesis (50). It appears from our data that this 'switch' towards a more active immunomodulatory epithelial cell phenotype during cancer promotion may be occurring much earlier than currently suggested. Characterization of the chemokine receptor profile on these two cell lines may glean important insights into the ability of these cell lines to differentially respond to signals coming from apposing immune cells.

These data highlight the importance of examining the effects of immunomodulatory factors on early events in cancer progression using a continuum model system. Acquired mutations in Apc may provide an opportunity for the specific promotional effects by adipoctye-derived factors such as leptin. This susceptibility would then only impact those individuals 'at risk' for colorectal cancer. However, current technologies cannot determine risk this early in colon cancer carcinogenesis. We argue that our model provides an excellent opportunity to examine the effects of these adipocyte-derived factors in a reductionist system. We identified a novel differential effect of leptin on models of normal and preneoplastic colon epithelial cells to elaborate immune-epithelial cell cross-talk in the colon. Taken together with our previous data, leptin promotes the proliferation of initiated

IMCE ($Apc^{Min/+}$) cells via a trans-IL-6 mechanism, induces the production of chemokines which may activate macrophages and promote immune cell chemotaxis. We underscore the importance of how mutations in *Apc* can lead to significant differences in the response to systemic signals (e.g. leptin) and the elaboration of paracrine signals (e.g. chemokines) from colon epithelial cells to mucosal immune cells while maintaining a non-tumorigenic phenotype. These data provide a rational basis for concluding that initiated epithelial cells participate in cross-talk with immune cells that may influence the promotional phase of carcinogenesis. These data, taken together with our other published data, provide further biological plausibility for a role for systemic mediators of inflammation associated with obesity involved in the promotion of preneoplastic colon epithelial lesions via a leptin-mediated mechanism.

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