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OVEREXPRESSION OF THE OBESE GENE IN THE GENETICALLY OBESE JCR:LA-CORPULENT RAT

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Expression of the obese (*ob*) gene in JCR:LA-cp rats was examined. A 360 bp fragment of the conserved region of the gene was obtained by RT-PCR using total RNA isolated from adipose tissues of Sprague-Dawley (SD), JCR:LA-cp obese and lean rats. The three gene fragments were sequenced and shown to be identical. They were over 90% identical to the mouse *ob* gene sequence. The amplified fragments encode for 120 amino acids and have a glutamine residue at position +49. The gene was shown to be expressed only in adipose tissues, both white and brown. A ten-fold increase in *ob* mRNA was detected in white adipose tissues of obese animals compared to the lean ones of the JCR-LA:cp strain of rat. *Ob* gene was expressed in adipocytes and preadipocytes from the obese rat whereas in the lean and SD rats, *ob* gene expression was found in adipocytes only. No *ob* mRNA was detected in preadipocytes from the lean or SD rats, indicating a differentiation or maturation-dependent expression in normal rats.

Obesity is a nutritional disorder that affects over 20% of adults in North America (1). It results from a chronic imbalance between energy intake and expenditure (2, 3). The condition is an important public health concern as it is associated with a variety of cardiovascular and metabolic disorders and is a major risk factor for non-insulin dependent diabetes mellitus (NIDDM) (2). The genetic and environmental factors that are involved in the development of obesity have yet to be identified. Recently, Zhang et al. (4) isolated the obese gene, a gene linked to obesity in mice. They showed that the gene is expressed only in white adipose tissues and in a mutated form, is linked to severe obesity and diabetes in mice. The *ob* protein has been shown to induce a reduction in food intake in mice and an increase in their energy use by accelerating their metabolism and making them more active (5, 6, 7). The overall effect is a reduction in body weight and fat content of the animal. The expression of the gene in human fat has also been shown (8, 9) and regional differences in the gene expression within the different fat tissues in an individual have been reported (9). A strong correlation was found between

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ob gene expression and body mass index (BMI), though no mutation was detected in the gene of the obese individuals tested (8). The expression of the gene in rat has not been reported so far. The aim of the present study was to determine if there is any correlation between obesity and the expression of the *ob* gene in genetically obese JCR:LA-corpulent (cp) rats. This is a strain of rat that develops insulin resistance, hyperinsulinemia and hyperlipoproteinemia spontaneously in animals homozygous for the cp gene (cp/cp) whereas heterozygotes (cp/+) and homozygous normal (+/+) remain lean (10). For such analysis, a probe was developed by RT-PCR, using RNA isolated from white fat of Sprague-Dawley rats. The probe was used to determine the expression of the gene in various tissues of both the lean and obese rat. The association of *ob* gene expression with adipocyte differentiation was also examined.

Materials and Methods

Animals

Obese and lean JCR:LA-corpulent rats were bred at the University of Alberta. Sprague-Dawley males were bought from Charles River. After transfer to the animal facilities at the Loeb Institute for Medical Research, the animals were maintained on standard rat chow (Agway, RMH 4018; 5% fat, 18% protein by weight) in wire-bottom cages at 22°C on a 12h light-dark cycle. Three-month-old male rats were sacrificed under light halothane anesthesia. White adipose tissues from the retroperitoneal and epididymal fat pads, brown adipose tissues from the subscapular brown pads and organs were rapidly excised, homogenised in guanidine thiocyanate (GTC) solution and stored at -70°C.

RNA isolation and Northern blotting

Total RNA was extracted by the method of Chomczynski and Sacchi (11). Total RNA (10 µg) was denatured in formamide and formaldehyde at 55°C for 15 minutes and separated by electrophoresis in 1% agarose gels containing formaldehyde. Separated RNA species were transferred onto Zeta probe blotting membrane (Bio-Rad laboratories) using 1X TAE (0.04 M Tris-acetate and 0.001 M EDTA) and baked for 2h at 80°C under vacuum. The blots were hybridised using 5 X 10^6 cpm of probe, generated using the Prime-a-Gene labeling kit (Promega Inc.) and [^{32}P] α-dCTP (3000 Ci/mmol). Hybridisation was carried out overnight at 42°C. A PhosphorImager (Bio-Rad GS-525) was used for quantitation of Northern blots. Autoradiography was carried out overnight at -70°C using Kodak X-ray films with intensifying screens.

Preparation of a rat *ob* cDNA fragment

A 360 bp fragment of the *ob* gene was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of total RNA obtained from adipocytes of SD, JCR-LA:cp corpulent and lean rats. Based on the mouse *ob* gene published sequence (4), two oligonucleotide primers from the conserved region of the gene (sense, 5'-CACCAAAACCCTCATCAAGACC-3' and antisense, 5'-CAGCCTGCTCAAAGCCACCACC-3') were synthesized (Oligonucleotide Synthesis Laboratory, Queen's University). 100 ng of total RNA was reverse transcribed using random hexamers as primer and amplified by PCR using the specific primers, as described previously (4). The fragment was then cloned into the T/A cloning kit (Invitrogen) and sequenced by the dideoxy chain termination method (12), using a sequenase kit (U.S. Biochemical).

Isolation of adipocytes and preadipocytes

Adipocytes and preadipocytes were isolated as previously described (13). Briefly, white adipose tissues from the epididymal and retroperitoneal fat pads were digested with collagenase (1mg/mL; type IV, Sigma) in Hank's balanced salt solution (Gibco) and 5% bovine serum albumin (Fraction V, Sigma) with shaking at 37°C for 45 min. The cell suspension was filtered through a 250-µm mesh Nitex filter (B and SH Thompson and Co. Ltd.) and centrifuged. The floating fat cell layer was used as the source of mature adipocytes. The pellet was resuspended, filtered through a 25 µm mesh filter and recentrifuged. The pellet obtained consisted mainly of preadipocytes. For isolation of preadipocytes from lean and SD rats, two animals were used for each experiment.

Results

Isolation of *ob* gene fragment.

Figure 1 shows a 360 bp gene fragment obtained by RT-PCR using primers derived from the published sequence of the mouse *ob* gene (4) with total RNA isolated from white adipose tissue of the SD rat. The gene fragment was cloned and sequenced in both directions. The sequence (data not shown) was found to be over 90% identical to that of the mouse. The translated sequence encoded 120 amino acids, starting at position 31 up to and including the amino acid at position 150. It has earlier been shown that the *ob* gene product can be one of two variants differing by only one amino acid, a glutamine residue, at position +49 (3,9). Our gene product contains a glutamine residue at position +49, implying a product of 167 amino acids. Similar experiments were carried out using total RNA isolated from adipose tissues obtained from obese and lean animals of the JCR-LA:cp strain. The sequences in both groups were identical to that of the SD rat.

Expression of *ob* gene in different tissues of obese and lean rats.

To establish whether there is a difference in expression of the *ob* gene between the obese and lean animals of the JCR-LA:cp strain, Northern blotting analysis, using the cloned rat cDNA fragment as probe, was carried out. An RNA species of 4.5 kb was detected in adipose tissues only (Fig. 2). No signal was detected in any other organ examined

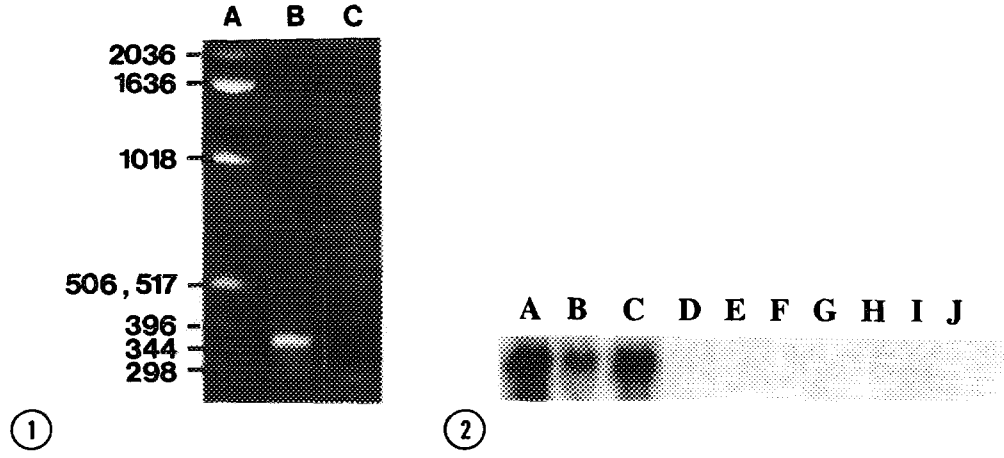

Figure 1. Amplification of *ob* gene fragment of SD rat. RT-PCR were carried out using random hexamers for first-strand cDNA synthesis and specific primers (sense 5' CACCAAAACCCTCATCAAGACC-3' and antisense 5' CAGCCTGCTCAAAGCCACCACC-3') for the PCR on 100 ng of total RNA isolated from white adipose tissues of SD rat (Lane B) or H_2O only (Lane C). Lane A shows part of the 1 Kbp ladder marker.

Figure 2. *Ob* gene expression in tissues and organs of JCR-LA:cp obese and lean rats. Northern blot analysis of total RNA was carried out using a fragment of the *ob* gene as probe. Total RNA was isolated from white adipose tissues (A & C), brown adipose tissues (B & D), liver (E & F), muscle (G & H) and testis (I & J) obtained from obese (A, B, E, G and I) and lean rats (C, D, F, H and J). 10 μg of total RNA was loaded in each lane and ^{32}P-labelled *ob* gene fragment was added at a concentration of 3-5 X 10^6 cpm/mL. Three sets of experiments were carried out using one lean and one obese rat for each set.

including liver, muscle and testis. There was a ten-fold increase in the amount of *ob* mRNA detected in the white adipose tissue of the obese rat relative to that of the lean animal (Fig. 2). In brown adipose tissue, the message was detected only in the obese animals. There was thirty-fold more *ob* message in the white fat than in the brown fat of the obese animal. No *ob* message was detected by Northern analysis in the lean counterpart, even when the amount of RNA was increased to 20 μg per lane (Fig. 3). The *ob* gene message was, however, detected by RT-PCR, in brown adipose tissues of these same animals (data not shown).

***Ob* gene expression in adipocytes and preadipocytes.**

To further analyse the expression of the *ob* gene in adipose tissues, preadipocytes and adipocytes were separated from adipose tissues of the obese and lean animals of the JCR:LA-corpulent strain as well as from Sprague-Dawley rats. Total RNA was isolated from the two cell types and analysed by Northern blotting. The results, as shown in Figure 4, indicated that *ob* gene mRNA was detected in the mature fat cells of all animals tested; whereas, in preadipocytes, the message was detected only in those obtained from the obese rat. More mRNA was detected in the fat cells of the obese animal than those of the lean or the SD rat. No *ob* gene mRNA was detected in preadipocytes from SD rats or JCR:LA-corpulent lean animals.

Discussion

To our knowledge, this is the first report describing the expression of the *ob* gene in rats. Using the mouse published sequence (4), we have amplified a fragment of the rat gene and used it as a probe in a series of experiments to examine the expression and possible role of the *ob* gene product in obesity of the JCR-LA:cp strain of rat. Sequences of

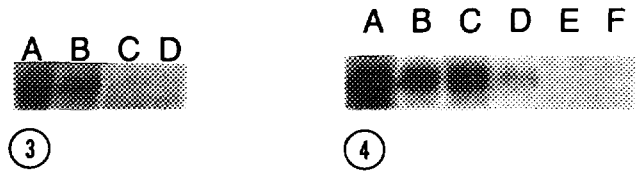

Figure 3. *Ob* gene expression in brown adipose tissues of obese and lean rat of the JCR-LA:cp rats. Northern blot analysis of total RNA was carried out using a fragment of the *ob* gene as probe. Total RNA was isolated from brown adipose tissues of the obese (A & B) and lean animals (C & D). 20 μg of total RNA were loaded in lanes A and C and 5μg were loaded in lanes B and D. ^{32}P-labelled *ob* gene fragment was added at a concentration of 3-5 X 10^6 cpm/mL. Three sets of experiments were carried out using one lean and one obese rat for each set.

Figure 4. Expression of *ob* gene in adipocytes and preadipocytes. Northern blot analysis of total RNA was carried out using a fragment of the *ob* gene as probe. Total RNA was isolated from adipocytes (A, B & C) and preadipocytes (D, E & F). Adipocytes and preadipocytes were isolated from obese (A & D) and lean rats (B & E) as well as from Sprague-Dawley rats (C & F). 5 μg of total RNA was loaded per lane. ^{32}P-labelled *ob* gene fragment was added at a concentration of 3-5 X 10^6 cpm/mL. Three sets of experiments were carried out using one lean and one obese and one SD rat for each set.

amplified *ob* gene fragments of obese and lean rats of JCR-LA:cp strain are identical to each other and to that of the SD rat suggesting that obesity in our rat model is not due to a mutation within the amplified fragment of the gene. In both obese and lean rats, a full size *ob* mRNA was detected indicating that there was no premature termination of transcription of the gene. Furthermore, a ten-fold increase in the amount of *ob* RNA was detected in the obese rat compared to the lean one, a finding consistent with previous reports in mice and humans (4, 8). Taken together, these results indicate that obesity in our model is not directly influenced by the level of gene expression, assuming that an active protein is synthesized. It has, however, recently been shown that the OB protein can cause a reduction in body fat and weight in obese mice (5, 6, 7) This would suggest that in our model the defect is found downstream of *ob* gene expression, possibly at the level of the *ob* receptor or post-receptor signalling pathway.

We have demonstrated by Northern blot that the *ob* gene is expressed in adipose tissue only, a finding consistent with other published results (4, 8, 9, 14). The *ob* mRNA was detected in both white and brown fat of the obese animal. In the JCR:LA-corpulent model, the *ob* mRNA in brown adipose tissue of the lean rat could be detected by RT-PCR, not Northern blotting, indicating that significantly less message was present in the brown fat of the lean animal compared to the obese one. Similar findings have been reported in mice (14). Our results show preferential expression of the gene in mature fat cells suggesting the involvement of a differentiation and/or maturation step in switching on the *ob* gene. It has been reported that the expression of the gene is differentiation dependent in 3T3-F442A preadipocytes (14). *Ob* gene was expressed following the differentiation of 3T3-F442A preadipocytes into adipocytes and not before. Similarly, no ob mRNA was detected when preadipocytes from lean and SD rats were used. However, ob gene expression was detected in preadipocytes from the obese rat of JCR-LA:cp strain, indicating either an upregulation of the gene expression to detectable levels or an early switch on of the gene. The aberrant expression of the gene within the white adipose tissues of the obese animal and the lack of response to the gene expression within those animals provide us with a model to study both the regulation of the ob gene at the cellular level and the response to the expression of the gene at the macromolecular level.

Acknowledgments

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