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THE EFFECT OF OVERWEIGHT AND OBESITY ON ADIPONECTIN LEVELS
AND ITS ASSOCIATION TO COLON AND PANCREATIC CANCERS

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ABSTRACT

Obesity is a growing problem in this nation as well as the rest of the world. Many factors are involved in the development and progression of obesity. After the discovery of adiposity hormones, much research has been conducted to understand the mechanisms of these hormones. Adiponectin is the adiposity hormone highlighted in this paper. Recent studies have shown a protective effect of adiponectin on pancreatic and colorectal cancers. However, no studies had investigated the effects of adiponectin in vitro. Our proposed hypothesis was that adiponectin would not change cell proliferation, but lower levels of adiponectin would increase tumor cell growth. We used an MTT assay to measure cell proliferation for 72 hours. The growth of both pancreatic tumor cells (Panc.02) and colon cancer cells (MC38) did not significantly change with the introduction of adiponectin at physiological levels. In addition, cells growing in fetal calf serum proliferated twice as fast as those in serum free media. In conclusion, low levels of adiponectin did not promote tumor cell growth and high levels of adiponectin did not decrease cell growth.
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BACKGROUND AND SIGNIFICANCE

Obesity is one of the most widespread public health problems that plagues this nation and the world. In the United States, more than two thirds of adults and one third of children and adolescents are considered overweight or obese (Nguyen et al. 2012). These statistics have more than doubled since the 1970’s. Obesity has been linked to many chronic problems including heart disease, diabetes, and cancer. This severe increase in the prevalence of obesity is of particular concern among young individuals. Children are now experiencing the effects of diseases that were only common among adults just a few decades ago. For example, many overweight children are now being diagnosed with Type 2 Diabetes (Lytle et al. 2012). This life-threatening problem involves many aspects of our daily lives including environment, genetics, culture and psychosocial factors.

Obesity is defined as having a very high relation of body fat to lean body mass. Body mass index (BMI) is used to determine an individual’s weight status. BMI is the measure of an individual’s weight in relation to his or her height, which is calculated by weight (kg) divided by height (m) squared. This measurement is often used because it is non-invasive and is fairly easy to calculate. Adults with a BMI $\geq 30$kg/m$^2$ are considered obese (Nguyen, 2012). The severity of this problem has led to the research of adipose tissue. It is now known that adipose tissue is active and releases hormones, such as leptin and adiponectin (APN) (Assad et al. 2012). Leptin is a pro-inflammatory adipokine that increases as body mass increases (Assad et al. 2012). Adipokines are cell-signaling molecules secreted by adipose tissue. Therefore, an increase in leptin was thought to contribute to the complications associated with obesity. However, the answer proved to not be so simple. The mechanisms underlying the regulation of energy intake and storage...
are complex. Numerous adipokines have been discovered, and they have the ability to modulate metabolic and inflammatory processes. These adipokines are believed to be key contributors in the pathophysiology behind obesity. Adiponectin is an adipokine of particular interest because it has been shown to produce anti-inflammatory effects (Assad et al. 2012). Due to the inflammatory state of obesity, the benefits of adiponectin are worth further investigating.

Unlike leptin, adiponectin levels are lower in obese individuals than individuals with a normal amount of body fat and adiponectin inhibits inflammation. Due to this distinction, adiponectin has been further studied as an anti-inflammatory and insulin-sensitizing component. Exactly how adiponectin targets inflammation and its role in
adipose tissue has yet to be fully determined. However, studies have indicated higher levels create beneficial effects.
Background of Adiponectin

Adiponectin is currently known to be the most abundant protein secreted by white adipose tissue. Adiponectin is known for its perplexing involvement in obesity-related disorders, as well as pathologies such as cancer where evidence has been reported supporting its relationship to obesity (Brochu-Gaudreau et al. 2010). Adiponectin also appears to be secreted in humans via cells such as bone marrow, osteoblasts, fetal tissue, myocytes, salivary gland epithelial cells, and cardiomyocytes (Brochu-Gaudreau et al. 2010). The exact regulation of adiponectin expression has yet to be fully determined. Some possible regulation mechanisms of adiponectin include three potential CCAAT-enhancer-binding proteins. CCAAT-enhancer-binding proteins promote the expression of certain genes. These binding proteins may regulate adiponectin because they are induced during the early stages of adipogenesis. In addition, adiponectin production can be downregulated by prolactin and growth hormone (Brochu-Gaudreau et al. 2010).

Adiponectin is now being widely studied because of its anti-inflammatory effects. Specifically, adiponectin seems to increase the action of the anti-inflammatory cytokines interleukin (IL) 10, as well as decrease the effects of pro-inflammatory IL-6. IL-10 decreases inflammation, which prevents disease, and IL-6 increases inflammation, promoting disease. Due to the role of adipose tissue in maintaining hormone balance and energy homeostasis, obesity has also been a topic of interest when studying adiponectin. Adiponectin is negatively correlated to body fat mass. Therefore, unlike other adiposity hormones such as leptin, adiponectin decreases as body fat mass increases. Weight loss usually results in an increase of adiponectin levels. Adiponectin has also been studied
regarding its insulin-sensitizing effect. Plasma adiponectin levels have been shown to be lower in men and women with diabetes. In addition, high circulating levels of adiponectin have been associated with a lower risk for developing Type 2 Diabetes (Brochu-Gaudreau et al. 2010); and adiponectin is negatively correlated with insulin and plasma glucose levels.

The protective effect of adiponectin on obesity-related diseases has been well documented in many published scientific journals. Recent studies have shown the potential of adiponectin to be used as a therapeutic option when examining obesity-related disorders.
**Effect of Overweight and Obesity on Adiponectin Levels**

As mentioned previously, the accumulation of excess adipose tissue is inversely related to adiponectin levels. Weight loss usually returns adiponectin levels to normal in obese individuals (Brochu-Gaudreau et al. 2010). Accumulating evidence also indicates a direct link of adiponectin to obesity-related disorders, such as insulin resistance, type 2 diabetes, and hypertension (Brochu-Gaudreau et al. 2010). One study examined the adiponectin and resistin response to the onset of obesity in rats by feeding a cafeteria diet. The cafeteria diet included foods such as bacon, biscuits, cheese, chocolate and salted peanuts. The group of rats fed the cafeteria diet had significantly higher body fat, specifically visceral fat. The group fed the cafeteria diet also had a decrease in production of adiponectin after the fifteen-day period (Ribot et al. 2008). Therefore, serum adiponectin appears to be an early response to obesity when consuming a high-fat diet.

Studies have also investigated the effect of diet in obese and overweight individuals. The goal of these studies is to uncover additional information about other factors that may influence adiponectin levels in obese or overweight individuals. One study researched the relationship between glycemic load and disease markers, particularly adiponectin. This study was a randomized, crossover feeding study meant to compare the effects of a low glycemic load diet as opposed to a high glycemic load diet on adiponectin levels. Healthy, non-smoking men and women (18-45 years old) completed a 3-day food recall to assess normal eating habits and were then placed on one of two 7-day diet rotations. The two diets were designed to be the same macronutrient composition, only differing in high or low glycemic loads. After measuring adiponectin and leptin levels, only adiponectin presented a significant difference. The group
consuming a low glycemic load seemed to have higher serum adiponectin levels. C-reactive protein (CRP) was also measured to indicate a response to inflammation. In this study, CRP levels had decreased. The results in this study coincide with those studies demonstrating that a low glycemic load diet decreases inflammation (Neuhouser et al. 2012). This established relationship between glycemic load and inflammation seems to also expand on the role of adiponectin in inflammation. Numerous studies since the 19th century have pointed to an association between cancer and inflammation (Ben-Neriah et al. 2011). The anti-inflammatory effect of adiponectin has led to an interest in examining the hormone's effect on certain types of cancer.
Adiponectin and Pancreatic Cancer

There is increasing evidence that alterations in the adiponectin levels play a role in the incidence, as well as survival rate of pancreatic cancer. Stolzenberg-Solomon et al. (2008) investigated the protective effects of adiponectin in a case-control study of male smokers aged 50-69. After 19 years, 311 of the participants had developed pancreatic cancer. The results showed that higher adiponectin levels were inversely related to pancreatic cancer (Stolzenberg-Solomon et al. 2008). The authors adjusted the results for smoking, blood pressure, and C-peptide levels. This relationship was most significant among those cases diagnosed 5 or more years after the beginning of the study. These results demonstrate a protective effect of adiponectin on the incidence of pancreatic cancer. Other studies have not found a significant impact of adiponectin on pancreatic cancer.

The European Prospective Investigation into Cancer and Nutrition (EPIC) cohort has been used to study an individual’s risk for developing pancreatic cancer based on adiponectin levels. EPIC is a large cohort study conducted in ten European countries. The study includes more than 500,000 men and women, aged 35 to 70 years. Blood samples were taken from 452 patients with pancreatic cancer and 452 matched controls. Waist circumference and BMI were inversely related to adiponectin levels. Overall, the results of this particular study showed no significant effects of adiponectin on pancreatic cancer. There was some data that suggests lower adiponectin concentrations could be associated with a higher risk for pancreatic cancer among those who had never been smokers, as well as a decrease risk for pancreatic cancer among individuals with higher levels of
circulating adiponectin (Grote et al. 2011). Studies have also investigated the role of obesity along with adiponectin in the development of pancreatic cancer.

In addition to human epidemiologic studies, preclinical animal model studies have also been conducted to study the relationship between obesity, adiponectin, and pancreatic cancer risk. To this end, lean and obese mice were inoculated with murine pancreatic cancer cell line. After five weeks of tumor growth, mice were sacrificed and tumors dissected. Mortality was greater and tumor size was larger in obese mice than lean mice. Furthermore, serum adiponectin was greater in lean mice than in obese mice. Also, tumor proliferation showed a strong negative correlation to circulating adiponectin levels (Zyromski et al. 2009). These findings lend support to the hypothesis that circulating adipokines may be involved in the increased risk for certain cancers in obese individuals.

Another study using mice to evaluate the effects of obesity and adipokines on pancreatic tumor cell growth, included diet in the study design. Thirty female mice were fed either a control diet (10% fat) or a 60% fat diet. The mice were inoculated with murine pancreatic tumor cells at eleven weeks. The group fed a high fat diet gained a significant amount of weight. Mice heavier than 23.1 g were considered overweight. The mice deemed overweight, had a significant increase in pancreatic tumor growth. There was not a significant difference in circulating adiponectin levels in lean and obese mice (White et al. 2010). This similarity may exist due to the small weight differential between the two groups. This study also implicates that obesity plays a role in the development of pancreatic tumor cell growth. However, the role of adiponectin in pancreatic tumor cell proliferation has yet to be fully determined.
Adiponectin and Colon Cancer

Chronic states of inflammation, such as Irritable Bowel Disease, have been shown to increase the risk of colorectal cancer (Vinuesa et al. 2012). Due to this association, other studies have been done to investigate a possible relationship between adiponectin and colon cancer risk. Many studies have examined the benefits of adiponectin on the prevalence and treatment of colon cancer. One study in particular examined adiponectin and its anti-inflammatory effects in regards to colon cancer. Mice were injected with intestinal tumor cells, and mice with disruptions in adiponectin were more likely to develop more intestinal tumors (Mutoh et al. 2011). Saxena et al. (2012) examined the relationship between adiponectin deficiency and inflammation-induced colon cancer. Both Adiponectin knockout (KO) and wild type (WT) mice were used to induce colon cancer and inflammation. Mice were sacrificed on day 153 and tumor area and number were counted. Adiponectin KO had more severe symptoms, as well as a greater number of tumors and larger areas of tumors (Saxena et al. 2012). These results indicate that an adiponectin deficiency may contribute to the development of colorectal cancer.

These findings have led researchers to question the role of adiponectin directly on tumor cell growth, including the relationship between proliferation and apoptosis of colonic and pancreatic tumor cells. To date, no one has directly examined the effects of adiponectin on tumor cell growth or proliferation in vitro. Studies to date have always had the confound of other obesity-related factors on board. Therefore, the goal of the present study was to determine if adiponectin could alter the proliferation of murine colon and pancreatic tumor cells. The working hypothesis was that adiponectin would have no effect on pancreatic and colon tumor cell growth at normal physiological levels;
however; as adiponectin levels decreased, an increase in tumor cell growth would occur.

The hypothesis was tested by growing colon and pancreatic cancer cells *in vivo.*

Adiponectin was then added at increasing levels from 0 to 20 µg to measure cell proliferation.
MATERIALS AND METHODS

Cell Culture Reagents

The MC38 murine colonic adenocarcinoma cell line was induced by subcutaneous injection with 1,2-dimethylhydrazine in C57BL/6 mice and was transplanted by subcutaneous injection (Fox, 1990). These cell lines were maintained in DMEM with 10% fetal bovine serum, 2 mm glutamine, 0.1 mm nonessential amino acids, 0.1 mm sodium pyruvate, and 50 µg/ml. Separate cultures of MC38 were also grown in DMEM culture media without fetal bovine serum, containing 10% recombinant serum replacement (X-vivo) 2 mm glutamine, 0.1 mm nonessential amino acids, 0.1 mm sodium pyruvate, and 50 µg/ml gentamicin sulfate.

The murine ductal adenocarcinoma cell line Panc02 was established through the induction of pancreatic tumors with 3-methylcholanthrene and serial subcutaneous transplantation in C57BL/6 mice (Corbett et al. 1984). Panc02 cells were cultured in McCoy’s 5A medium supplemented with 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 2 mmol/L l-glutamine, 10 mmol/L HEPES, 300 µg/mL G418 sulfate, and 10% heat-inactivated fetal bovine serum.

Separate cultures of Panc.02 were also grown in McCoy’s 5A culture media without fetal bovine serum, 10% recombinant serum replacement, X-vivo, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 2 mmol/L l-glutamine, 10 mmol/L HEPES, 300 µg/mL G418 sulfate.

Recombinant mouse globular adiponectin was purchase from Adipobiosciences and added to cell culture assays starting at 20 µg/ml.
Proliferation Assay

An MTT assay was used to examine cell proliferation for 72 hours. Cell concentration ranged from 0 to 5000 with MC38 cells, and 0 to 5000 with Panc.02 cells. For the remaining experiments, MC38 tumor cells were plated at a density of 1000 cells per well, and Panc.02 tumor cells were plated at 2500 per well. This difference in cells per well is due to the difference in cell growth between the two tumor cells. The Panc.02 rate of cell division is 1 division every 18 to 24 hours, and MC38 division is 1 division every 12 to 16 hours. Cells need to be plated at different cell densities in order to optimize growth. Cells were incubated with varying doses of adiponectin ranging from 0 to 20 ug/mL. The MTT Assay was performed according to the manufacturer’s instructions (Trevigen). Wavelength absorbance was measured at 570 and 650 nm and the average of triplicate readings was calculated.
Statistical Analysis

All data are presented as the mean plus or minus the standard error of the mean. Differences in tumor cell proliferation based on cell density were determined using one-way analysis of variance (ANOVA), followed by Fisher Least Significant Difference post-hoc tests and a Bonferroni correction for multiple comparisons where appropriate. Differences in cell proliferation and adiponectin levels and media conditions were evaluated using two-way ANOVA, followed by Fisher Least Significant Difference post-hoc tests and a Bonferroni correction for multiple comparisons where appropriate. All analyses were conducted using GraphPad software and statistical significance was accepted at the $P \leq 0.05$ level.
RESULTS

Figure 1 – Panc.02 cells were diluted starting at 5000 cells per well and optical density was measured in a standard MTT assay. Cell proliferation increased as the number of Panc.02 cells increased in both FCS-containing and serum-free media.

Our results showed that as the number of Panc.02 cells increased, the optical density also increases in both fetal calf serum-containing and in control media. Cells were grown in X-Vivo media (serum replacement media) in order to control for naturally present adiponectin in fetal calf serum. The results in Figure 1 demonstrate that Panc.02 cells proliferate twice as well in fetal calf serum than in X-Vivo. Therefore, X-Vivo does not adequately mimic fetal calf serum. The significant increase in cell growth in fetal calf serum demonstrates that fetal calf serum is a media that supports the growth of Panc.02.
cells. Therefore, this media will promote the growth of Panc.02 and create an adequate environment to test the proposed hypothesis.

Figure 2 – Adiponectin was introduced in increasing concentrations to the Panc.02 cells in both FCS-containing and the control media. In both media, cell proliferation did not significantly change as adiponectin levels were increased.

Adiponectin was added at concentrations from 0 to 20 µg/ML to the Panc.02 cells in FCS and X-Vivo. This experiment again shows that Panc.02 cells in X-Vivo grow half as well as those in fetal calf serum. The results indicate that adiponectin concentrations at physiological levels do not alter cell proliferation in FCS or X-Vivo. Adiponectin has no significant effect on Panc.02 cell proliferation at these levels. Adiponectin does not appear to directly influence murine pancreatic cancer growth.
Figure 3 – MC38 cells were diluted and optical density was measured at varying cell numbers. As the number of MC38 cells increased, cell proliferation also increased in fetal calf serum.

The data for MC38 cells shows an increase in cell density as cell proliferation increases. These results demonstrate that media containing fetal calf serum serves as a better growth medium for MC38 cells as compared to X-Vivo (serum free) media.
Figure 4 – Adiponectin was added to the MC38 cells at varying concentrations. Both FCS and X-Vivo containing media were used to measure MC38 cell proliferation. MC38 proliferation did not significantly change when increasing adiponectin concentration. The results in Figure 4 demonstrate that adiponectin does not alter MC38 cell proliferation in FCS and in serum free media. No significant change in cell proliferation was found when increasing adiponectin concentrations. The results from Figure 2 and 4 demonstrate that adiponectin does not influence murine pancreatic or colon cancer growth.
DISCUSSION

The data in Figures 2 and 4 demonstrates that as adiponectin concentrations increase, proliferation rates stay constant in both pancreatic and colon cancer cells. This suggests that a change in adiponectin from normal, physiological levels of 20 µg/mL to lower levels often seen in obesity (1 µg/mL) does not affect tumor cell growth.

However, it does appear that cell concentration and the type of media play a key role in cell proliferation. Our results demonstrate that cells need nutrients in fetal calf serum to grow, and that tumor cells are not receiving adequate nutrients from serum free media to maintain adequate cell growth.

Since adiponectin did not directly influence pancreatic and colon tumor cell growth, this suggests that there are factors other than adiponectin involved in the relationship between obesity and tumor growth. Perhaps other anti-inflammatory proteins have a greater impact on an inflammatory response to cancer.

In the study conducted by Stolzenberg-Solomon et al, higher levels of adiponectin had protective effects on the development of pancreatic cancer. Although this study accounted for factors such as obesity, there may be other physiological components involved in the development of pancreatic cancer. Adiponectin could be involved in the protective effects found in this study, but adiponectin does not appear to be the only factor involved.

Saxena et al had investigated the benefits of adiponectin in the inflammatory state of colon cancer in mice. This particular study focused on the possible complications of an adiponectin deficiency. Therefore, this study may implicate that a deficiency in adiponectin may promote colon cancer but adequate levels may have little to no effect.
Our data has shown that adiponectin alone does not directly affect tumor cell growth. Adiponectin is most likely a crucial part of a larger inflammatory process. Therefore, further research should be done to isolate other factors involved in the inflammatory response to obesity.
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