Published in final edited form as:

Mol Carcinog. 2010 July ; 49(7): 700–709. doi:10.1002/mc.20644.

Adipokine regulation of colon cancer: adiponectin attenuates interleukin-6-induced colon carcinoma cell proliferation via STAT-3

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Abstract

Obesity results in increased circulating levels of specific adipokines which are associated with colon cancer risk. The disease state is associated with increased leptin, insulin, IGF-1, and IL-6. Conversely, adiponectin levels are decreased in obese individuals. Previously, we demonstrated adipokine-enhanced cell proliferation in preneoplastic, but not normal, colon epithelial cells, demonstrating a differential effect of adipokines on colon cancer progression *in vitro*. Using a model of late stage carcinoma cancer cell, namely murine MC-38 colon carcinoma cells, we compared the effect of obesity-associated adipokines (leptin, insulin and IGF-1 and IL-6) on MC-38 cell proliferation and determined whether adiponectin (full length or globular) could modulate adipokine-induced cell proliferation. We show that insulin and IL-6, but not leptin and IGF-1, induce proliferation in MC-38 cells. Adiponectin treatment of MC-38 cells did not inhibit insulin-induced cell proliferation but did inhibit IL-6-induced cell proliferation by decreasing STAT-3 phosphorylation and activation. Nitric oxide (NO) production was increased in MC-38 cells treated with IL-6; co-treatment with adiponectin blocked IL-6 induced iNOS and subsequent NO production. These data are compared to previously reported findings from our laboratory using the YAMC (model normal colon epithelial cells) and IMCE (model preneoplastic) cells. The cell lines are utilized to construct a model summarizing the hormonal consequences of obesity and the impact on the differential regulation of colon epithelial cells along the continuum to carcinoma. These data, taken together, highlight mechanisms involved in obesity-associated cancers and may lead to potential targeted therapies.

Keywords

adiponectin; colon; IL-6; cancer; MC-38

INTRODUCTION

Several epidemiological studies associate cancer risk and obesity, namely colon cancer [1-3]. The clinical presentation of obesity related metabolic dysregulation includes increased abdominal fat, elevated levels of triglycerides and low-density lipoprotein cholesterol, low levels of high-density lipoprotein cholesterol, elevated blood pressure, insulin resistance, and prothrombotic and proinflammatory states [4]. This increased abdominal fat produces various growth factors and cytokines that play a role in the low-grade, chronic inflammatory state that is linked to obesity and subsequent cancer risk. Colon cancer is linked to other

inflammatory conditions, such as inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease [5] so it is certainly plausible that the increased inflammation that occurs with obesity underlies the obesity-colon cancer association [6]. The risk of colon cancer is much higher in these patients compared to the general population [5,7].

Obesity is a risk factor for developing diabetes, specifically type 2 diabetes [8]. A primary mechanism thought to influence the increased risk of colon cancer in the obese population is hyperinsulinemia in conjunction with insulin resistance [9,10]. In the progression of diabetes, insulin levels are high during the early phases and act as a mediator for cancer development. Giovannucci puts forth a model illustrating the relationship between insulin, IGF-1 and IGF binding proteins [11]. This model integrates the potential effects of nutrition, insulin, IGF-1, and carcinogenesis based on three principles: "(1) insulin and the IGF-1 axis function in an integrated fashion to promote cell growth and survival; (2) chronic exposure to these growth properties enhances carcinogenesis; (3) factors that influence bioactive IGF-1 will affect cancer risk" [11].

Leptin is classically associated with food intake and energy balance [12,13]. A wide variety of immune, epithelial, normal and tumor cell types, including colon epithelial cells, express the leptin receptor, which mediates many of leptin's biological effects [14]. Leptin may act locally within the gastrointestinal tract to influence intestinal functions, such as nutrient absorption or systemically, away from its source. Leptin induces tumor cell proliferation [15-18]; however, it is unclear if elevated leptin levels have pathophysiologic implications in colorectal cancer.

Adiponectin, another adipocyte-derived hormone, is thought to play a key role in dysregulated obesity. Opposite to leptin, circulating levels of adiponectin are decreased in obese individuals as well as those with type 2 diabetes [19]. Reduced levels of adiponectin are associated with cancer risk [9,19]. Adiponectin exists as a full-length protein of 30 kDa (full length adiponectin (fAdipo) or high molecular weight; HMW). The protein circulates in trimeric, hexameric and higher order complexes. The majority of circulating levels are in this full length form with concentrations in the range of 1-15 µg/ml [20]. However, globular adiponectin (gAdipo), formed through proteolytic cleavage, also has biological activity [21]. The role of both gAdipo and fAdipo in cancer risk is unclear; however, recently gAdipo was shown to inhibit leptin-stimulated cell proliferation in four different oesophageal adenocarcinoma cell lines [21].

Downstream pro-inflammatory mediators such as IL-6 increase with obesity [8,22]. IL-6 levels have an inverse correlation with BMI, thus demonstrating the chronic pro-inflammatory state that is obesity [23]. IL-6 is secreted by diverse cell types under homeostatic and inflammatory conditions [24] and is associated with the pathogenesis of IBD [25,26]. Although IL-6 is homeostatic at low levels in intestinal epithelial cells, overproduction leads to degradation of the epithelial barrier. In addition, IL-6 stimulates cell proliferation in dysplastic and neoplastic cell lines [27-29].

Previously, our laboratory demonstrated that leptin and IL-6 induced phenotypes consistent with the hallmarks of cancer in a model of preneoplastic but not normal colon epithelial cells [30,31]. These phenotypes included evidence of proliferation and sustained angiogenesis [30]. Additionally we found that leptin induces cell proliferation in the preneoplastic IMCE (*Apc^{Min/+}*) cells but not the normal YAMC (*Apc^{+/+}*) cells by inducing autocrine IL-6 production and NF-κB-dependent trans IL-6 signaling [32]. In the current study, we further investigate the *stage-specific* effects of adipokines on colon colon carcinogenesis by examining the effects of leptin, IGF-1, IL-6, insulin and adiponectin on the syngenic MC-38 carcinoma cell line. We purposely selected a syngenic (C57BL/6) mouse colon

adenocarcinoma cell line to compare to the “model normal” colon epithelial cell line YAMC. This allows for the best possible comparisons for interpretation of data not complicated with significant genetic and species differences. Colon carcinoma cell proliferation was induced by both insulin and IL-6. Adiponectin (full length and globular) did not reduce insulin-induced cell proliferation. In contrast, adiponectin did block IL-6-induced cell proliferation and NO production.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Recombinant murine leptin, globular adiponectin (gAdipo), IGF-1, IL-6 and murine IL-6R antibody, were purchased from R&D Systems (Minneapolis, MN). Murine full length adiponectin (fAdipo) was purchased from BioVendor (Candler, NC). Murine insulin was purchased from Sigma (St. Louis, MO). STAT-3 Inhibitor (PpYLKTK-mts) was purchased from Calbiochem (San Diego, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise noted.

Cells and Cell Culture Conditions

The murine carcinoma-38 (MC-38) colon cancer cell line was derived from a murine colon tumor, grade III carcinoma, which was chemically induced in the C57BL/6 female mouse [33]. This cell line was kindly gifted by Dr. Hursting (University of Texas-Austin) and cultured in DMEM (Gibco; Rockville, MD) supplemented with 10% fetal bovine serum (Gibco; Rockville, MD) and 1% penicillin/streptomycin at 37°C with 5% CO₂ [34].

Leptin, IL-6, IGF-1, Insulin and Adiponectin Proliferation Assays

MC-38 cells were grown in 96-well plates as described above. Briefly, approximately 1,500 cells/well were seeded in 96-well plates (Corning Costar; Lowell, MA). Cells were treated (8 wells per treatment) with leptin (0.01, 0.1, 1 or 50 ng/ml), IL-6 (0.01, 0.1, 10, 25, 50 ng/ml), insulin (0.001, 0.01, 1, 10, 100 µg/ml), IGF-1 (0.1, 1, 50, 100, 200 ng/ml), or adiponectin (0.001, 0.01, 0.1, 1 µg/ml) for 24 hrs. Co-treatment experiments were carried out using insulin (1 or 10 µg/ml) in combination with either globular adiponectin (1 or 10 µg/ml) or full-length adiponectin (1 or 10 µg/ml). The antibody neutralization experiments were carried out using a monoclonal anti-murine IL-6R antibody (1 µg/ml) or STAT-3 inhibitor (0.01, 0.1, 1, 10, or 100 mM) with antibody co-treatment for 24 hrs.

Cell proliferation was measured after 24 hrs of treatment using the commercial CellTiter96 Aqueous kit according to manufacturer's instructions (Promega; Madison, WI). Briefly, 20 µl/well of CellTiter96 Aqueous One solution reagent was added to the 96-well plate containing the cells in 100 µl of culture media and incubated for 1 hr at 37°C in 5% CO₂. Upon completion of the assay procedure the plate was read at 490 nm using the Synergy HT plate reader (Bio-Tek; Winooski, VT).

Western Blotting

Cells were treated with IL-6 alone or co-treated with IL-6 (50 ng/ml and adiponectin (1 µg/ml) for 0, 15, 30, or 60 min, then prepared for western blot. Briefly, cells were washed twice with cold phosphate-buffered saline, scraped into 1 ml sample buffer (sodium dodecyl sulfate Reducing Buffer; 0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulfate and 0.5% bromophenol blue in ddH₂O), sonicated and boiled. Samples were then loaded on an equal protein basis of 40 µg per lane, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to an Immobilon-FL polyvinylidene difluoride membrane (Millipore Corporation; Billerica, MA). Membranes were probed with primary antibodies

against STAT-3 (1:2000), pSTAT-3 (1:1000), iNOS (1:1000), gp130 (1:2000), IL-6R, adiponectin receptor 1 and 2, insulin receptor alpha and beta, and IGF-R1 (1:1000) with shaking overnight at 4°C. Actin (1:1000) was also run for normalization. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise specified. Incubation with the primary antibody was followed by appropriate infrared-labeled second antibodies: 1) goat anti-rabbit (1:1000; Invitrogen, Eugene, OR), 2) goat anti-mouse (1:1000; Rockland, Gilbertsville, PA) and 3) donkey anti-goat (1:1000, Li-Cor, Lincoln, NE). Signal was detected using the Odyssey Infrared Imaging System (LI-COR Biosciences; Lincoln, NE). The images were collected using the direct infrared fluorescence detection and converted to black and white for publication using the manufacturers' software. The system has a wide linear detection range allowing for comparisons of weak and strong bands from the same blot. Blots shown are from one experiment representative of the three.

STAT-3 Activation

MC-38 cells were treated with IL-6 alone (50 ng/ml) or IL-6 co-treated with anti-IL-6R antibody (1 µg/ml) for 0, 15, 30, and 60 min and STAT-3 activation was measured using the TransAM™ STAT-3 Transcription Factor Assay Kit according to the manufacturer's instructions (Active Motif; Carlsbad, CA). Briefly, the TransAM™ STAT-3 kit contains a 96-well plate on which has been immobilized oligonucleotide containing the STAT-3 consensus binding site (5'-TTCCCGGAA-3'). The active form of STAT-3 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used to detect STAT-3 recognizes an epitope on STAT-3 that is accessible only when STAT-3 is activated and bound to its target DNA. Substrate-activated HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by spectrophotometry at 450 nm.

Nitric Oxide Assay

Cells were treated with IL-6 (50 ng/ml) for 3, 6, 12, and 24 hrs and nitric oxide (NO) production measured. Cells were then treated with IL-6 alone (1 or 50 ng/ml) and in co-treatment with adiponectin (IL-6 at 50 ng/ml, adiponectin at 1 µg/ml) and NO production measured after 24 hrs. The production of nitric oxide in the cell culture media was measured according to the manufacturer's instructions using a total nitric oxide kit (R&D Systems; Minneapolis, MN) via a Griess reaction. Briefly, samples were diluted 2-fold and 50 µl of diluted sample was added to the well. Next, 25 µl of NADH and nitrate reductase was added to each well and the plate was incubated for 30 min at 37°C. 50 µl of Griess reagent I and II were added to each well and the plate was incubated for 10 min at room temperature. Upon completion of the assay procedure, the plate was read at 450 nm wavelength using a Synergy HT plate reader (Bio-Tek; Winooski, VT).

Statistical Analysis

The experiments were repeated three times and data shown are representative of all three. The data shown is the mean ± standard error. Differences in proliferation were compared using ANOVA in combination with Tukey's multiple comparisons test. Pair-wise differences were compared using ANOVA in combination with Tukey's multiple comparisons test. The Prism® software package (Graph Pad; San Diego, CA) was utilized for this analysis.

RESULTS

Effect of leptin, IL-6, IGF-1 and insulin on MC-38 cell proliferation

The effects of several commonly elevated hormones associated with obesity, including leptin, IL-6, IGF-1 and insulin on MC-38 cell proliferation were assessed using a commercial CellTiter96 cell proliferation kit as described in the materials and methods. As shown in Figure 1A, treatment of cells with leptin over a dose range (0.01-50 ng/ml) did not induce cell proliferation in MC-38 cells. IL-6, however, significantly induced proliferation in a dose-dependent manner at 10 and 25 ng/ml doses ($p < 0.05$ compared to control), with the largest increase at the 50 ng/ml dose ($p < 0.01$ compared to control, Fig 1B). Similar to that observed with leptin, IGF-1 failed to induce cell proliferation in MC-38 cells over the dose range tested (0.1-200 ng/ml, Fig 1C). Treatment with insulin also significantly increased MC-38 cell proliferation in a dose dependent fashion (at dose 1 $\mu\text{g/ml}$, $p < 0.05$) with the highest increase at the 10 and 100 $\mu\text{g/ml}$ doses ($p < 0.01$, Fig 1D). With variable responsiveness of MC-38 cells to adipokines, receptors for IL-6 (IL-6R and gp130), adiponectin (AdipoR1 and AdipoR2), leptin (Ob-R), insulin (INSR-a and INSR-b) and IGF-1 (IGF-R1) were determined by western blot (Fig 1E). MC-38 cells had detectable levels of all receptors tested with exception of the leptin receptor Ob-R.

Effect of full-length and globular forms of adiponectin on MC-38 cell proliferation and insulin-induced MC-38 cell proliferation

The effect of both full-length and globular forms of adiponectin was tested on MC-38 cell proliferation using the previously mentioned commercial cell proliferation assay kit. Treatment of MC-38 cells with either form of adiponectin alone did not result in alteration of cell proliferation at any dose tested (0.001-1.0 $\mu\text{g/ml}$, Fig 2A,B). Similar to that seen in figure 1D, insulin significantly increased MC-38 cell proliferation compared to control ($p < 0.05$) in a dose-dependent fashion, however no inhibitory effect was observed with the addition of full-length adiponectin (Fig 2C) or the globular form of adiponectin (Fig 2D), as the insulin-induced proliferation levels still remain significantly increased ($p < 0.05$) with their addition.

Treatment of MC-38 cells with IL-6 leads to STAT-3 activation

MC-38 cells were treated with IL-6 (50 ng/ml), collected at various time points (0, 15, 30, 60 min post treatment), and the total cell lysates were probed using western blot. IL-6 - induced phosphorylation of STAT-3 at 15 and 30 min which was resolved by the 60 min time point (Fig 3A). Treatment of MC-38s with IL-6 caused a significant increase in STAT-3 activation in an inverse time-dependent manner with highest levels peaked at 15 min ($p < 0.01$ compared to 0 min) and gradually decreased after 30 and 60 min ($p < 0.05$ compared to 0 min) (Fig 3B). Co-treatment of MC-38 cells with IL-6 and a neutralizing anti-IL-6R antibody blocked this STAT-3 activation, showing no significant difference in activation levels compared to time 0 at any time point tested (Fig 3B). MC-38 cells were again treated with IL-6 and shown to induce cell proliferation similar to what was previously observed (Fig 3D, $p < 0.05$ compared to unstimulated). This proliferation was inhibited over a dose range (0.1-100 mM) of a specific STAT-3 inhibitor, with the 0.1 mM STAT-3 inhibitor having a p value < 0.05 and the higher doses (10 and 100 mM) showing further significance ($p < 0.01$) when compared to IL-6 alone stimulation (Fig 3D). As evident in figure 3C, this inhibitor was shown to have no intrinsic toxicity over a dose range in this cell type.

Effect of full-length and globular adiponectin on IL-6-induced cell proliferation in MC-38 cells

The effect of both full-length and globular forms of adiponectin were tested on IL-6-induced MC-38 cell proliferation using the above mentioned commercial cell proliferation assay kit. We previously showed that treatment of MC-38 cells with either form of adiponectin alone did not result in increased cell proliferation at any dose tested (0.001-1.0 $\mu\text{g/ml}$, Fig 2A,B). Similar to figure 1B, proliferation of MC-38 cells significantly increased with IL-6 treatment (50 ng/ml, $p < 0.05$, Fig 4A). This IL-6-induced proliferation significantly decreased from co-treatment with either the full-length or globular form of adiponectin (1 $\mu\text{g/ml}$, $p < 0.01$ when compared to IL-6 alone, Fig 4A). The anti-proliferative effect of adiponectin, in the face of IL-6, was partially blocked by the addition of neutralizing anti-AdipoR1 or AdipoR2 antibodies (Fig 4B). Similar to that observed in figure 3A, treatment of cells with IL-6 (50 ng/ml) induced phosphorylation of STAT-3 at 15 and 30 min time points, which was attenuated when cells were co-treated with IL-6 and globular adiponectin (Fig 4C). In addition, the co-incubation with anti-AdipoR1 or anti-AdipoR2 partially recovered IL-6 induced STAT3 phosphorylation nuclear translocation. As we observed no difference between full-length and globular adiponectin in reducing cell proliferation we utilized only globular adiponectin for further studies. In addition, globular adiponectin was the only adiponectin utilized in the previous studies with IMCE and YAMC cells and proliferation (only commercially available at the time). We wanted to compare the response to prior published observations.

Effect of IL-6 and adiponectin co-treatment on markers of inflammation in MC-38 cells

MC-38 cells were treated with IL-6 over time and in dose response manner and nitric oxide was measured at the specified times post treatment. Nitric oxide (NO) production was increased due to IL-6 (50 ng/ml) treatment over time (Fig 5A) and dose (1 and 50 ng/ml, Fig 5B). This increase in IL-6 driven NO production was attenuated by co-treatment with adiponectin (1 $\mu\text{g/ml}$, Fig 5B). IL-6-driven, MC-38 cell protein expression of inducible nitric oxide synthase (iNOS) was increased with IL-6 (50 ng/ml) treatment and was subsequently decreased by the addition of adiponectin (1 $\mu\text{g/ml}$) to the IL-6 treated cultures (Fig 5C).

DISCUSSION

Obesity is a complex condition which increases the risk of a number of diseases including colon cancer. The obese state is associated with increased circulating levels of adipokines. This dysregulated physiological state is likely to have profound effects on cell number homeostasis of normal, preneoplastic and tumor cells. Previously, our laboratory showed that model normal and preneoplastic colon epithelial cells respond very differently to these signals. In the current study, we examined the effect of adipokines on cell proliferation of a syngenic carcinoma cell line (MC38). We hypothesized that as cells acquire mutations consistent with carcinogenesis, there would be a different response by the cells depending on "stage". NCI Think Tanks in Cancer sites that the identification of cancer prevention targets begins with the critical comparison of the response of normal vs tumor cells to a stimulus [35]. We show for the first time that insulin and IL-6, but not leptin and IGF-1, induced proliferation in MC-38 cells through a STAT-3 mediated pathway. Adiponectin does not inhibit insulin-induced cell proliferation but does inhibit IL-6-induced cell proliferation in MC-38 cells by decreasing STAT-3 phosphorylation and activation. The authors acknowledge that the generalization of these data are limited by the use of a single colon adenocarcinoma cell line. However, we purposely selected a syngenic (C57BL/6) mouse colon adenocarcinoma cell line to compare to the colon epithelial cell line YAMC

and IMCE. This allowed for the best possible comparisons for interpretation of data not complicated with significant genetic and species differences.

Obesity is associated with increased levels of certain adipokines including insulin, IGF-1 and leptin [9,36]. We showed that insulin increased cell proliferation in the MC-38s (Fig 1D). High circulating levels of insulin increase hepatic synthesis of IGF-1 and decrease production of IGFBP-1. This in turn increases the biologic activity of the IGF-1 [9,36]. IGF-1 enhances growth of various cell cancer cell lines and is identified as a “cell cycle progression factor” as it can stimulate cell progression through the cell cycle from G1 to S phase. Cells over-expressing IGF-1R show decreased apoptosis. Further, both insulin and IGF-1 increase cancer cell proliferation and decrease apoptosis *in vitro* [36]. Previously, we showed that IGF-1 can increase cell proliferation in a pre-neoplastic, but not normal, colon cancer epithelial cells, thus showing stage specific effects [30]. Here, however, using a cell line in a different (later) stage, the murine colorectal adenocarcinoma (MC-38) cell line, IGF-1 failed to elicit an increase in cell proliferation (Fig 1C). Again, this lends support to the idea of stage specific effects of certain obesity-associated inflammatory markers, in this case IGF-1, on colon cancer progression.

Development of leptin resistance is common in obese individuals. We have previously demonstrated that leptin stimulates proliferation of the pre-neoplastic and neoplastic, but not normal, cell culture models [30]. Previous studies have demonstrated increased proliferation in tumor cell lines due to leptin treatment [15-18]. Here we failed to observe a leptin-induced proliferation in this cell line, potentially due to a lack of the leptin receptor (Figure 1E) in this cell type.

In contrast to the other adipokines discussed, serum adiponectin levels are decreased in obesity and increase with weight loss [9]. It is also inversely associated with insulin resistance and hyperinsulinemia, which are risk factors for development of colon cancer [9]. Further, TNF- α and IL-6 present during inflammation, can reduce adipocyte mRNA expression of adiponectin [37]. As far as its relationship to leptin, adiponectin can desensitize colon cells to leptin-induced proliferative signals [38]. We previously demonstrated that treatment of IMCE (*Apc^{Min/+}*) cells with adiponectin blocked leptin-induced proliferation and attenuated signaling pathways that were upregulated by leptin, including I κ B- α phosphorylation, I κ B- α degradation and decreased NF- κ B p65 DNA activation and binding [9,38]. The role of adiponectin in colitis remains unclear. Adiponectin receptors are present on murine intestinal epithelial cells and adiponectin protected mice from DSS-induced colitis [39]. Another study found that adiponectin knockout mice were protected from chemically induced colitis [40].

IL-6 is associated with cancer; IL-6 protein and mRNA are upregulated in both serum and tumor samples of humans and mice with colon cancer [5]. In colon cancer patients, elevated circulating IL-6 levels have been observed [22]. Lastly, IL-6 enhances proliferation of human colon carcinoma cells *in vitro* and attenuation of the IL-6 signaling during the late stages of colon cancer development results in slowed tumor growth [5]. Consistent with these observations, IL-6 significantly induced cell proliferation in MC-38 cells (Fig 1B).

Classically, IL-6 signals through the receptor, gp130, which is found on many cell types. IL-6 can also bind to its soluble IL-6 receptor (sIL-6R) “trans-signaling” [41] which then induces dimerization of gp130 chains, resulting in activation of JAKs. JAK activation causes phosphorylation of gp130 which leads to the recruitment and activation of STAT-3 and to a lesser extent, STAT-1 [22]. This IL-6 mediated STAT-3 activation plays a crucial role in tumorigenesis [22]. Inhibition of IL-6 dependent trans-signaling decreases STAT-3 phosphorylation and cell proliferation [41]. Hyperactivation of STAT-3 promotes tumor

growth through direct effects as well as indirectly by regulating anti-tumor responses [41]. Treatment of MC-38 cells with IL-6 resulted in phosphorylation of STAT-3 (Fig3A) and this IL-6-induced STAT-3 activation was attenuated when IL-6 was blocked, demonstrating a clear link between them (Fig 3B). This is further supported by the increased cell proliferation due to IL-6 is decreased when STAT-3 is blocked (Fig 3D).

Treatment of MC-38 cells with IL-6 results in increased proliferation, which was reduced to untreated levels through co-treatment with adiponectin (Fig 1A). The phosphorylation of STAT-3 by IL-6 treatment was also attenuated when co-treated with adiponectin (Fig 4B). This IL-6/STAT-3 trans-signaling pathway plays a role in inflammatory bowel diseases like Crohn's disease and ulcerative colitis which leads to an increased risk of developing colon cancer [7]. Enhanced production of reactive oxygen and nitrogen species and long-term exposure of the tissue to this species can result in chronic tissue damage and regeneration which can promote "cancer-initiating genetic alterations" [7]. Treatment with IL-6 increased NO production over time in MC-38 cells (Fig 5A). However, co-treatment with adiponectin attenuated IL-6-induced NO production (Fig5B). This is further supported by the fact that iNOS, which induces NO production, is shown to increase with IL-6 treatment and decrease when co-treated with adiponectin (Fig 5C). The two NF- κ B dependent cytokines, IL-6 and TNF- α , cross-regulate each other during development of these IBD-related cancers, enhancing both inflammation and tumorigenesis [5].

Our data illustrate a potential role of adipokines in colon epithelial cell homeostasis (Fig 6). The increased circulating levels of leptin, IL-6, and free insulin that are observed in obese individuals act on cells differently depending on progression through the process of carcinogenesis. In a normal cell (YAMC (*Apc*^{+/+})), apoptosis is increased while proliferation is reduced, resulting in a normal phenotype. In a preneoplastic cell (IMCE (*Apc*^{Min/+})), dysregulation occurs leading to: increased cell proliferation, NF- κ B triggering of the IL-6R signaling pathway, and greater VEGF production leading to increased angiogenesis. Cumulatively, this can lead to the promotion of a transformed colon epithelial cell. In the case of the colon adenocarcinoma cell (MC-38), IL-6 and insulin induce proliferation and damage is done to tissue by IL-6-induced NO. This results in the rapid expansion of tumors. It is further hypothesized that adiponectin is an overarching inhibitor in these processes. We have clearly demonstrated that co-treatment of the MC38 cells with adiponectin attenuates the proliferative, phosphorylative, and NO inducing effects of IL-6. Taken together, this work further illustrates the mechanistic basis for the increased obesity-associated cancer risk and may lead to potential targeted therapies to decrease this risk.

Acknowledgments

Research supported in part by the National Cancer Institute Grant Number R03CA130033 and by the Michigan Agricultural Experiment Station.

REFERENCES

1. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *Lancet Oncol.* 2002; 3(9): 565–574. [PubMed: 12217794]
2. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer.* 2004; 4(8):579–591. [PubMed: 15286738]
3. Gunter MJ, Leitzmann MF. Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes. *J Nutr Biochem.* 2006; 17(3):145–156. [PubMed: 16426829]
4. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama.* 2001; 285(19):2486–2497. [PubMed: 11368702]

5. Grivennikov S, Karin E, Terzic J, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*. 2009; 15(2):103–113. [PubMed: 19185845]
6. Munkholm P. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther*. 2003; 18(Suppl 2):1–5. [PubMed: 12950413]
7. Atreya R, Neurath MF. Signaling molecules: the pathogenic role of the IL-6/STAT-3 trans signaling pathway in intestinal inflammation and in colonic cancer. *Curr Drug Targets*. 2008; 9(5):369–374. [PubMed: 18473764]
8. Greenfield JR, Campbell LV. Relationship between inflammation, insulin resistance and type 2 diabetes: 'cause or effect'? *Curr Diabetes Rev*. 2006; 2(2):195–211. [PubMed: 18220627]
9. Ealey KN, Archer MC. Elevated circulating adiponectin and elevated insulin sensitivity in adiponectin transgenic mice are not associated with reduced susceptibility to colon carcinogenesis. *Int J Cancer*. 2009; 124(9):2226–2230. [PubMed: 19123482]
10. LeRoith D, Novosyadlyy R, Gallagher EJ, Lann D, Vijayakumar A, Yakar S. Obesity and type 2 diabetes are associated with an increased risk of developing cancer and a worse prognosis; epidemiological and mechanistic evidence. *Exp Clin Endocrinol Diabetes*. 2008; 116(Suppl 1):S4–6. [PubMed: 18777452]
11. Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. *Horm Metab Res*. 2003; 35(11-12):694–704. [PubMed: 14710348]
12. Vendrell J, Broch M, Vilarrasa N, et al. Resistin, adiponectin, ghrelin, leptin, and proinflammatory cytokines: relationships in obesity. *Obes Res*. 2004; 12(6):962–971. [PubMed: 15229336]
13. Stattin P, Lukanova A, Biessy C, et al. Obesity and colon cancer: does leptin provide a link? *Int J Cancer*. 2004; 109(1):149–152. [PubMed: 14735482]
14. Sweeney G. Leptin signalling. *Cell Signal*. 2002; 14(8):655–663. [PubMed: 12020765]
15. Li L, Gao Y, Zhang LL, He DL. Concomitant activation of the JAK/STAT3 and ERK1/2 signaling is involved in leptin-mediated proliferation of renal cell carcinoma Caki-2 cells. *Cancer Biol Ther*. 2008; 7(11)
16. Perera C, Chin H, Duru N, Camarillo I. Leptin regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin regulated mammary tumor growth and progression. *J Endocrinol*. 2008
17. Miyazaki T, Bub JD, Iwamoto Y. c-Jun NH(2)-terminal kinase mediates leptin-stimulated androgen-independent prostate cancer cell proliferation via signal transducer and activator of transcription 3 and Akt. *Biochim Biophys Acta*. 2008; 1782(10):593–604. [PubMed: 18718531]
18. Ogunwobi OO, Beales IL. Cyclo-oxygenase-independent inhibition of apoptosis and stimulation of proliferation by leptin in human colon cancer cells. *Dig Dis Sci*. 2007; 52(8):1934–1945. [PubMed: 17406816]
19. Sugiyama M, Takahashi H, Hosono K, et al. Adiponectin inhibits colorectal cancer cell growth through the AMPK/mTOR pathway. *Int J Oncol*. 2009; 34(2):339–344. [PubMed: 19148467]
20. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev*. 2005; 26(3):439–451. [PubMed: 15897298]
21. Ogunwobi OO, Beales IL. Globular adiponectin, acting via adiponectin receptor-1, inhibits leptin-stimulated oesophageal adenocarcinoma cell proliferation. *Mol Cell Endocrinol*. 2008; 285(1-2): 43–50. [PubMed: 18313838]
22. Bromberg J, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer Cell*. 2009; 15(2):79–80. [PubMed: 19185839]
23. Pischon T, Nothlings U, Boeing H. Obesity and cancer. *Proc Nutr Soc*. 2008; 67(2):128–145. [PubMed: 18412987]
24. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol*. 2003; 149:1–38. [PubMed: 12687404]
25. Brown KA, Back SJ, Ruchelli ED, et al. Lamina propria and circulating interleukin-6 in newly diagnosed pediatric inflammatory bowel disease patients. *Am J Gastroenterol*. 2002; 97(10):2603–2608. [PubMed: 12385446]
26. Seegert D, Rosenstiel P, Pfahler H, Pfefferkorn P, Nikolaus S, Schreiber S. Increased expression of IL-16 in inflammatory bowel disease. *Gut*. 2001; 48(3):326–332. [PubMed: 11171821]

27. Schneider MR, Hoefflich A, Fischer JR, Wolf E, Sordat B, Lahm H. Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. *Cancer Lett.* 2000; 151(1):31–38. [PubMed: 10766420]
28. Culig Z, Steiner H, Bartsch G, Hobisch A. Interleukin-6 regulation of prostate cancer cell growth. *J Cell Biochem.* 2005; 95(3):497–505. [PubMed: 15838876]
29. Huang SP, Wu MS, Shun CT, et al. Interleukin-6 increases vascular endothelial growth factor and angiogenesis in gastric carcinoma. *J Biomed Sci.* 2004; 11(4):517–527. [PubMed: 15153787]
30. Fenton JI, Hord NG, Lavigne JA, Perkins SN, Hursting SD. Leptin, insulin-like growth factor-1, and insulin-like growth factor-2 are mitogens in ApcMin/+ but not Apc+/+ colonic epithelial cell lines. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(7):1646–1652. [PubMed: 16030096]
31. Fenton JI, Hursting SD, Perkins SN, Hord NG. Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis.* 2006; 27(7):1507–1515. [PubMed: 16597643]
32. Birmingham JM, Busik JV, Hansen-Smith FM, Fenton JI. Novel mechanism for obesity-induced colon cancer progression. *Carcinogenesis.* 2009
33. Corbett TH, Griswold DP Jr, Roberts BJ, Peckham JC, Schabel FM Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. *Cancer Res.* 1975; 35(9):2434–2439. [PubMed: 1149045]
34. Pajtasz-Piasecka E, Szyda A, Rossowska J, et al. Loss of tumorigenicity of murine colon carcinoma MC38/0 cell line after transduction with a retroviral vector carrying murine IL-12 genes. *Folia Biol (Praha).* 2004; 50(1):7–14. [PubMed: 15055737]
35. Sogn JA, Anton-Culver H, Singer DS. Meeting report: NCI think tanks in cancer biology. *Cancer Res.* 2005; 65(20):9117–9120. [PubMed: 16230364]
36. Hursting SD, Lashinger LM, Wheatley KW, et al. Reducing the weight of cancer: mechanistic targets for breaking the obesity-carcinogenesis link. *Best Pract Res Clin Endocrinol Metab.* 2008; 22(4):659–669. [PubMed: 18971125]
37. Huang XF, Chen JZ. Obesity, the PI3K/Akt signal pathway and colon cancer. *Obes Rev.* 2009
38. Fenton JI, Birmingham JM, Hursting SD, Hord NG. Adiponectin blocks multiple signaling cascades associated with leptin-induced cell proliferation in Apc Min/+ colon epithelial cells. *Int J Cancer.* 2008; 122(11):2437–2445. [PubMed: 18338750]
39. Nishihara T, Matsuda M, Araki H, et al. Effect of adiponectin on murine colitis induced by dextran sulfate sodium. *Gastroenterology.* 2006; 131(3):853–861. [PubMed: 16952554]
40. Fayad R, Pini M, Sennello JA, et al. Adiponectin deficiency protects mice from chemically induced colonic inflammation. *Gastroenterology.* 2007; 132(2):601–614. [PubMed: 17258715]
41. Bollrath J, Phesse TJ, von Burstin VA, et al. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell.* 2009; 15(2):91–102. [PubMed: 19185844]

Figure 1.

The effect of leptin, IL-6, IGF-1 and insulin on the proliferation of MC-38 cells and specific adipokine receptor expression. Cells were treated with (A) leptin (0.01-50 ng/ml), (B) IL-6 (0.01-50 ng/ml), (C) IGF-1 (0.1-200 ng/ml), or (D) insulin (0.001-100 μ g/ml) for 24 hrs and proliferation measured. (E) Characterization of specific adipokine receptors in MC-38 cells. Western blot analysis was performed and protein levels for IL-6 (IL-6R and gp130), adiponectin (AdipoR1 and AdipoR2), leptin (Ob-R), insulin (INSR-a and INSR-b), IGF-1 (IGF-R1) and actin loading control were determined. CON; control. Results are representative of 3 separate experiments. * = $p < 0.05$ (compared to control); ** = $p < 0.01$ (compared to control).

Figure 2.

The effect of adiponectin treatment on the proliferation of MC-38 cells. Cells were treated with (A) globular adiponectin (gAdiponectin, 0.001-1 µg/ml) or (B) full-length length adiponectin (fAdiponectin, 0.001-1 µg/ml) for 24 hrs. (C) Cells were treated with insulin (1 and 10 µg/ml) alone, fAdiponectin (1 and 10 µg/ml) alone, or in combination for 24 hrs. (D) Cells were treated with insulin (1 and 10 µg/ml) alone, gAdiponectin (1 and 10 µg/ml) alone, or in combination for 24 hrs. Con; Control, $*=p<0.05$ (compared to control). Results are representative of 3 separate experiments.

Figure 3.

IL-6 treatment of MC-38 cells specifically activates STAT-3. (A) The effect of IL-6 (50 ng/ml) on STAT-3 activation measured by western blot. Cells were treated overnight in serum-free medium prior to exposure to IL-6, and then treated with IL-6 for 0-60 min. At the indicated times, cellular extracts were prepared and western blot analysis was performed. Actin and STAT-3 shown as total protein loading controls. (B) IL-6 (50 ng/ml) treatment or co-treatment with anti-IL-6R of MC-38 cells and activation of nuclear STAT-3 in MC-38 cells using the TransAM™ STAT-3 Transcription Factor Assay. Cells were treated with IL-6 alone (50ng/ml) or in combination with anti-IL-6R antibody (1 µg/ml) and STAT-3 activation measured at indicated times. *= $p < 0.05$ (compared to IL-6/anti-IL-6R treated cells at same time point), **= $p < 0.01$ (compared to IL-6/anti-IL-6R treated cells at same time point). (C) Toxicity of a STAT-3 inhibitor on MC-38 cells. Cells were treated with a STAT-3 inhibitor (0.01-100 mM) for 24 hrs and cell proliferation was measured as described in the methods section. (D) Effect of STAT-3 inhibitor on IL-6-induced cell proliferation in MC-38s. Cells were treated with either IL-6 (50 ng/ml) alone, STAT-3 inhibitor (0.1-100 mM) alone or in combination for 24 hrs. *= $p < 0.05$ (compared to IL-6 alone treatment); **= $p < 0.01$ (compared to IL-6 alone treatment); ◆= $p < 0.05$ compared to untreated control. Results are representative of 3 separate experiments.

Figure 4.

Adiponectin (globular and full-length) treatment attenuates IL-6-induced signaling in MC-38 cell. (A) The effect of 24 hr treatment of IL-6 alone (50 ng/ml), gAdipo alone (1 μ g/ml), fAdipo alone (1 μ g/ml), or in combination, on MC-38 cell proliferation. $*=p<0.05$ (compared to untreated control), $**=p<0.01$ (compared to IL-6 alone treatment). (B) Co-treatment with neutralizing anti-adiponectin receptor 1 (AdipoR1) or receptor 2 (AdipoR2) on the effect of 24 hr cell proliferation induced by IL-6 $*=p<0.05$ (compared to untreated control), $**=p<0.01$ (Different from both IL-6 treatment and CON). (C) The effect of IL-6 (50 ng/ml) alone or IL-6 co-treatment with gAdiponectin, anti-AdipoR1 or R2 (1 μ g/ml) on STAT-3 activation measured by western blot. MC-38 cells were treated for 0-60 min, nuclear extracts collected and membranes probed for pSTAT-3. STAT-3 and actin loaded for control. gAdipo; globular adiponectin. fAdipo; full-length length adiponectin. Results are representative of 3 separate experiments.

Figure 5.

IL-6-induced nitric oxide production is inhibited by gAdiponectin treatment in MC-38 cells. (A) The effect of IL-6 treatment on nitrous oxide (NO) production in MC-38 cells. Cells were treated with 50 ng/ml IL-6 and NO measured after indicated times between 3 and 24 hrs. * = $p < 0.05$ (compared to 3 hr time) (B) The effect of IL-6 (1 and 50 ng/ml) and gAdiponectin (1 μ g/ml) on NO production. Cells were treated with above described concentrations and NO measured after 24 hrs. CON; control. * = $p < 0.05$ (compared to control); ** = $p < 0.01$ (compared to control); *** = $p < 0.01$ (compared to IL-6 50 ng treatment). (C) IL-6 treatment or co-treatment with adiponectin of MC-38 cells and phosphorylation (activation) of iNOS. Cells were incubated overnight in serum-free medium and then treated with either IL-6 alone (1 ng/ml or 50 ng/ml) or in combination with adiponectin (1 μ g/ml, IL-6 at 50 ng/ml). Cellular extracts were then prepared and western-blot analysis was performed. Actin shown as total protein control. Results are representative of 3 separate experiments.

Figure 6.
Hypothesized role of Adipokines on Colon Epithelial Cell Homeostasis