Adiponectin and metformin additively attenuate IL1β-induced malignant potential of colon cancer

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Abstract

Both adiponectin (AD) and metformin (Met) have been proposed to downregulate cell proliferation of colon cancer cells, but whether their effect might be additive has not been studied to date. Genetic studies in humans have suggested an important role for interleukin 1β (IL1β) in cancer pathogenesis. Direct evidence that IL1β contributes to the development of colon cancer has not yet been fully confirmed and no previous studies have evaluated how IL1β may interact with AD and/or Met to regulate malignant potential and intracellular signaling pathways in human and mouse colon cancer cells. We conducted \textit{in vitro} studies using human (LoVo) and mouse (MCA38) colon cancer cell lines to evaluate whether AD and Met alone or in combination may antagonize IL1β-regulated malignant potential in human and mouse colon cancer cell lines. IL1β increased malignant potential and regulated the expression of tumor suppressor (p53) and cell cycle regulatory genes (p21, p27, and cyclin E2) in human and mouse colon cancer cell lines. These effects were reversed by co-administration of AD and Met and were additively altered by AD and Met in combination in a STAT3- and AMPK/LKB1-dependent manner. We also observed using fluorescence activated cell sorter analysis that IL1β-regulated cell cycle progression is altered by AD and Met alone or in combination. Our novel mechanistic studies provide evidence for an important role for IL1β in colon cancer and suggest that AD and/or Met might be useful agents in the management or chemoprevention of IL1β-induced colon carcinogenesis.

Key Words
- adiponectin
- metformin
- interleukin 1β
- colon cancer
- malignant potential
- LKB1

Introduction

Colon cancer is one of the most prevalent malignancies, ranking as the second leading cause of death from cancer in the United States (Jemal \textit{et al}. 2008). Several epidemiological studies have linked colorectal cancer risk with obesity (Calle \& Kaaks 2004, Gunter \& Leitzmann 2006). A primary mechanism thought to influence the increased risk of colon cancer in the obese population is metabolic dysfunction, including dyslipidemia and hyperinsulinemia, in the context of insulin resistance (LeRoith \textit{et al}. 2008, Ealey \& Archer 2009). More recently, abnormal adipokine levels associated with intra-abdominal obesity have also been proposed to play a role at different stages of obesity-induced cancers (Dalamaga \textit{et al}. 2012).

Adiponectin (AD), one of the most abundant proteins in serum, is mainly secreted by white adipose tissue and acts through autocrine/paracrine and endocrine pathways...
(Ealey & Archer 2009). Low AD levels are correlated in epidemiology studies with an increased risk for several obesity-associated malignancies including endometrial, breast, prostate, and especially colon cancer (Wei et al. 2005, Moon et al. 2011, 2013, Dalamaga et al. 2012). Metformin (Met), an insulin sensitizer, acts mainly by inhibiting gluconeogenesis and hepatic glucose output, reduces blood glucose levels and results in a secondary decline in insulin levels (Algire et al. 2010, Wu et al. 2012). Retrospective studies among diabetic patients receiving Met therapy have provided preliminary evidence that this compound favorably influences cancer outcomes (Wu et al. 2012). Also, recent meta-analysis demonstrated that use of Met reduces, while use of sulfonylureas appears to increase, the risk for cancer in individuals with type 2 diabetes (Thakkar et al. 2013). Interleukin1-β (IL1β) is produced by activated macrophages as a pro-protein, is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis in cancers (Snider et al. 2010).

While genetic studies in humans have suggested an important role for IL1β in cancer (Kato et al. 2001, Lee et al. 2006), evidence that IL1β contributes directly to colon cancer pathogenesis is lacking. Also, the exact signaling pathways mediating IL1β’s action in colon cancer development remain unknown. Moreover, no previous study has evaluated IL1β signaling and/or has studied how IL1β interacts with AD and/or Met to alter signaling pathways in colon cancer cells. Hence, we conducted in vitro studies using human and mouse colon cancer cell lines i) to determine whether IL1β may regulate malignant potential, ii) to investigate whether AD and Met in combination may additively (or synergistically) reduce malignant potential, and iii) to evaluate whether AD and Met alone or in combination may antagonize IL1β-regulated malignant potential in human and mouse colon cancer cell lines and whether this effect may be mediated through the same or different intracellular signaling pathways.

Materials and methods

Materials

AD was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Met was purchased from Sigma–Aldrich. IL1β was purchased from EMD Millipore Corporation (Billerica, MA, USA). Mouse monoclonal p-STAT3 (target site at Tyr 705), rabbit polyclonal STAT3, rabbit polyclonal p-AMPK (target site at Tyr 172), rabbit polyclonal AMPK, mouse monoclonal p-LKB1 (target site at Ser 431), goat polyclonal LKB1, rabbit polyclonal p21, rabbit polyclonal p27, mouse monoclonal p53, goat polyclonal cyclin E2, and mouse monoclonal β-actin were purchased from Santa Cruz Biotechnology.

Cell culture

Human LoVo colon cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse MCA38 was provided by Dr Nicholas Restifo, National Cancer Institute, National Institute of Health. The LoVo and MCA38 cells were authenticated using a STR analysis. The LoVo and MCA38 cells that propagated between ten and 40 times were used. The LoVo and MCA38 cells were grown in a DMEM. All cells were supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in an atmosphere of 5% CO2 in air and sub-cultured beyond 80% confluency.

Proliferation assay

The cells were trypsinized and seeded in 96-well plates at a density of 5 × 10^3 cells/well. Cells were left to adhere overnight and then treated with AD, Met, and IL1β alone or in combination for 24 h. At the end of the incubation period, the effect of AD, Met, and IL1β on cell proliferation was assayed with an MTT assay (Invitrogen). Cells were washed with PBS and incubated with 100 μl serum-free medium and 10 μl Vybrant MTT solution for 2 h. Formazan crystals were dissolved overnight at 37 °C with the addition of 100 μl 10% SDS in 0.01 M HCl per well and absorbance was measured at an optical density of 570 nm in a PowerWave XS (BIOTEK, Winooski, VT, USA).

Clonogenic assay

The cells were grown in the media to 60% confluency and treated with AD, Met, and IL1β alone or in combination for 24 h. After 24 h, 500 cells were re-seeded into a 100 mm culture dish and incubated for 12 days. Fresh media were changed every 3 days. At day 12, the media were removed, added to 2 ml clonogenic reagent (50% ethanol + 0.25% 1,9-dimethyl-methylene blue), and left at room temperature for 45 min. After 45 min, the cells were washed with PBS and the blue colonies were counted.

Adhesion assay

The cells were pretreated with AD, Met, and IL1β alone or in combination for 24 h and plated (5 × 10^4 cells/well) in
10 μg/cm² fibronectin-coated (Sigma) wells in 96-well plates that were then incubated at 37 °C (5% CO₂) for 60 min. Adherent cells were fixed with 3% paraformaldehyde for 10 min, washed with 2% methanol for 10 min, and stained with 0.5% crystal violet in 20% methanol for 10 min. The stain was eluted and absorbance was measured at 540 nm.

**Invasion assay**

For an *in vitro* model system for metastasis, we performed a Matrigel invasion assay using a Matrigel invasion chamber from BD BioCoat Cellware (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

**Flow cytometric analysis of cell cycle**

The cells were collected by centrifugation and fixed with 70% ethanol at 4 °C overnight. The 70% ethanol was then removed by centrifugation, and the DNA of the cells was stained with PI staining solution (Sigma–Aldrich) according to the manufacturer's protocol. The samples were then analyzed by fluorescence activated cell sorter (FACS).

**Introduction of small interfering RNA**

The small interfering RNA (siRNA) of *STAT3*, *AMPK*, and *LKB1* and scrambled siRNA (ssiRNA) were purchased from Santa Cruz. The cells were seeded onto six-well tissue culture plates and grown to 60% confluency in the absence of antibiotic before transfection. Immediately prior to transfection, the culture medium was removed and the cells were washed once with PBS and then transfected with either ssiRNA or siRNA using Fugene 6 transfection reagent in Opti-MEM medium according to the manufacturer's instructions (Roche). After transfection, cultures were incubated at 37 °C for 5 h and then placed in fresh culture medium. Cells were studied after an additional 24 h.

**Protein extraction**

The collected cells were suspended in a lysis buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 0.1% Igepal and then incubated for 30 min at 4 °C. The suspension was centrifuged for 10 min at 17,226 g and the supernatant was saved as the protein extract.

**Western blotting**

After SDS–PAGE, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH, USA). The membranes were blocked for 1 h in TBS containing 5% nonfat dry milk and 0.1% Tween 20. Incubation with primary antibodies was performed in TBS containing 5% nonfat dry milk overnight and then incubated with HRP secondary antibodies for 2 h. After incubation with antibodies, membranes were washed with TBS containing 0.1% Tween 20. ECL was used for detection. Measurement of signal intensity on nitrocellulose membranes after western blotting with various antibodies was performed using ImageJ processing and analysis software.

**Statistical analysis**

All data were analyzed using Student's *t*-test and/or one-way ANOVA followed by *post-hoc* test for multiple comparisons. All analyses were performed with SAS (SAS version 9.1; SAS Institute, Cary, NC, USA).

**Results**

**IL1β and Met regulate cell proliferation**

We observed that cell proliferation is increased by ~20–35% at high physiological/pharmacological doses, i.e., 10–50 ng/ml of IL1β when compared with control in human LoVo and mouse MCA38 colon cancer cell lines (Fig. 1A and B). By contrast, cell proliferation was decreased by ~10–60% at physiological and high physiological/pharmacological doses, i.e., 1–50 μg/ml Met when compared with control in human LoVo and mouse MCA38 colon cancer cell lines (Fig. 1C and D).

**IL1β-induced malignant potential is attenuated by AD and Met alone or in combination**

Based on Fig. 1 and our previous report (Moon *et al*. 2013), we chose a representative concentration of IL1β (50 ng/ml), Met (10 μg/ml), and AD (20 μg/ml) respectively. We then studied in detail whether AD, Met, and IL1β alone or in combination may regulate malignant potential in human LoVo and mouse MCA38 colon cancer cell lines. We observed that IL1β increases malignant potential, i.e., cell adhesion (Fig. 1E and F), invasion (Fig. 1G and H), and/or colony formation (Fig. 1I and J) of human LoVo and mouse MCA38 colon cancer cell lines. By contrast, IL1β-induced malignant potential is attenuated by co-administration of AD and/or Met, and these are...
Interleukin 1β (IL1β) and metformin (Met) regulate cell proliferation and malignant potential. The cells were cultured as described in detail in the ‘Materials and methods’ section. The cells were incubated with IL1β (A and B) and/or Met (C and D) at indicated concentration for 24 h, and cell viability was then measured by MTT assay as described in detail in the ‘Materials and methods’ section. The cells were incubated with adiponectin (AD, 20 µg/ml), IL1β (50 ng/ml), and Met (10 µg/ml) alone or in combination, and cell adhesion (E and F), invasion (G and H) and colony formation (I and J) were then performed as described in detail in the ‘Materials and methods’ section. All data were analyzed one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± S.D. Means with different letters are significantly different, P<0.05, whereas means with similar letters are not different from each other.
additively reduced by AD and Met in combination (Fig. 1E, F, G, H, I, and J).

IL1β-regulated activation of STAT3/AMPK signaling is altered by AD and Met alone or in combination

We observed that IL1β increases p-STAT3 and decreases p-AMPK expression in human LoVo and mouse MCA38 cell lines (Fig. 2A, B, C, and D). By contrast, these are altered by co-administration of AD and/or Met and are additively reversed by AD and Met in combination (Fig. 2A, B, C, and D). When the levels of STAT3 mRNA were downregulated via siRNA, the inhibitory and/or stimulatory effect of AD, Met, and/or IL1β on cell proliferation was abolished (Fig. 2E and F). Under the same condition, the regulation of malignant potential by AD, Met, and IL1β alone or in combination was also blocked by knockdown of STAT3 (Supplementary Figure 1, see section on supplementary data given at the end of this article) and/or AMPK (Supplementary Figure 2) in human LoVo and mouse MCA38 colon cancer cell lines.

IL1β-regulated malignant potential is attenuated by AD and Met alone or in combination in a LKB1-dependent manner

IL1β decreased p-LKB1 expression in human LoVo and mouse MCA38 colon cancer cell lines (Fig. 3A and B). By contrast, these are increased by co-administration of AD and/or Met and are additively enhanced by AD and Met in combination (Fig. 3A and B). Also, the expression of p-AMPK by AD, Met and IL1β alone or in combination is blocked by LKB1 siRNA administration in all colon cancer cell lines (Fig. 3C and D). In addition, AD-, Met-, and/or IL1β-regulated malignant potential of human and mouse colon cancer cell lines was also blocked by LKB1 siRNA administration (data not shown).

IL1β-regulated expression of tumor suppressor and cell cycle regulatory genes is altered by AD and Met alone or in combination

IL1β-regulated expression of tumor suppressor and cell cycle regulatory genes was altered by co-administration of AD and/or Met and that all these are additively regulated by AD and Met in combination (Fig. 4A, B, C, and D). We also observed that all these are abolished by LKB1 siRNA administration (data not shown).

IL1β-regulated cell cycle progression is altered by AD and Met alone or in combination

Treatment with AD and Met attenuated IL1β-increased G1 and/or S phase, thereby reducing cell growth, as judged by the appearance of a Sub-G1 peak during cell cycle progression in all colon cancer cells (Table 1). Also, we observed that all these are additively regulated by AD and Met in combination. Finally, we observed that all these are abolished by LKB1 siRNA administration (data not shown).

Discussion

We have demonstrated for the first time that AD directly regulates malignant potential and controls metabolic (AMPK/S6), inflammatory (STAT3/VEGF), and cell cycle (p21/p27/p53/cyclins) signaling pathways in both mouse and human colon cancer cell lines in a LKB1-dependent manner (Moon et al. 2013). Met has also been shown to display significant growth inhibitory effects in several cancer cells and mouse tumor models such as breast, prostate, ovarian, and glioma (Zakikhani et al. 2006, Dowling et al. 2007, Ben Sahra et al. 2008). While genetic studies in humans have suggested an important role of IL1β in cancers (Kato et al. 2001, Lee et al. 2006), direct evidence that IL1β contributes to the pathogenesis of colon cancer has not yet been published. Here, we show that AD and Met alone or in combination have beneficial effects on IL1β-regulated malignant potential in human and mouse colon cancer cells via STAT3 and AMPK/LKB1 signaling pathways.

It has been shown that AD regulates cell cycle arrest in certain cancer cell lines such as hepatoma HepG2, prostate carcinoma PC-3, and breast cancer MCF-7 by upregulating tumor suppressor genes such as p53, p21, and p27 (Xiang et al. 2004, Rattan et al. 2005, Imamura et al. 2011). Also, it has been demonstrated that the effects of Met on cancer cell proliferation are associated with decreased expression of cyclins and increased expression of p27 (Isakovic et al. 2007, Gotlieb et al. 2008). Moreover, it has been shown that Met decreases cell proliferation in ovarian cancer cell lines accompanied by decreased cyclins and increased p21 protein expression (Rattan et al. 2011). Also, it has been demonstrated that Met activates p53 in breast cancer cell lines (Malki & Youssef 2011). Similar to these reports, we demonstrated that the expressions of tumor suppressor genes are directly regulated by AD and/or Met administration in human and mouse colon cancer cell lines. We also observed that both AD and Met decrease the expression of the cell cycle regulatory gene, cyclin E2, which helps to drive the progression from G1 to S phase (Oshita et al. 2002).
in human and mouse colon cancer cell lines. Indeed, we observed using a FACS analysis that both AD and Met increase Sub-G1 peak during cell cycle progression, thereby reducing cell growth. By contrast, IL1β decreased the expression levels of tumor suppressor and increased the expression levels of cell cycle regulatory genes in human and mouse colon cancer cell lines. We also confirmed using FACS analysis that IL1β increases G1 and/or S phase. Also, IL1β-regulated the expression of tumor suppressor and cell cycle regulatory genes (with cell cycle progression) are

Figure 2
Interleukin 1β (IL1β)-regulated malignant potential is attenuated by adiponectin (AD) and metformin (Met) alone or in combination in a STAT3- and/or AMPK-dependent manner. The cells were cultured as described in detail in the ‘Materials and methods’ section. (A, B, C and D) The cells were stimulated with IL1β (50 ng/ml), AD (20 μg/ml), and Met (10 μg/ml) alone or in combination for 30 min and western blotting was then performed as described in detail in the ‘Materials and methods’ section. (E and F) The cells were transfected with STAT3 siRNA as described in detail in the ‘Experimental Procedures’ section. The cells were then stimulated with IL1β (50 ng/ml), AD (20 μg/ml), and Met (10 μg/ml) alone or in combination for 24 h and MTT assay was then performed as described in detail in the ‘Materials and methods’ section. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n = 3) ± S.D. Means with different letters are significantly different, P < 0.05, whereas means with similar letters are not different from each other.
A number of immunosuppressive factors produced by tumor cells in a STAT3-dependent manner are angiogenic factors (Melani et al. 2003, Yu et al. 2007) and a role of constitutively activated STAT3 in promoting tumor angiogenesis and metastasis of tumor cells has been well documented (Moon et al. 2013). In agreement with these previous studies, we identified that AD and/or Met treatment significantly downregulates the expression levels of p-STAT3 in human and mouse colon cancer cell lines. Also, we observed that IL1β-increased STAT3 activation is attenuated by co-administration of AD and is further decreased by AD and Met in combination. Finally, we observed that the suppression of STAT3 expression levels by STAT3 siRNA administration blocks colon cancer cell viability as well as malignant potential, suggesting that the STAT3 pathway is essential for AD-, Met-, and/or IL1β-regulated colon cancer growth.

Although AD and Met have been shown to downregulate cancer cell proliferation via AMPK signaling (Yamauchi et al. 2002, 2003, Isakovic et al. 2007, Kubota et al. 2007, Gotlieb et al. 2008), it remains unclear whether IL1β-regulated malignant potential in colon cancer cells may be mediated by AMPK. We herein observed that the suppressive effect of AD and/or Met on colon cancer cell proliferation as well as malignant potential is mediated by AMPK signaling. Also, IL1β-decreased AMPK activation is attenuated by co-administration of AD and/or Met, and these are further decreased by AD and Met in combination. These results suggest that AD’s and/or Met’s effect on IL1β-increased malignant potential is mediated by AMPK activation in both human and mouse colon cancer cell lines.

Figure 3
Interleukin 1β (IL1β)-regulated LKB1 activation is altered by adiponectin (AD) and metformin (Met) alone or in combination. The cells were cultured as described in detail in the ‘Materials and methods’ section. (A and B) The cells were stimulated with IL1β (50 ng/ml), AD (20 μg/ml), and Met (10 μg/ml) alone or in combination for 30 min and western blotting was then performed as described in detail in the ‘Materials and methods’ section. (C and D) The cells were transfected with LKB1 siRNA as described in detail in the ‘Materials and methods’ section. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n=3) ± S.D. Means with different letters are significantly different, P<0.05, whereas means with similar letters are not different from each other.
Figure 4

Interleukin 1β (IL1β)-regulated expression of tumor suppressor and cell cycle regulatory genes is altered by adiponectin (AD) and metformin (Met) alone or in combination in a LKB1-dependent manner. The cells were cultured as described in detail in the 'Materials and methods' section. (A, B, C and D) The cells were stimulated with IL1β (50 ng/ml), AD (20 μg/ml), and Met (10 μg/ml) alone or in combination for 24 h and western blotting was then performed as described in detail in the 'Materials and methods' section. All figures showing quantitative analysis include data from at least three independent experiments. All density values for each protein band of interest are expressed as a fold increase. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n = 3) ± S.D. Means with different letters are significantly different, P < 0.05, whereas means with similar letters are not different from each other.
Table 1  IL1β-regulated cell cycle progression is altered by adiponectin and metformin alone or in combination

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<th>Sub-G₁</th>
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<td>11.2 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

The cells were treated with interleukin 1β (IL, 50 ng/ml), adiponectin (AD, 20 μg/ml), and metformin (Met, 10 μg/ml) alone or in combination for 24 h, and FACS analysis was then performed as described in detail in the 'Materials and methods' section. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means (n = 3) ± s.d. Means with different letters are significantly different, P < 0.05, whereas means with similar letters are not different from each other with respect to the column.

Tumor suppressor effects of LKB1 are due to its ability to activate the master metabolic regulator AMPK (Hardie 2005). In a manner comparable to a previous study on AD signaling in human endometrial cancer cell lines (Moon et al. 2011), we found that AD and/or Met increases activation of the tumor suppressor gene LKB1 in human and mouse colon cancer cells. In contrast to AD and Met, we observed that IL1β decreases LKB1 activation. Hence, it is possible to speculate that the regulation of the tumor suppressor gene LKB1 by AD, Met, and/or IL1β would further contribute to the control of cellular activities in colon cancer cells. Hence, we hypothesized that AD-, Met-, and/or IL1β-regulated cell proliferation, malignant potential, cell cycle progression, and/or intra-cellular signaling pathways could be mediated by LKB1 in colon cancer cells. Indeed, we observed that LKB1 gene knockdown results in abrogation of AD-, Met-, and/or IL1β-regulated cellular activities above in human and in mouse cancer cell lines. We also observed that AD-, Met-, and/or IL1β-regulated AMPK activation is blocked by LKB1 siRNA administration. These results suggest that LKB1 is important in AD-, Met-, and/or IL1β-regulated development of human and mouse colon cancer cells.

In summary, we observed for the first time that IL1β-regulated cellular activities including cell proliferation, malignant potential, apoptosis, and cell cycle regulatory genes are attenuated by co-administration of AD and/or Met mainly via downregulation of STAT3 and upregulation of AMPK signaling pathways in human and mouse colon cancer cell lines. In addition, we observed that these are additively regulated when AD and Met are administered in combination. Importantly, our data demonstrate for the first time that AD, Met, and/or IL1β regulates the expression of the tumor suppressor gene LKB1 and that LKB1 is required for AD-, Met-, and/or IL1β-mediated AMPK activation in human and mouse colon cancer cell lines. These novel mechanistic studies provide evidence for a causal role of AD, Met, and/or IL1β in colon cancer development. Finally, in vivo studies in rodents, and hopefully later in humans, could further elucidate the role of AD’s or AD analogs and/or Met’s potential use as therapeutic agents to be used alone or in combination for colon cancer. If these data are confirmed and extended by future studies, AD and Met, or their analogs alone or in combination, could potentially prove to be a useful agent in the management or chemoprevention of IL1β-induced colon carcinogenesis.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0240.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Author contribution statement

All authors participated in the study design, performance, and coordination. All authors contributed to writing the manuscript. All authors read and approved the final manuscript.

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