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Obesity Provides a Permissive Milieu in Inflammation-Associated Carcinogenesis: Analysis of Insulin and IGF Pathways

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Summary

Current dogma suggests that the positive correlation between obesity and cancer is driven by white adipose tissue that accompanies obesity, possibly through excess secretion of adipokines. However, recent studies in fatless A-Zip/F-1 mice, which have undetectable adipokine levels but display accelerated tumor formation, suggest that adipokines are not required for the enhanced tumor development. The A-Zip/F-1 mice are also diabetic and display elevated circulating levels of other molecules frequently associated with obesity and carcinogenesis: insulin, insulin-like growth factor-1, and inflammatory cytokines. Therefore, we postulate that the pathways associated with insulin resistance and inflammation, rather than adipocyte-derived factors, may represent key prevention or therapeutic targets for disrupting the obesity-cancer link.

Keywords

Cancer; Diabetes; Lypodystrophy; Lipoatrophy; Insulin; Inflammation

1. Introduction

Obesity is a well-established risk factor for several cancers. Estimates from an American Cancer Society study, the largest prospective analysis to date of the weight/cancer relationship, suggest that 14% of all cancer deaths in men and 20% of all cancer deaths in women from a range of cancer types are attributable to excess body weight (1). According to the Centers for Disease Control and Prevention (CDC), the prevalence of obesity among US adults continues to rise every year, thus it is likely that the harmful impact of obesity on cancer will continue to rise as many of these obese individuals age (2). In fact, in some European countries, there has been a clear increase in the incidence of cancers linked to obesity (3).

The mechanism through which obesity increases cancer risk remains unknown. Some of the hypotheses that have been proposed to explain the association of obesity with cancer propose that excess body fat in obese individuals may increase the risk of breast cancer through: (1) excess production of biologically active adipokines (4), (2) the induction of insulin resistance (5), or (3) the promotion of inflammation (6). There is also evidence suggesting that obesity may alter the risk of breast cancer through hormonal factors, such as estrogen and insulin-like growth factor-1 (IGF-1) (7, 8). Unfortunately, the contribution of these proposed factors have on cancer development have not been well delineated.

Contrary to these proposed hypotheses, our recent studies using the A-Zip/F-1 mice (9) that have no white adipose tissue and undetectable leptin, adiponectin and other adipokines, showed that these mice are actually more susceptible to papilloma formation in a classical two-stage skin carcinogenesis experiment; these findings have been confirmed in a separate report (10, 11). Furthermore, A-Zip/F-1 mice crossed to the C3(1)/T-antigen mammary tumor transgenic mouse model developed large tumors at an accelerated rate. (10). Taken together, these findings cast a doubt on previous studies implicating adipokines in cancer risk and indicate that adipokines are not required for enhancement of tumor development. Despite their lack of white adipose tissue, the A-Zip/F-1 mice are diabetic with high circulating levels of insulin, IGF-1, and pro-inflammatory cytokines characteristic of diet-induced obesity. In addition, these mice have activation of several carcinogenesis-related signaling pathways, particularly those downstream of the insulin and IGF-1 receptors and the ErbB/ras and PI3K/Akt pathways (10).

The A-Zip/F-1 mice have elevated levels of several cytokines, including IL-1- α , IL-4, and IL-6; however, TNF- α was not elevated. It is important to note that IL-4 is the prototypical Th2-type cytokine. A Th2-type immune response typically will result in reduced inflammatory monokines such as IL-1- α and TNF- α ; however, Th2-type inflammation, which is particularly prominent in allergic and fibrotic diseases, is mediated in part through alternative cytokine effectors such as IL-13 (12). IL-1- α and TNF- α mediated inflammation is typically generated from a Th1-type T cell response that is largely dependent upon IFN- γ secretion (13). Finally, IL-6 has been shown recently to be a crucial molecule for promoting inflammation: that is, IL-6 is necessary for the induction of Th17 cells that secrete the proinflammatory molecule, IL-17 (14).

In short, these data suggest that the A-Zip/F-1 mice express an inflammatory state of mixed phenotype characterized by increases in Th2 biology (IL-4), Th17 biology (IL-6), and Th1 biology (increased IL-1- α). Further experiments will need to be performed to better characterize this relatively complex inflammatory state. We speculate that the mixed pattern of inflammation may reflect a longitudinal disease process whereby Th1 cells are initially activated, with subsequent regulation of Th1-mediated inflammation by a Th2 cell response; this type of Th1→Th2 inflammatory evolution appears to be operational in other disease states, such as graft-versus-host disease (GVHD) (15). On the basis of these results our central hypothesis is that insulin resistance and inflammation, rather than adipose tissue and its myriad of secreted factors, are the key targets for disrupting the obesity-cancer link.

Possible break

Obesity is associated with insulin resistance, which is a state of reduced tissue responsiveness to the physiological actions of insulin. Consequently, this results in a compensatory rise in plasma insulin; such obesity-induced insulin resistance increases the likelihood of developing Type 2 diabetes. Both insulin resistance and Type 2 diabetes have been linked to increased cancer risk (8, 16). In animal studies we have shown that both IGF-1 and insulin are elevated in obese mice (17). IGF-1 is a polypeptide with 70 amino acids (18) that is involved in cell proliferation, differentiation, and apoptosis of many cancer cells (19–21). In circulation, about 70–80% of the IGF-1 is found in a high molecular weight

complex with IGFBP-3 and an acid-labile subunit (18). Approximately 20% of the IGF-1 is associated with other systemic IGFbps and less than 5% of the total IGF-1 circulates unbound (18). Because both IGF-1 and insulin are elevated in obese mice, it is unclear if the increased cancer risk associated with obesity is due to the tumor-enhancing effects of insulin via the insulin receptor on cancer cells or, alternatively, due to the indirect effects of insulin via the stimulation of IGF-1. With respect to body fat in women, evidence shows that IGF-1 levels correlate directly with BMI in lean subjects (BMI up to 25); whereas, at higher BMI levels, this relationship is inverse (22, 23). As a result, the exact relationship between obesity, IGF-1, and cancer is unclear. However, in some cancers such as breast cancer, the mammary gland is surrounded by adipose tissue, and adipose tissue is the second major source of IGF-1 (24), thus it is likely that the mammary tissue of obese women may be exposed to higher levels of IGF-1 than that of lean women. Others have shown that mice with low IGF-1 levels are less susceptible to mammary and colon cancer (25–27) and others have shown that supplementing IGF-1 to animals with low IGF-1 reverses cancer susceptibility (26, 27).

We have examined the effects of high fat diet on several serum markers in mice. In rodents insulin resistance can be measured using the insulin-tolerance test (ITT) and glucose-tolerance test (GTT). The GTT assays measure how quickly injected glucose is cleared from the blood (Fig. 1A). The ITT assays test how quickly endogenous glucose is cleared from the blood in response to insulin administration (Fig. 1B). Growth factors such as IGF-1 can be measured using commercially available Radio Immuno Assays (RIA) (Fig. 2A). IGF-1 binding proteins (IGFBPs) in the serum of mice can be measured using the ligand assay (18) (Fig. 2B). Insulin along with other adipokines can be measured using the Luminex-based bead array method using a LINCplex simultaneous multianalyte detection system (Linco Research, Inc., St. Charles, MO).

2. Materials

2.1. Glucose Tolerance Test (Fig. 1a)

1. Glucose (D-glucose from Mallinckrodt #4912).
2. Glucometer Elite (Fisher cat #23-021402).
3. Glucometer Elite Strips (Fisher cat #23-025711).
4. Capillary tubes (Fisher cat #22-362574).
5. ½-cc Insulin syringe.
6. Timer.
7. Balance.

2.2. Insulin Tolerance Test (Fig. 1b)

1. Insulin (Humulin (Lilly HI-210)).
2. Glucometer Elite (Fisher cat #23-021402).
3. Glucometer Elite Strips (Fisher cat #23-025711).

4. Capillary (Fisher cat #22-362574).
5. ½-cc Insulin syringe.
6. Timer.
7. Balance.

2.3. IGF-1 Measured Using a RIA Kit (Fig. 2a)

1. Mouse/rat IGF-I standards.
2. Control (low and high IGF-1).
3. Mouse IGF-I¹²⁵ radioactive labeled with 125 iodine.
4. Mouse IGF-I Antiserum.
5. Precipitating reagent.
6. Extraction solution.
7. Neutralizing solution.
8. Mouse/rat IGF-I control.
9. Gamma counter.

2.4. IGF-1 Binding Proteins Measured by Western Ligand Blotting Assay (Fig. 2b)

1. Western blotting assay reagents.
2. I¹²⁵-IGF-I.
3. Loading buffer in nonreducing conditions.
4. 2 µl of serum.

2.5. Insulin, Adipokines, and Cytokines Measured by a Luminex-Based Bead Array Assay (Table 1)

1. Beads coupled with capture antibody.
2. Detection antibody.
3. Standard.
4. Quality controls.
5. Serum diluent.
6. Bead diluent.
7. Streptavidin-PE.
8. Assay buffer.
9. Wash buffer.
10. 96-Well microtiter filter plate.

3. Methods

3.1. GTT and ITT Assays

The GTT is carried out after an overnight fast of the animals, usually 10–15 h of food deprivation (17). The next day mice are injected with 20% glucose (2 g/kg). Blood glucose levels are measured at baseline (0 min), then at 15, 30, 60, and 120 min using the Glucometer Elite. Approximately half drop of blood is placed into each Glucometer Elite Strip. Blood is collected from the mouse tail. Figure 1A plots from a GTT assay carried out in mice fed a low fat (5% fat) diet and a high fat (35%) diet for 20 weeks. In this study the final body weight of the mice consuming the low fat diet was 26 ± 2 g and the final body weight for mice consuming the high fat diet was 36 ± 3 g. The composition of the low and high fat diets has been previously published in (17). The GTT results in Fig. 1A show that mice consuming the high fat diet had impaired glucose tolerance.

The ITT is carried out after 6–7 h fasting of the mice (17). After depriving the mice of food, they are injected with insulin (0.75 U/kg). Glucose is measured in a similar fashion as the GTT at baseline (0 min), then at 15, 30, 60, and 120 min using the Glucometer Elite. As with the GTT, approximately half drop of blood is placed into each Glucometer Elite Strip. The ITT data plotted in Fig. 1B come from the same mice in which the GTT was performed.

3.2. Serum IGF-1 and IGFBPS

Diagnostic Systems Laboratories, Inc. sells commercially available RIA kits to measure IGF-1 in the serum of mice and rats (10). The DSL-2900 Mouse RIA Kit contains sufficient reagents for 100 tubes. There are other nonradioactive kits but we consider this the best kit with the best sensitivity. The DSL kit measures IGF-1 in serum. A step by step method can be found at <http://www.dslabs.com/home/default.aspx>. Serum is collected from nonfasted deprived mice. The serum can be stored at 2–8°C for up to 24 h and frozen at –20°C or lower for up to 6 months. Once the serum is stored at –20°C or lower, avoid repeated freezing and thawing of samples. When collecting the serum avoid inducing hemolysis of the red blood cells, since hemolysis can interfere with the measurement of IGF-1. Before measuring IGF-1 in the serum, IGF-1 needs to be extracted with the solution provided in the kit. The extraction procedure yields approximately 90–100%. The extracted samples are then used in the RIA assay.

Unknown sample concentrations are calculated using a log-linear fit standard curve. Using this assay we calculated the IGF-1 levels plotted in Fig. 2A, in mice consuming a low and a high fat diet. The figure shows that obese mice have higher serum levels of IGF-1 than mice consuming low fat diet.

Serum IGFBPs levels can be analyzed by Western ligand blotting assay (18) using I¹²⁵-IGF-I (Amersham Life Science, Buckinghamshire, UK); for this 2 µl of serum are mixed with 2 µl of protein-loading buffer in nonreducing conditions and boiled for 3 min. Serum samples are then separated on 4–20% gradient SDS-PAGE followed by Western blotting. Membranes are then blocked with tris-buffered saline (TBS) 1% bovine serum albumin and incubated with I¹²⁵-IGF-I (1.5×10^6 cpm in TBS 0.1% tween-20) overnight at 4°C followed

by three washes of TBS 0.1% Tween-20. Signals can be quantified by phosphoimaging. Figure 2B below shows the four IGFBPs that can be detected in the serum of FVB/N mice.

3.3. Insulin and Adipokines

In mice, new technologies such as the Luminex-based bead array assays allow the measurement of insulin and adipokines in small quantities of serum (10, 17). This is important since usually one can get small serum amount from mice. Numerous vendors sell Luminex-based bead array assays, we prefer kits that are sold by Linco Research, Inc. (St. Charles, MO). Using 10 μ l of serum, one can measure up to seven factors that include, leptin, resistin, insulin, IL-6, MCP-1, PAI-1, and TNF- α . To be able to use this kit a Luminex-based simultaneous multianalyte detection system is needed. Usually these assays are done in a 96-well plate and can be used to measure unknown sample concentrations in ~38 samples in duplicates.

In our work with the fatless A-ZIP/F-1 mice we measured insulin, leptin, resistin, IL-6, MCP-1, PAI-1, and TNF- α serum levels using a Luminex-based bead array from Linco Research, Inc. (St. Charles, MO). Table 1 shows the serum levels of these factors. TNF- α was not detectable in the serum of A-ZIP/F-1 mice. However, as expected, circulating fat-derived adipokines, such as leptin and resistin were undetectable or very low in A-ZIP/F-1 mice. In contrast, they were detectable in wild mice. A-ZIP/F-1 mice are diabetic, hence the high levels of insulin.

4. Notes

1. It is essential that in the GTT assay the mice be deprived of food for at least 10 h.
2. For the ITT, mice can be food deprived for 7 h.
3. For both the GTT and ITT it is recommended that each experimental group has at least ten animals per group; we have found that this numbers gives the best results.
4. Fasting decreases systemic IGF-1 levels in the serum of mice, thus we recommend that they be measured in both conditions. This may provide a more adequate picture of the levels of IGF-1 in the mice.
5. IGFBPs are also modified by fasting the mice, thus it is recommended that they be measured in both conditions.
6. Fasting of the A-ZIP/F-1 mice dramatically reduces the insulin levels. For example, during the feed period insulin levels can be between 100 and 200 times higher than after fasting, which Table 1 shows them to be 3.7 ng/ml. Because of the effects of fasting on hormones, we usually measured all these factors in both nonfasting and fasting conditions.

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Fig. 1. Diet-induced obesity led to a state of insulin resistance. **(A)** Shows the result of a GTT assay in mice consuming a low and a high fat diet; obese mice have a state of glucose intolerance. **(B)** Shows the ITT results in the same mice, the figure shows that obese mice are insulin resistant. * $P < 0.05$, is the statistical difference between low fat and high fat mice. Figures taken from (17) with permission.

Fig. 2.

Serum IGF-1 and IGFBPs in FVB/N mice. **(A)** IGF-1 serum levels were measured with RIA kit from DSL. The figure shows that mice consuming high fat diet have higher levels of IGF-1. **(B)** IGFBPs serum levels were measured with Western ligand blotting assay. We have not measured IGFBPs in mice consuming low or high fat diet. However, the figure shows that these proteins can be measured in the serum of mice. **(B)** Taken from (18) with permission. * $P = 0.05$, is the statistical difference between low fat and high fat mice.

Table 1

Serum levels of insulin, adipokines, and cytokines in A-ZIP/F-1 and wild-type female mice. Serum levels of these factors were measured in mice that were fasted overnight and measured with a Luminex-based bead array from Linco Research Inc.

	Wild-type (ng/ml)	A-ZIP/F-1 (ng/ml)
Insulin	0.47 ± 0.15	3.7 ± 0.81 *
Leptin	2.68 ± 1.62	0.03 ± 0.01 *;
Resistin	5.90 ± 0.33	0.11 ± 0.02 *;
Adiponectin	4.35 ± 0.82	0.06 ± 0.01 *;
MCP-1	43.74 ± 6.30	86.99 ± 18.45 *;
IL-6	41.55 ± 4.02	75.31 ± 12.37 *;

* P = 0.05, statistical difference between A-ZIP/F-1 and wild-type mice. Figures taken from (10) with permission