Galectin-3 Stimulates Preadipocyte Proliferation and Is Up-regulated in Growing Adipose Tissue

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Abstract

KIWAKI, KOHJI, COLLEEN M. NOVAK, DANIEL K. HSU, FU-TONG LIU, AND JAMES A. LEVINE. Galectin-3 stimulates preadipocyte proliferation and is up-regulated in growing adipose tissue. *Obesity.* 2007;15:32–39.

Objective: Some cytokines and mediators of inflammation can alter adiposity through their effects on adipocyte number. To probe the molecular basis of obesity, this study determined whether galectin-3 was present in adipose tissue and investigated its effects on fat cell number.

Research Methods and Procedures: In the first study, obesity-prone C57BL/6J mice were fed with high-fat (58%) diet. Epididymal fat pads were collected at Day 0, Day 60, and Day 120 after the start of high-fat feeding.

Results: Levels of adipocyte galectin-3 protein, determined using Western blot analysis, increased as the mice became obese. Galectin-3 mRNA and protein were then detected in human adipose tissue, primarily in the preadipocyte fraction. It was found that recombinant human galectin-3 stimulated proliferation of primary cultured preadipocytes as well as DNA synthesis through lectin-carbohydrate interaction.

Discussion: Galectin-3, which has been known to play a versatile role especially in immune cells, might play a role also in adipose tissue and be associated with the pathophysiology of obesity.

Key words: C57BL/6J mice, diet, obesity phenotypes

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Introduction

Obesity affects one-third of the American population and is associated with substantial comorbidity and mortality in the Western world (1). The manipulation of adipose cell number may be a mechanism through which obesity might be treated. Cytokines have been shown to affect adipocyte proliferation (2). For example, tumor necrosis factor-α induces apoptosis in human adipocytes and preadipocytes (2). Other cytokines may also be present in adipose tissue and affect adipocyte number (3).

Galectin-3 is a member of a growing family of β-galactoside-binding animal lectins (4,5). This 30-kDa protein is composed of a carboxyl-terminal carbohydrate recognition domain and amino-terminal tandem repeats (6,7). Like other galectins, galectin-3 does not contain a classic signal sequence. Consistent with this, it is localized primarily in the cytoplasm but is also present in the nucleus under specific conditions (8). A number of studies, however, have demonstrated secretion of galectin-3 (9). Extracellularly, galectin-3 can exert a number of different functions in a fashion that is dependent on its carbohydrate-binding activity, as demonstrated by the use of recombinant galectin-3 (reviewed in Reference 10). One of the well-established functions of galectin-3 is to stimulate the growth of fibroblasts when added exogenously to cells (11). Because the fibroblast lineage includes adipogenesis, we wondered whether this lectin might be important in adipocyte biology. In other tissues, galectin-3 is up-regulated under diabetic conditions (12,13), and galectin-3 deficiency is associated with increased diabetic glomerulopathy (13,14).

Previous studies have demonstrated a variety of biological activities for this lectin, including activation of cells (15–20), modulation of cell adhesion (21–23), induction of pre-mRNA splicing (24), and regulation of apoptosis (25,26). Galectin-3 normally distributes in the epithelia of many organs and various inflammatory cells, including macrophages, as well as dendritic cells and Kupffer cells (27). The expression of galectin-3 is up-regulated during

inflammation (27), cell proliferation (28,29), and cell differentiation (18,30) and through *trans*-activation by viral proteins (19). Its expression is also affected by neoplastic transformation (19,31–39). Increased galectin-3 expression has also been noted in human atherosclerotic lesions (11). In fibroblast culture, exogenous galectin-3 has been reported to stimulate cell proliferation (40). These findings indicate that galectin-3 expression is affected during these physiological and pathological responses and suggest that galectin-3 may have the capacity to alter adipocyte cell number.

To examine the biological underpinnings of obesity, several genetic models of obesity have been developed in rodents, including *ob/ob* mice. This mouse model is characterized by hyperphagia, increased adiposity, hyperglycemia, insulin resistance, and decreased thermogenesis. Gene expression profiling of adipose tissue from *ob/ob* mice has given us insight into the molecular events important in the development of obesity (41,42). There is a limitation in extrapolating to human obesity, however, because leptin deficiency does not generally account for obesity in humans, which is thought to be a leptin-resistant state (43). The C57BL/6J (B6)[1] mouse is an obesity-prone strain: these mice remain lean and otherwise normal on low-fat diets, but when raised on a high-fat diet, they develop severe obesity (44–46). Additionally, B6 is the background strain in which the *ob/ob* mutation is commonly placed. In the following studies, we investigated the possible role of galectin-3 in adipocytes in B6 mice. To investigate the molecular basis of diet-induced obesity, we fed the B6 mice a high-fat diet and examined galectin-3 protein levels. The ability of galectin-3 to alter proliferation of primary cultured human preadipocytes was examined as well.

Research Methods and Procedures

Animals

Twenty-one male 4-week-old B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and individually housed in cages with a 12:12 hour light:dark cycle at 22 ± 2 °C. Standard laboratory rodent chow (Laboratory Rodent Diet 5001; PMI Nutrition International, St. Louis, MO) and water were allowed ad libitum. From 6 weeks of age (Day 0), all mice were given a high-fat diet (D12331; Research Diets, Inc., New Brunswick, NJ) containing 16.4% protein, 25.5% carbohydrate, and 58.0% fat. Animals were divided into three groups ($n = 7$). The three groups of animals were killed on Days 0, 60, and 120, respectively; their epididymal fat pads were removed and immediately stored at −72 °C in liquid nitrogen. All procedures involving mice were approved by the Mayo Foundation Institutional Animal Care and Use Committee.

Human Subjects

Fat tissue was collected from two subjects during liposuction surgery for management of obesity. Tissues were in a non-malignant condition. The protocol was approved by the Mayo Foundation Institutional Review Board.

Isolation of Adipocytes and Preadipocyte Culture

Human subcutaneous adipose tissue from liposuction was separated into preadipocytes and adipocytes with collagenase digestion and centrifugation. Fat tissue was minced and then digested in Hanks' balanced salt solution containing 1 mg/mL collagenase and 7.5% fetal bovine serum (FBS) in a 37 °C shaking water bath until fragments were no longer visible. Digests were filtered and centrifuged at 800*g* for 10 minutes. Adipocytes were collected from the top layer. The preadipocyte fraction (the bottom layer) was treated with an erythrocyte lysis buffer. The cells were then plated by using Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% FBS at a density of 4×10^4 cells/cm^2 (47).

Western Immunoblotting for Galectin-3

Samples from adipose tissue homogenates, adipocytes, and preadipocytes were prepared using three freeze-thaw cycles, sonication, and lipid separation by floatation. For Western immunoblot analysis, 10 μg of protein were loaded in each lane. The amount of protein was measured using a dye-binding method (Bio-Rad Laboratories, Hercules, CA). The samples and a size marker were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinyidene fluoride membrane (Bio-Rad Laboratories). The membrane was exposed to Ponceau S stain for 5 minutes to confirm efficient protein transfer. After 2 hours of blocking in 3% gelatin in Tris-buffered saline, the membrane was exposed to murine anti–galectin-3 (human/mouse/rat) monoclonal antibody (Research Diagnostics, Flanders, NJ) for 90 minutes and to a secondary antibody conjugated to alkaline phosphatase for 45 minutes. The alkaline phosphatase was detected using a colorimetric method (Bio-Rad Laboratories). In addition to the Ponceau S stain, we used immunostaining for glyceraldehyde 3-phosphate dehydrogenase as a control for the amount of sample loaded.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Human Galectins

Preadipocytes from human adipose tissue were seeded in complete growth medium (DMEM/F-12, 10% FBS) at 1.0×10^4 cells/cm^2. After 24 hours, cultures were subjected to serum starvation by removing the medium, washing twice with phosphate-buffered saline (PBS), and incubating in starvation medium (DMEM/F-12, 0.2% FBS) for 72

[1] Nonstandard abbreviations: B6, C57BL/6J; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; rh-galectin-3, recombinant human galectin-3; BrdU, bromodeoxyuridine.

hours. Cells were stimulated to re-enter the cell cycle by addition of complete growth medium for 18 hours. Cells were harvested before (FBS−) and after (FBS+) the stimulation with complete growth medium. Cultured preadipocyte samples were frozen with liquid nitrogen immediately after collection and stocked at −80 °C. Total RNA was isolated from samples by homogenization (30 seconds), isovolume chloroform extraction, and application of the spun (10,000*g* for 10 minutes) supernatant to RNeasy columns (Qiagen, Valencia, CA). Treatment with DNase I (Qiagen) was done on RNeasy column according to the manufacturer's protocol. RT was carried out on 200 ng of DNase-treated RNA using oligo$(dT)_{20}$ in the presence and absence of reverse transcriptase (ThermoScript RT-PCR system; Invitrogen, Carlsbad, CA). PCR was carried out using 10% of the RT products. The amplification parameters were 94 °C (2 minutes), then 30 cycles at 94 °C (30 seconds), 60 °C (30 seconds), and 72 °C (1 minute). The products were separated using agarose gel (1.5%) electrophoresis, stained with ethidium bromide, and photographed using ultraviolet light. For human galectin-1 and -3, published primer sequences (48) were used. These primers were designed such that the sequence stretch between primer positions included a sizable fraction of the introns to minimize the risk of amplification of residual genomic DNA sequences. The sequences of primers are as follows: galectin-1 (product size, 323 base pairs) sense AACCTGGAGAGTGCCTTCGA, antisense GTAGTTGATGGCCTCCAGGT; galectin-3 (product size, 719 base pairs) sense ATGGCAGACAATTTTTCGCTCC, antisense ATGTCACCAGAAATTCCCAGTT.

Cell Proliferation Assay

Recombinant human galectin-3 (rh-galectin-3) was prepared as described previously (49).

Analysis of DNA Synthesis. Preadipocytes were plated at 6×10^3 per well in DMEM/F-12/10% FBS on 96-well plates and incubated for 24 hours. Subsequently, the cells were washed three times with PBS and serum-starved into quiescence with DMEM/F-12/0.5% FBS for 24 hours. rh-galectin-3 was added at varying concentrations in the presence or absence of 50 mM lactose (a disaccharide competitive for carbohydrate-binding by galectin-3) and further incubated for 48 hours. No non-galectin-binding disaccharides were included as control groups. Cultures were then labeled for 24 hours with bromodeoxyuridine (BrdU) at 10 μM. BrdU incorporation was measured by colorimetric immunoassay (Roche, Mannheim, Germany).

Analysis of Cell Number. Cell proliferation was assayed according to dose- and time-dependence. Cells (2×10^4) were plated in 0.5 mL DMEM/F-12/10% FBS per well of a 24-well plate. After 24 hours, the cells were washed three times with PBS, and the medium was replaced with fresh DMEM/F-12/0.5% FBS. The cells were starved for 24 hours to synchronize the cultures and then stimulated with rh-galectin-3 at varying concentrations in the presence or absence of 50 mM lactose for 7 days. Fresh factor was added every 2 days, and the cells were quantified after trypsinization using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA). Synchronized cells were cultivated with 3 μM rh-galectin-3 in the presence or absence of 50 mM lactose (competitive binding control) for the indicated times (3, 5, and 7 days) and then quantified with a Coulter counter.

Statistical Analysis

Results are expressed as the means ± standard error of the mean. Within-group differences were compared using a paired *t* test, and significance was defined as $p < 0.05$.

Results

We fed 21 male B6 mice with a high-fat diet (58% fat) from 6 weeks of age. The animals were divided into three groups, and their epididymal fat pads were collected at three different time-points: on the first day of the high-fat diet (Day 0, 6 weeks of age), on the 60th day after the start of the high-fat diet (Day 60, 15 weeks of age), and on the 120th day (Day 120, 23 weeks of age). On Days 0, 60, and 120, animals weighed 20.5 ± 0.7 g (standard deviation), 40.0 ± 3.1 g, and 52.1 ± 2.9 g, respectively.

Galectin-3 Protein Expression Levels in Mouse and Human Adipose Tissue

To examine protein levels of this gene, the adipose tissue samples were analyzed by Western immunoblotting. Protein expression levels of galectin-3 also increased as the B6 mice became obese (Figure 1A).

To investigate galectin-3 expression in human adipose tissue, subcutaneous adipose tissue was collected from liposuction surgery. The removed adipose tissue was divided into preadipocyte and adipocyte fractions by enzymatic digestion and centrifugation and then analyzed by Western immunoblotting. Galectin-3 protein was found in human adipose tissue and expressed predominantly in preadipocytes. The band of galectin-3 from the mature adipocyte fraction of human adipose tissue was faint (Figure 1B).

Galectin Gene Expression in Growing and Quiescent Preadipocytes

Preadipocytes from human adipose tissue were cultured in medium containing 10% FBS for 24 hours, and then FBS was withdrawn for 72 hours. Preadipocytes were stimulated to re-enter the cell cycle by addition of FBS for 18 hours. Galectin-1 and -3 gene expression were analyzed by RT-PCR. The band of galectin-3 was very faint on serum withdrawal and became strong on serum addition (Figure 2).

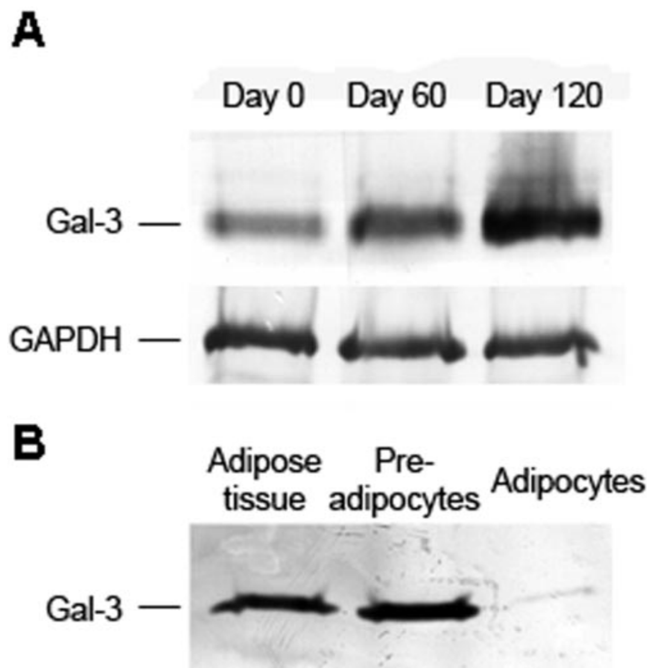

Figure 1: (A) Galectin-3 (Gal-3) protein levels (compared with glyceraldehyde 3-phosphate dehydrogenase, GAPDH) increased during development of diet-induced obesity in adipose tissue of B6 mice. Epididymal fat tissue was collected from B6 mice at Days 0, 60, and 120 after starting a high-fat diet. (B) Galectin-3 protein was found in human adipose tissue, expressed predominantly in preadipocytes.

Stimulation of Preadipocyte Proliferation by rh-galectin-3

To investigate of effect of galectin-3 on preadipocyte proliferation, cells were cultured with rh-galectin-3 in the presence or absence of 50 mM lactose, a disaccharide competitive for carbohydrate binding by galectin-3. Incubation of preadipocytes with rh-galectin-3 stimulated DNA synthesis in a dose-dependent manner, as indexed by BrdU incorporation. Maximal response was observed at 3 μM. On the addition of lactose, the stimulatory effect of rh-galectin-3 on DNA synthesis was significantly reduced (Figure 3). Consistent with the result of DNA synthesis, rh-galectin-3 increased preadipocyte number in a dose-dependent manner, and this effect was significantly attenuated with 50mM lactose (Figure 4A). Figure 4B depicts the time course of preadipocyte proliferation with 3 μM rh-galectin-3. rh-galectin-3 induced cell proliferation progressively.

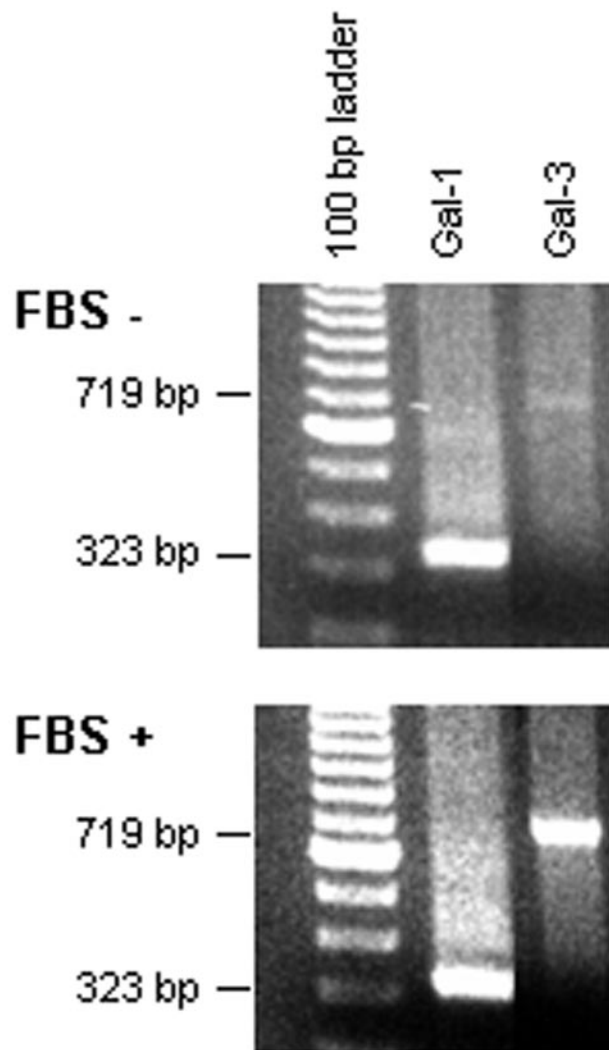

Figure 2: Galectin-1 and -3 gene expression in growing and quiescent preadipocytes. Cultured preadipocytes from human adipose tissue were harvested before (FBS−) and after (FBS+) stimulation to re-enter the cell cycle with complete growth medium. Galectin-3 was expressed in human adipose tissue, predominantly in preadipocytes. The band of galectin-3 was very faint on serum withdrawal and became strong on serum addition. bp, base pairs.

Discussion

Galectins are widely distributed in the body and, accordingly, have several functions. In this study, we demonstrate that both mouse and human adipose tissues synthesize galectin-3 and, moreover, that galectin-3 up-regulation, indicated by increases in both mRNA and protein levels, is associated with tissue growth induced by a high-fat diet. Galectin-3 is expressed predominantly in the preadipocyte fraction in human adipose tissue. In addition, exogenous galectin-3 stimulates cell proliferation in primary human preadipocyte cultures and stimulates DNA synthesis in a dose-dependent manner through lectin-carbohydrate interaction, as it can be significantly inhibited by lactose, a disaccharide competitive for carbohydrate-binding by galectin-3. Taken together, these data suggest that endogenous galectin-3 may play a role in adiposity and, potentially, in obesity.

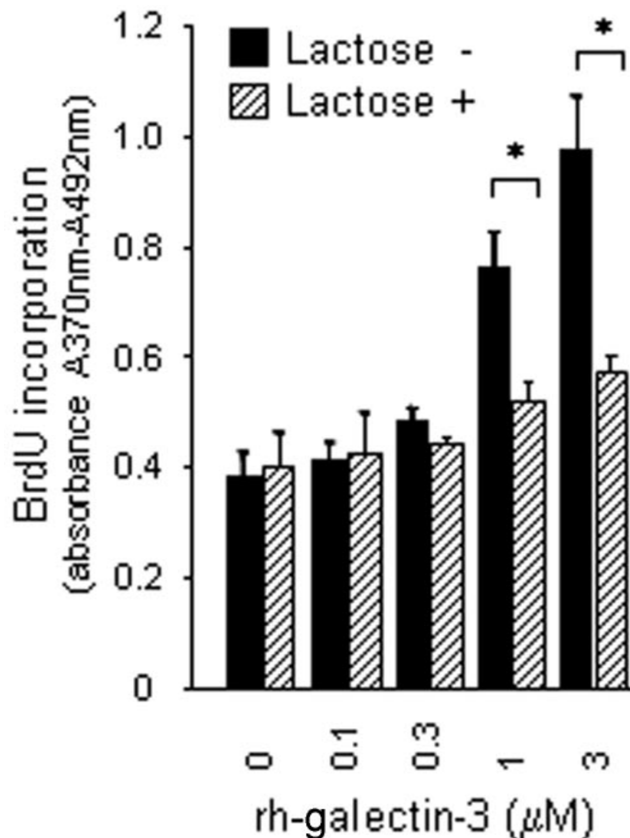

Figure 3: Effect of rh-galectin-3 on DNA synthesis in primary cultured preadipocytes. Synchronized preadipocytes from human adipose tissue were stimulated with rh-galectin-3 of varying concentrations in the presence or absence of 50 mM lactose, and BrdU incorporation was measured (means ± standard error, in triplicate). *, significant difference between lactose+ and lactose−; $p < 0.05$.

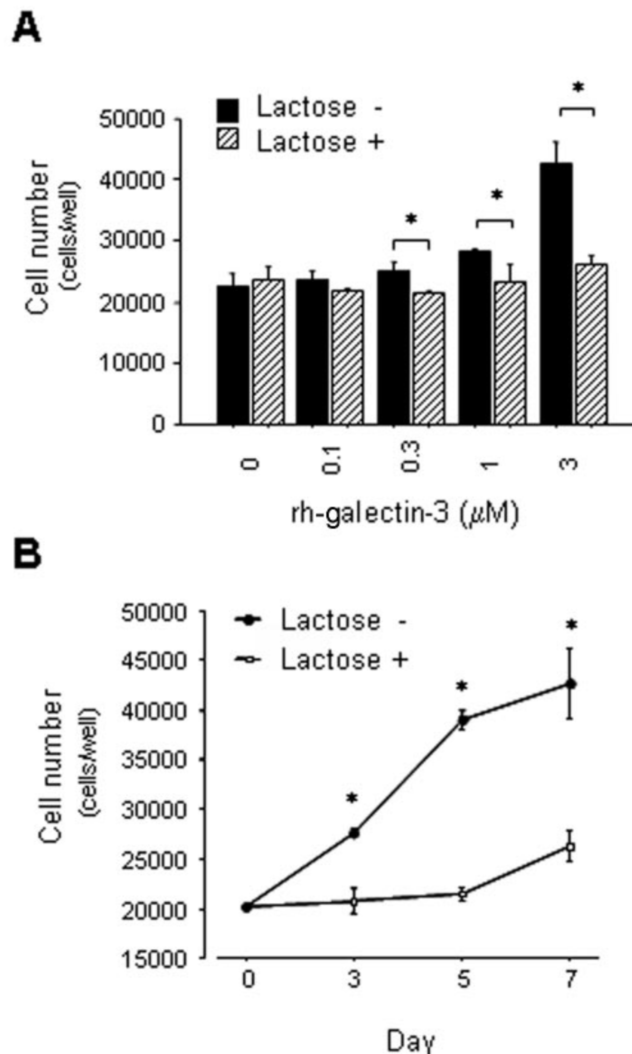

Figure 4: Effect of rh-galectin-3 on proliferation of primary cultured preadipocytes. (A) Preadipocyte proliferation by rh-galectin-3 was concentration-dependent. (B) Time course of rh-galectin-3–induced preadipocyte proliferation. Synchronized cells cultured with 3 μM rh-galectin-3 showed proliferation (increase in cell number) in the absence (lactose−) but not in the presence (lactose+) of lactose. *, significant difference between lactose+ and lactose−; $p < 0.05$.

Galectin-3 expression in adipose tissue showed a progressive increase during development of high-fat diet-induced obesity in B6 mice. Unlike the results of Nadler et al. (42), our data demonstrate that the expression of galectin-3, which increases adipocyte proliferation, is increased in association with obesity. Our results are not surprising, given the role of galectin-3 in cell growth (50) and its anti-apoptotic actions (51). In general, galectins play a role in cell proliferation and have both apoptotic and anti-apoptotic effects (52,53). Galectin-12, which is also expressed in adipocytes, is important in differentiation and is associated with apoptosis in adipocytes (53,54). Adipose tissue is deposited in all mammals and grows by way of increases in adipocyte size (hypertrophy) and number (hyperplasia). Because adipocytes exhibit finite size, the several-fold increase in adipose tissue mass that occurs throughout life is accounted for primarily by hyperplasia. Adipocyte hyperplasia results from the recruitment of new adipocytes from pluripotent precursor cells (preadipocytes), as adipocytes are unable to divide (55,56). Little is known about the mechanism of adipocyte hyperplasia.

Galectin-3 stimulated differentiation of preadipocytes to lipid-laden adipocytes. The concentrations of galectin-3 needed to stimulate cell proliferation were analogous to other activities previously demonstrated for this lectin, such as activation of inflammatory cells (15,17,18) and monocyte migration (57). Thus, these concentrations seem to be consistent for physiological functioning. This is probably related to the concentrations of galectin-3 that are required for the dimerization or oligomerization of the lectin to take

place. Galectin-3 is known to exist at relatively high concentrations in the cytosol of many cell types [e.g., 5 μM in a human colon adenocarcinoma cell line, T84 (58)]. Finally, although we included lactose as a competitive binding control, we did not include a non–galectin-3-binding disaccharide control such as sucrose in our studies on preadipocyte differentiation.

We propose that the mechanism through which galectin-3 mediates preadipocyte proliferation is comparable to the effects of galectin-3 in other cell systems. Inohara et al. (40) reported that galectin-3 was a mitogen capable of stimulating fibroblast cell proliferation in a paracrine fashion through interaction with cell surface glycoconjugates. Moreover, in 3T3 fibroblasts, which can differentiate into adipocyte phenotypes, the levels of both galectin-3 mRNA and protein are greatly up-regulated when quiescent cells are stimulated by serum to reenter the cell cycle; galectin is up-regulated in a manner comparable to other mitogen-activated genes, including the oncogenes c–*fos* and c-*myc* (29). Our current results in human primary preadipocyte cultures are consistent with those reports. Adipocytes are terminally differentiated cells that originate from the stromovascular cells. Serum-derived growth factors are believed to be facultative for adipocyte differentiation. Thus, when looking at adipocyte differentiation as an effect of a given putative mediator, it helps to de-differentiate the cells by starving them of medium. Therefore, it is likely that, in vivo, galectin-3 acts to promote preadipocyte differentiation.

Although mice lacking galectin-3 do not differ in body weight from wild-type mice (59), this does not preclude a role for galectin-3 in weight gain. It is possible that galectin-3 is present inside cells and is secreted from those cells under specific conditions, such as inflammation. Under these conditions, galectin-3 may be released from cells—either adipocytes or other cell types, such as inflammatory cells—and stimulate the growth of adipose tissue.

In summary, galectin-3 is found in adipose tissue, increases in association with obesity, and stimulates adipocyte proliferation. It is not known what role galectin-3 plays in the increase in adiposity associated with obesity. Regardless, cytokines such as galectin-3 may be potential targets for the treatment of obesity.

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