ORIGINAL ARTICLE

Obesity, But Not High-Fat Diet, Promotes Murine Pancreatic Cancer Growth

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Abstract

Background Obesity accelerates pancreatic cancer growth; the mechanisms underlying this association are poorly understood. This study evaluated the hypothesis that obesity, rather than high-fat diet, is responsible for accelerated pancreatic cancer growth.
Methods Male C57BL/6J mice were studied after 19 weeks of high-fat (60 % fat; $n=20$) or low-fat (10 % fat; $n=10$) diet and 5 weeks of Pan02 murine pancreatic cancer growth (flank).
Results By two-way ANOVA, diet did not ($p=0.58$), but body weight, significantly influenced tumor weight ($p=0.01$). Tumor weight correlated positively with body weight ($R^2=0.562$; $p<0.001$). Tumors in overweight mice were twice as large as those growing in lean mice (1.2 ± 0.2 g vs. $0.6\pm.01$ g, $p<0.01$), had significantly fewer apoptotic cells than those in lean mice (0.8 ± 0.4 vs 2.4 ± 0.5; $p<0.05$), and greater adipocyte volume (3.7 vs. 2.2 %, $p<0.05$). Apoptosis ($R^2=0.472$; $p=0.008$) and serum adiponectin correlated negatively with tumor weight ($R=0.45$; $p<0.05$).
Conclusions These data suggest that body weight, and not high-fat diet, is responsible for accelerated murine pancreatic cancer growth observed in this model of diet-induced obesity. Decreased tumor apoptosis appears to play an important mechanistic role in this process. The concept that decreased apoptosis is potentiated by hypoadiponectinemia (seen in obesity) deserves further investigation.

Keywords Obesity · Pancreatic cancer · Diet-induced obesity · Adipokines · High-fat diet

Background

Obesity is a well-accepted risk factor for development and accelerated growth of numerous cancers, including pancreatic cancer.[1] Multiple mechanisms by which obesity influences cancer development and growth are currently being investigated actively. For example, the hormonal, inflammatory, and immunological changes observed in the obese state all likely contribute in part to altered tumor biology.[2] In addition, the specific influence dietary fat (that is to say both percentage as well as type of fat) has been an area of our group's active interest.[3]

We have developed a model of murine pancreatic cancer in obesity using congenitally obese mice and flank-implanted murine pancreatic cancer cells.[2] In this model, congenitally obese mice developed larger pancreatic tumors, more metastases, and increased mortality compared to lean animals. Similar results were observed in a diet-induced obesity model: Overweight animals developed significantly larger tumors than lean animals.[3] A question raised by these studies was whether accelerated tumor growth seen in obese animals was due to diet or body habitus. Therefore, the current study was undertaken to evaluate the hypothesis that obesity, rather than high-fat diet is responsible for accelerated pancreatic cancer growth.

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Materials and Methods

Animals and Diets All experiments were approved by the Indiana University Animal Care and Use Committee. Thirty male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in standard conditions. At 5 weeks of age, 20 mice were fed a high-fat diet (20 % protein, 20 % carbohydrate, and 60 % fat), and 10 mice were fed a low-fat diet (20 % protein, 70 % carbohydrate, and 10 % fat; Research Diets, Inc., New Brunswick, NJ, USA; D12492 and D12450B).

Tumor Model Our tumor model was used as previously described.[2,3] Briefly, 19-week-old mice were inoculated in the flank with 2.5×10^5 PAN02 murine pancreatic cancer cells suspended in 150 μl of phosphate-buffered saline. Mice were monitored daily; mouse weight and tumor size were recorded weekly. Tumor volume was calculated as previously described.[2,3] After 5 weeks of tumor growth, tumors were carefully dissected from the surrounding tissue, weighed, and measured. Serum was collected. The tumor was preserved in 10 % formalin for subsequent histologic evaluation. Spleens of 18 mice were collected for flow cytometry.

Tumor Biology Tumor sections (5 μm) were stained for proliferation [proliferating cell nuclear antigen (PCNA); Invitrogen, Carlsbad, CA, USA) and apoptosis (Apoptag peroxidase, Millipore, Billerica, MA, USA). The number of positively stained cells per 10 high-powered fields (HPF, ×400) of each tumor sample was recorded by three trained observers who were unaware of treatment protocol.

Biochemical Analysis Enzyme-linked immunosorbent assay was utilized to determine serum concentrations of adiponectin, leptin, and insulin (Millipore). Serum glucose was assessed by colorometric assay (Stanbio Laboratory, Boerne, TX, USA).

Flow Cytometry Splenic lymphocytes were dissociated by mechanical injection with Hank's balanced salt solution (Invitrogen), centrifuged for 5 min at $600 \times g$, and resuspended. Red cells were lysed, and 100-μl aliquots (5×10^5 cells) were incubated for 20 min with antibody against helper T cells (CD4$^+$), cytotoxic T cells (CD8$^+$), total T cells (CD3$^+$), and total B cells (CD45R$^+$/B220$^+$); antibodies were obtained from Becton Dickinson Biosciences (San Jose, CA, USA). Flow cytometry was performed with the Epics Profile II flow cytometer (Coulter Corp, Hialeah, FL, USA) and analyzed with the Corel Quatro Pro 8 program (Orem UT).

Tumor Microenvironment Formalin-fixed tumor cells were stained with hematoxylin and eosin for analysis of the tumor
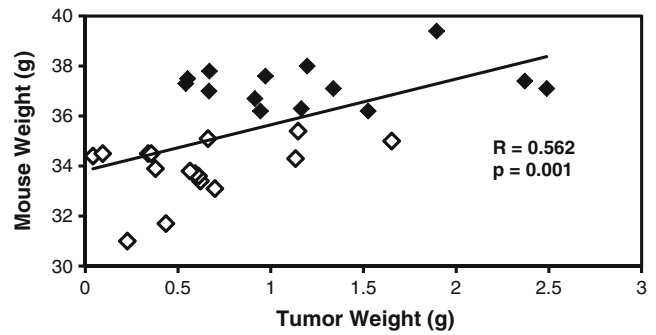

Fig. 1 In all mice, a strong positive correlation was observed between tumor weight and mouse body weight. Lean mice (*empty diamond*); overweight mice (*filled diamond*) ($p=0.0012$)

adipocyte histology. Three observers blinded to treatment group estimated the percentage of tumor occupied by adipocytes in 10 HPF per tumor section (×200 magnification). Tumor-infiltrating lymphocytes were assessed by immunohistochemistry, as previously described.[3] Two observers unaware of treatment protocol identified tumor infiltrating lymphocytes in 10 HPF per specimen using a 0–4+ quantitative scale.

Statistics Data are reported as mean±standard error of the mean. All statistics were performed using the SigmaStat software package (Jandel, San Rafael, CA, USA). Two-way ANOVA, Student's *t* test, Wilcoxon rank sum, and Pearson's correlation were applied as appropriate. A $p<0.05$ was accepted as statistically significant.

Results

Definition of Overweight and Lean At sacrifice, high-fat diet (HFD) mice weighed 35.4±0.4 g and low-fat diet (LFD) mice weighed 35.7±0.8 g ($p=0.94$). HFD mice consumed 3.4±0.07 g of chow per mouse daily, while LFD mice consumed 3.3±0.3 g of chow per mouse daily. Because of the increased calories in the HFD compared to the LFD (7 vs. 4.5 kcal/g), the HFD mice received 23.6 kcal/mouse/day compared to 14.7 kcal/mouse/day in the LFD mice. Mice were segregated by weight for
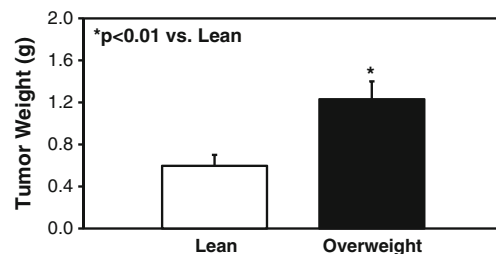

Fig. 2 Tumors growing in overweight mice ($n=14$) weigh significantly more than tumors growing in lean mice ($n=16$)
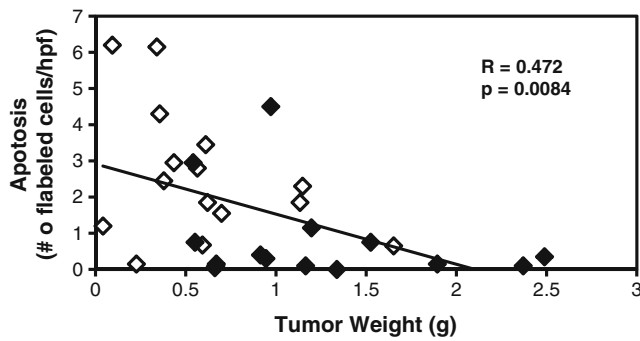
Fig. 3 A significant negative correlation was observed between tumor weight and apoptosis [lean mice (*empty diamond*); overweight mice (*filled diamond*)]
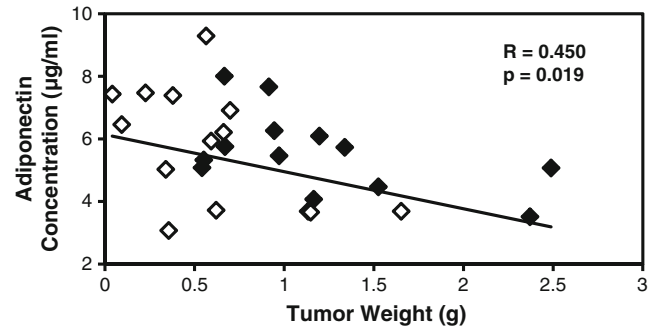

Fig. 4 A significant negative correlation was observed between tumor weight and circulating adiponectin [lean mice (*empty diamond*); overweight mice (*filled diamond*)]

further analysis as we have previously described.[3] Overweight (OW) was defined as weighing greater than the mean weight of the HFD mice ($\geq$35.4 g) and lean as weighing less than the mean weight of HFD mice. After this division, OW mice ($n=14$) weighed 37.3 ± 0.2 g and lean mice ($n=16$) weighed 33.9 ± 0.3 g ($p<0.001$). Nine of the mice in the OW group and 11 of the mice in the lean group were fed the HFD.

Body Weight, Not Diet, Affects Tumor Growth As we have previously observed with the diet-induced obesity model,[3] tumor weight correlated strongly and positively with body weight (Fig. 1; $R^2=0.562$; $p<0.001$). Tumors in OW mice were twice as large as those growing in lean mice (1.2 ± 0.2 g vs. 0.6 ± 0.1 g, $p<0.01$; Fig. 2), a difference that remained significant even when considering tumor weight as a percentage of body weight: Tumors in OW mice weighed 3.3 ± 0.4 % of body weight and tumors in lean mice weighed 1.7 ± 0.3 % ($p<0.01$).

To address the question of whether diet or body weight was responsible for these observed differences, two-way ANOVA was performed on tumor weight, using diet (HFD vs. LFD) and body weight (OW vs. lean) as independent variables. Diet did not influence tumor weight ($p=0.58$), but body weight significantly influenced tumor weight ($p=0.01$). No combined effect on tumor weight was observed.

Overweight mice gained significantly more weight compared to lean animals during the time of tumor inoculation until sacrifice (2.5 ± 0.5 vs 1.2 ± 0.3 g).

Tumor Biology Tumor proliferation was measured by the number of PCNA-labeled cells; proliferation in tumors of OW and lean mice was equivalent (139 ± 18 vs. 143 ± 14, $p=0.845$). In contrast, tumors growing in OW animals demonstrated significantly fewer apoptotic cells than those growing in lean mice (0.8 ± 0.4 vs. 2.4 ± 0.5; $p=0.01$). A significant negative correlation was observed between apoptosis and tumor weight ($R^2=0.472$; $p=0.008$; Fig. 3).

Serum Biochemistry Serum biochemistry results are presented in Table 1. The circulating concentration of glucose, insulin, leptin, and adiponectin was similar in OW and lean groups. Circulating adiponectin showed a strong and significant negative correlation with tumor weight ($R^2=-0.45$, $p=0.02$; Fig. 4). No significant correlation was observed between serum glucose, insulin, leptin, adiponectin, and either apoptosis or proliferation.

Peripheral Lymphocyte Profile Flow cytometry results are presented in Table 2. The peripheral (splenic) lymphocyte distribution [specifically: total T (CD3+), helper T (CD4+), cytotoxic T (CD8+), and B (CD45R+/B220+)] was similar in OW and lean mice.

Table 1 Serum biochemistry

	Insulin (ng/ml)	Glucose (mg/dl)	Leptin (ng/ml)	Adiponectin (µg/ml)
Overweight	0.27 ± 0.12	345 ± 40	5.20 ± 1.18	5.58 ± 0.35
Lean	0.28 ± 0.07	318 ± 18	4.01 ± 0.85	5.71 ± 0.51
p value	0.84	0.54	0.47	0.83

Table 2 Peripheral lymphocyte profile

	Helper T (%)	Cytotoxic T (%)	Total T Cells (%)	Total B cells (%)
Overweight	9.4 ± 0.7	6.0 ± 0.5	51.2 ± 2.5	32.9 ± 1.0
Lean	9.6 ± 0.6	6.4 ± 0.4	49.2 ± 2.3	32.0 ± 1.9
p value	0.8	0.5	0.6	0.7

Tumor Microenvironment Intratumoral adipocytes occupied a significantly greater percentage of the tumor microenvironment in tumors growing in OW mice relative to those growing in lean mice (3.7±0.7 vs. 2.2±0.3 %, $p<0.05$; Fig. 5). Tumor infiltrating T and B lymphocytes were observed in tumors from both overweight and lean mice in similar number (T lymphocytes, 1.31±0.18 vs. 1.54±0.16, $p=0.35$; B lymphocytes, 0.63±0.08 vs. 0.91 ±0.15, $p=0.33$).

Discussion

Perhaps the most important finding of this study is that animal body weight, and not high-fat diet, is responsible for the increased tumor growth seen in this diet-induced obesity model of pancreatic cancer. Tumor weight correlated significantly with animal weight, and tumors in "overweight" (OW) mice grew significantly larger than those in lean animals. Apoptosis was significantly decreased in tumors from OW mice, and apoptosis correlated inversely with tumor weight. Interestingly, the circulating concentration of the anti-inflammatory and antiapoptotic adipokine adiponectin demonstrated a significant inverse correlation with tumor weight. Overweight and lean mice manifest an equivalent peripheral (splenic) lymphocyte profile. Similar to our prior observations, tumor infiltrating T and B lymphocytes were present in tumors from both OW and lean mice. Finally, tumors growing in OW mice demonstrated significantly greater adipocyte volume in the tumor microenvironment relative to tumors growing in lean animals.

These experiments represent an extension of our ongoing work in a diet-induced obesity model of murine pancreatic cancer. Our primary goal was to determine whether high-fat diet or obesity (body weight) played a more important role in accelerated pancreatic cancer growth. The relationship between high-fat diet and obesity is complex and intimately intertwined; it is not surprising that experimental evidence addressing these indices in cancer is heterogeneous. For example, Boylan and Cohen[4] observed that high-fat diet did not affect murine mammary tumor size or development of lung metastasis, though this experimental diet contained only 23%fat. More recently, Ray *et al.*[5] reported that high-fat diet, but not increases in serum leptin, accelerated murine mammary tumor development. Furthermore, Cleary and colleagues demonstrated that obesity-susceptible mice (fed a 33 % fat diet) had decreased latency to spontaneous development of mammary tumors relative to obesity-resistant mice fed this high-fat diet and lean animals fed a chow (low fat) diet.[6] Finally, similar results were seen in a model of spontaneous pancreatic cancer: High-fat diet fed obese mice developed an increased number of precursor lesions (PanIN) compared to lean mice fed a chow diet.[7]

In our model of diet-induced obesity, mice gained only a modest amount of weight. It is possible that tumor cachexia played a role in this observation—at the time of sacrifice, half of our animals had lost weight from their peak weight. On the other hand, the concept of obesity-prone and obesity-resistant strains of rodent is well established,[8,9] and these factors clearly extend to tumor development and progression. For example, Hill-Baskin and colleagues[10] showed that C57BL/6 mice were susceptible to developing obesity, steatohepatitis, and hepatocellular carcinoma when fed a high-fat diet, changes that were not observed in A/J mice fed the same diet. Thus, the type of fat and percentage of fat present in a high-fat diet, in addition to rodent strain, all clearly influence tumor biology.

Our diet was chosen to maintain internal consistency with previously conducted studies from our laboratory. Establishing a standard for type and percentage of fat in

Fig. 5 Representative photomicrographs (×20) demonstrating adipocyte volume in lean (**a**) and overweight (**b**) mouse tumors. Tumors from overweight mice had significantly greater adipocyte volume compared to those growing in lean mice (3.7 ±0.7 % vs. 2.2±0.3 %; $p<0.05$)
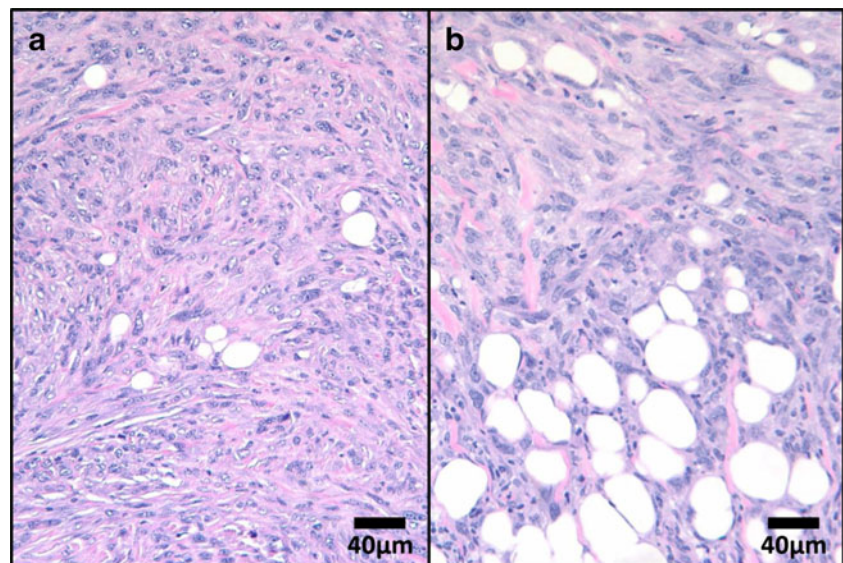
a "high-fat diet" presents challenges, but obviously would be helpful in interpreting results among various laboratories. The fact that moderate weight gain (over-weight) in our model clearly correlates with increased tumor size provides strong support for the concept that obesity in itself contributes to enhanced cancer growth. A logical next step would be to evaluate the effects of high- and low-fat diet on tumor growth in obese but stable weight mice.

The current experiments clearly show that decreased apoptosis is present in larger tumors growing in overweight mice. In addition, apoptosis correlated negatively with tu-mor weight, suggesting that increased tumor size is related to those decreases in apoptosis. In our previous diet-induced obesity experiments, we observed a twofold decrease in apoptosis in tumors growing in overweight mice, though this difference did not quite reach statistical significance.[3] Interestingly, larger sized tumors growing in congenitally obese mice did not demonstrate any difference in apoptosis relative to tumors growing in lean control animals,[2] again highlighting potential biological differences present in various experimental models.

The mechanisms by which obesity affects cancer growth are most certainly multifactorial. Recently, increasing atten-tion has been directed toward understanding the role of adipokines in this process.[11] The adipokine adiponectin is generally an anti-inflammatory molecule and, though pro-duced by adipocytes, paradoxically decreases with increas-ing obesity.[12,13] The role of adiponectin in tumor biology is emerging; while one in vitro study documented increased proliferation in a colon cancer cell line,[14] the vast majority of studies show that adiponectin has an antiproliferative effect.[15–18] Adiponectin receptors are present in the murine pancreas[19] as well as on Pan02 and human pancreatic cancer cell lines (unpublished data). In the current experiments, circulating adiponectin correlated negatively with tumor weight. This observation is in accordance with our previous finding that adiponectin correlated negatively with pro-liferation in pancreatic tumors growing in congenitally obese mice.[2] Adiponectin has been shown to influence apo-ptosis in some tumor models[18]; in fact, recent elegant work suggests that apoptosis modulation (through sphingolipid pathways) may be the common mechanism responsible for many of adiponectin's pleiotropic actions.[20] Further investiga-tion is clearly indicated to validate the intriguing possibility that adiponectin-mediated apoptosis influences accelerated pancreatic cancer growth in obesity.

Another logical link between obesity and altered tumor initiation/progression relates to the immunologic perturba-tion seen in obesity. Obesity clearly alters both immune profile and immune function,[21,22] and the immune system's influence on tumor biology is well recognized.[23] Interestingly, we have observed changes in B-lymphocyte number and function in both congenital and diet-induced

models of pancreatic cancer (unpublished data, manuscript under review). In the current study, no changes were seen in the peripheral immune profile, though it is likely that the small degree of weight gain in the overweight group was too little to cause a significant change. On the other hand, both T and B lymphocytes were observed in the tumor microenvironment, validating our previous observations as well as raising the question of the B lymphocytes' role in tumor immunity.

We observed a significantly greater volume of adipocytes in the tumor microenvironment of tumors growing in over-weight animals compared to those growing in lean mice. We have previously reported the observation of increased adi-pocyte volume in tumors growing in congenitally obese mice,[2] as well as the correlation between tumor microenvi-ronment adipocyte volume and tumor proliferation.[3] The concept that tumor microenvironment influences directly pancreatic cancer growth and metastases has been well established.[24] The idea, however, that adipocytes may im-pact the tumor microenvironment is novel, but quite logical considering the fact that adipocytes are biologically active cells, secreting over 100 proteins.[25] Definition of the local paracrine cytokine/adipokine milieu in the pancreatic cancer microenvironment clearly deserves further investigation.

In summary, these data suggest that body weight, and not a high-fat diet, is responsible for the accelerated pancreatic cancer growth seen in obesity. Furthermore, decreased tumor apoptosis in tumors growing in obese mice appears to play an important mechanistic role in this process. The concept that decreased apoptosis is potentiated by hypoadiponectinemia (as ubiquitously seen in obesity) certainly deserves closer scrutiny. Further expanding our understanding of these metabolic underpinnings holds the real potential to identify novel therapeutic targets for treating pancreatic cancer.

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