Regulation of Alström syndrome gene expression during adipogenesis and its relationship with fat cell insulin sensitivity

SARA ROMANO1, GABRIELLA MILAN1, CATERINA VERONESE1, GAYLE B. COLLIN2, JAN D. MARSHALL2, CINZIA CENTOBENE1, FRANCESCA FAVARETTO1, CHIARA DAL PRA1, ALESSANDRO SCARDA1, SONIA LEANDRI1, JÜRGEN K. NAGGERT2, PIETRO MAFFEI1 and ROBERTO VETTORI1

1Endocrine-Metabolic Laboratory, Internal Medicine 3, Department of Medical and Surgical Sciences, University of Padua, via Ospedale 105, I-35128 Padua, Italy; 2The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA

Received January 24, 2008: Accepted February 28, 2008

Abstract. Alström syndrome (ALMS) is an autosomal recessive genetic disease with characteristic phenotypical features including multi-organ fibrosis, insulin resistance, obesity and type 2 diabetes. ALMS1, a ubiquitously expressed gene mutated in ALMS patients, gives rise to a protein of unknown function localized to basal bodies of ciliated cells and centrosomes. Together with Bardet-Biedl syndrome, ALMS is a member of genetic ciliopathies, but the link between cilia/centrosome deficits and metabolic abnormalities remains to be determined. In this study for the first time we quantified Abms1 expression in a cellular model of adipogenesis during the differentiation of 3T3-L1 cells. An early decrease in Abms1 mRNA was observed during preadipocyte to adipocyte conversion. However, acute treatment of preadipocytes with the adipogenic factors did not result in significant change of Abms1 expression. In addition, to study the possible relationship between Abms1 and the degree of fat cell insulin sensitivity, as assessed with a chronic treatment with insulin or rosiglitazone respectively. In all these conditions Abms1 expression remained unchanged. In conclusion, our results suggest a role of the gene in the early phase of adipogenesis. Moreover, changes in fat cell insulin sensitivity do not imply any effect on Abms1 expression.

Introduction

Alström syndrome [ALMS (MIM #203800)] is a recessively inherited disorder characterized by early retinal photoreceptor degeneration, sensorineural hearing impairment, childhood obesity and severe insulin resistance followed by type 2 diabetes mellitus. Systemic fibrosis and multiple organ involvement including dilated cardiomyopathy, hepatic and renal failure may occur (1). ALMS is caused by mutations in ALMS1, a novel gene of unknown function on chromosome 2p13 which is ubiquitously expressed at low levels (2,3).

The analysis of transcripts in humans as well as in mice indicates the presence of different splice variants in several tissue types (2,4). It is possible that each protein isoform has a specific role in particular tissues and organs. Moreover, the mutations identified thus far cluster in distinct positions (mostly in exon 8, 10 and 16) and may explain the phenotypic variability among ALMS patients.

ALMS1 localizes to the centrosomes and basal bodies of ciliated cells and roles in cytoplasmic microtubular organization, intracellular transport and/or cilia assembly or function have been suggested (5,6). These observations led to the inclusion of ALMS in an emerging class of human genetic disorders, such as the Bardet-Biedl syndrome [BBS (MIM #209900)], called ‘ciliopathies’ (7,8). BBS shares with ALMS numerous features, in particular obesity and type 2 diabetes. A recent report investigated the temporal expression pattern of genes implicated in BBS in a cellular model of adipogenesis. Although not all BBS genes are expressed in adipose tissue, some of them showed increased mRNA levels in mature adipocytes compared to preadipocytes (9).

Although the major phenotypic features of ALMS, such as severe insulin resistance, obesity and type 2 diabetes, are observed in human and in murine models (4,10,11), there is little experimental evidence linking the Alms1 gene to the development of insulin resistance, to fat cell metabolism and adipose tissue growth and differentiation.

Adipose tissue insulin sensitivity is central in the regulation of glucose and free fatty acid uptake, inhibition of lipolysis and stimulation of fatty acid synthesis in adipocytes. In addition, insulin modulates adipose tissue growth and differentiation in concert with peroxisome proliferator-activated receptor (PPARγ), the key adipose tissue transcriptional regulator (12), by stimulation of glucose uptake together with translocation of glucose transporter 4 (GLUT4) from intracellular storage vesicles to the plasma membrane (13).
This complex insulin-stimulated intracellular trafficking is not completely clear and is specifically regulated in distinct cells and tissues (14). The organelles that are primarily involved in this process are skeletal muscle and adipose tissue and most studies about the mechanism of insulin-stimulated glucose uptake have been performed in 3T3-L1 adipocytes (15,16). Moreover, evidence indicates that cytoskeleton structures are implicated in insulin-induced glucose uptake and in GLUT4 translocation in fat cells (14,17). Therefore, the specific ALMS1 localization to centrosomes raises the possibility that ALMS1 could impact the GLUT4 translocation process and glucose uptake (6).

In order to study Alms1 transcripts during adipogenesis we utilized the established murine preadipocyte cell line 3T3-L1. We aimed also to investigate in 3T3-L1 preadipocytes the acute effect of the individual substances present in the adipogenic medium on Alms1 mRNA level. In addition, we evaluated whether modifications of insulin sensitivity could be associated with changes in Alms1 expression in fully differentiated 3T3-L1 adipocytes.

**Materials and methods**

**Cell culture.** 3T3-L1 preadipocytes (ATCC CL-173) were grown in 24-well cell culture plates (CellStar, Greiner Bio-One International AG, Germany) in standard medium (SM), consisting of Dulbecco’s Eagle’s medium (DMEM, Gibco, Invitrogen Life Technologies, Paisley, UK), 10% bovine serum (Gibco), 150 U/ml streptomycin, 200 U/ml penicillin, 2 mM glutamine and 1 mM HEPES (Gibco). At confluence, adipocytes were stimulated with 2 μM insulin, 1 μM dexamethasone, 3-isobutyl-1-methyl-xantine (IBMX, Sigma-Aldrich, St. Louis, MO, USA) and for the house-keeping β2-microglobulin mRNA content and absorbance was measured at 518 nm by spectrophotometer (Beckman Coulter, Inc., CA, USA).

**Treatments of 3T3-L1 preadipocytes and adipocytes.** The 3T3-L1 cell line was grown in SM for 2 days. Then, preadipocytes were stimulated with 2 μM insulin, 1 μM dexamethasone, 0.5 mM IBMX, AM, 10 μM rosiglitazone and AM with 10 μM rosiglitazone or DMSO (Sigma-Aldrich) in standard medium (SM), grown in 24-well cell culture plates (CellStar, Greiner Bio-One International AG, Germany) in standard medium (SM), consisting of Dulbecco’s Eagle’s medium (DMEM, Gibco, Invitrogen Life Technologies, Paisley, UK), 10% bovine serum (Gibco), 150 U/ml streptomycin, 200 U/ml penicillin, 2 mM glutamine and 1 mM HEPES (Gibco). At confluence, adipocytes were stimulated with 2 μM insulin, 1 μM dexamethasone, 3-isobutyl-1-methyl-xantine (IBMX, Sigma-Aldrich, St. Louis, MO, USA) and for the house-keeping β2-microglobulin mRNA content and absorbance was measured at 518 nm by spectrophotometer (Beckman Coulter, Inc., CA, USA).

**Gene expression.** Total RNA was extracted using the RNEasy mini kit (Qiagen GmbH, Hilden, Germany), treated with DNase Treatment & Removal Reagents (Ambion, Inc, Austin, TX, USA) and 2 μg was reverse-transcribed with 150 ng of random hexamers and 200 U of M-MLV RT (Promega Corp., Madison, WI, USA), according to the protocol recommended by the manufacturer.

PCR was carried out using Hot Star Taq DNA polymerase (Qiagen) in 25 μl of standard buffer with 1.5 mM MgCl₂, 200 μM of each dNTP and 0.5 μM of primers for different adipogenic markers, as Fatty acid binding protein 2 (Fabp-2), Fatty acid transporter (Cd36), Adiponectin (AdipoQ) and Liprotein lipase (Lip): primer sequences and amplification conditions are available upon request. Amplification reaction (10 μl) was separated by electrophoresis on agarose gels, visualized with ethidium bromide staining, using Image Master Total Lab 2.00 software. mRNA levels were expressed as the ratio of signal intensity for the target genes relative to that for 18S rRNA in arbitrary units (a.u.).

In regard to Alms1 transcripts, they were amplified using two primer sets, located in exon 8 (Alms1 ex8) and in exon 16 (Alms1 ex16). The oligonucleotide primers for mouse Alms1 ex8 (NM_145223) (forward 5'-GAA AAT ATG GCA CTG AAA CG-3' and reverse 5'-TTC CCA ATT TCC AAC ACC-3'), Alms1 ex16 (forward 5'-GTG GTG CCT GTG ATG CGG CAA AAG A-3' and reverse 5'-AGG CCC GGA GTG AAT GTG-3') and for the house-keeping β2-microglobulin gene (forward 5'-GCT TCA GTC GTC AGT G-3' and reverse 5'-CAG TTC ATG AGT TTC GGC TTC C-3') were designed using the Omega™ 2.0 software (Oxford Molecular Ltd., WI, USA). qPCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA) on a DNA Engine Opticon™ 2 Continuous Fluorescence Detection System (MJ Research, MA, USA). Each cDNA sample was assayed in duplicate and a no template control was included in every reaction. Each 30-μl reaction contained 5 μl first-strand cDNA (10 ng), 15 μl 2X SYBR Green PCR Master Mix (Applied Biosystems, Milan, Italy) and 300 nM of each forward and reverse primers. The activation step (95°C for 10 min) was followed by 45 cycles of 94°C x 15 sec, 53°C x 30 sec and 72°C x 30 sec for Alms1 ex8 (amplicon of 237 bp), by 45 cycles of 95°C x 15 sec and 60°C x 1 min for Alms1 ex16 (amplicon of 95 bp) and by 45 cycles of 95°C x 15 sec, 57°C x 30 sec and 72°C x 30 sec for β2-microglobulin (amplicon of 149 bp).

Standard curves were obtained using cDNA from 3T3-L1 cells by plotting values for log cDNA quantity (in a.u.) versus cycle threshold. For each sample, Alms1 expression values were normalized by β2-microglobulin mRNA content and reported as a.u. ratio.

**Assessment of glucose uptake.** Adipocytes were incubated at 37°C for 2 h in serum-free medium and then treated with Oil-Red O staining and triglyceride quantification. Cells were fixed in 10% formalin in PBS containing CaCl₂, 0.9 mM and MgCl₂, 0.5 mM (PBS-CM) for 1 h at 4°C. Adipocytes were stained with Oil-Red O (Sigma-Aldrich) in 40% isopropanol for 15 min at room temperature. After 3 washes in PBS-CM, Oil-Red O stain was extracted in 600 μl isopropanol and the absorbance was measured at 518 nm by spectrophotometer (Beckman Coulter, Inc., CA, USA).
different concentrations of insulin (0, 100 nM or 2 μM) for 1 h at 37°C. The assay was initiated by the addition of 300 μl of a solution of 50 μM D-glucose and 1.5 μCi/ml 2-Deoxy-glucose (Amersham-Biosciences, Piscataway, NJ, USA). After 15 min at 37°C, the assay was terminated by two rapid washes in ice-cold PBS. Cells were solubilized in 500 μl PBS with 0.1% Triton X-100 and the radioactivity was measured using a β-counter (Wallace-Perkin-Elmer, MA, USA). Total protein was quantified using a Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL, USA). The insulin-induced glucose uptake was normalized to total protein content and reported as percentage of the basal value.

Statistical analyses. Statistical analysis was performed using unpaired Student's t-test (two-tailed) and analysis of variance. Differences were considered significant at P<0.05.

Results

Adipogenic differentiation of 3T3-L1 cells. To assess the differentiation of 3T3-L1 we monitored morphological changes during the adipogenic process by microscopy. After 12 days in AM, we obtained fully differentiated adipocytes, as shown by Oil-Red O staining of lipid droplets in red (Fig. 1B).

Expression levels of well-known adipose-cell specific genes (Fabp-2, Cd36, AdipoQ and Lpl) were measured during the time course of adipogenic differentiation (Fig. 1C). These genes were absent or weakly expressed in 3T3-L1 preadipocytes and were highly up-regulated as a result of adipogenesis. Particularly, Fabp-2, Cd36 and AdipoQ levels increased the second day of the differentiation process, peaking at the fourth day after the addition of AM and then maintaining steady levels thereafter. Conversely, Lpl was expressed at low levels in preadipocytes and its expression increased steadily during the conversion to mature fat cells.

Alms1 expression during 3T3-L1 adipogenic differentiation. Modifications in Alms1 expression were quantified by RT-qPCR with 2 different primer sets, localized in exons 8 and 16, to measure the putative presence of different transcripts. A significant decrease in mRNA levels of Alms1 was observed throughout adipogenesis with both primers, beginning from the second day after the addition of AM. The expression levels in fully differentiated adipocytes were 20% of those observed in preadipocytes (Fig. 2).

Effect of different adipogenic substances on Alms1 expression in preadipocytes. We analyzed Alms1 transcripts by RT-qPCR, to investigate the possible role of the single AM components and of rosiglitazone in the reduction of Alms1 mRNA expression found in mature fat cells. No significant changes in Alms1 expression in 3T3-L1 preadipocytes were observed considering all the different treatments performed with both the primer sets used (Fig. 3).

Insulin sensitivity and Alms1 expression in 3T3-L1 mature adipocytes. To compare Alms1 expression and insulin resistance in mature adipocytes, insulin-induced glucose uptake assays were performed on 3T3-L1 cells and Alms1 mRNA levels were quantified. Mature adipocytes differentiated in the presence of rosiglitazone displayed greater insulin sensitivity with respect to control cells differentiated without the drug (600% vs 400% increase of basal glucose uptake upon stimulation with 2 μM of insulin). The number, size and morphology of adipocytes, estimated by microscopic examination and measured by Oil-Red O staining, were very similar, regardless of the presence of rosiglitazone during the differentiation and irrespective of the different treatments.
performed (Fig. 4A, inner panel). However, despite the protocol of differentiation used, mature adipocytes did not increase their insulin responsiveness with rosiglitazone treatment for 24 h (Fig. 4A and data not shown).

While 3T3-L1 adipocytes were generated by a 12 days culture in AM containing 2 μM insulin, it is important to consider that when insulin was not removed after the differentiation, the adipocytes were insulin resistant (Fig. 4A).

Nevertheless, despite the distinct conditions of insulin sensitivity generated, no significant difference in Alms1 mRNA expression was observed in adipocytes differently treated, independently of the primer sets used (Fig. 4B).

Discussion

In this study, we investigated Alms1 regulation in the 3T3-L1 cell line during the adipogenic process and its possible relationship with fat cell insulin sensitivity. Alms1 mRNA is predominantly expressed in preadipocytes, whereas it is clearly down-regulated in mature adipocytes, suggesting distinct functions for Alms1 in adipose tissue. On the contrary, the majority of BBS genes expressed in mouse adipose tissue, are reported to be up-regulated together with fat cell maturation (9). Despite these contrasting results, the genes involved in both syndromes showed the greatest variations during early adipogenesis. The progressive decrease of Alms1 expression during the early phase of adipogenic differentiation led us to speculate on a role of ALMS1, when the preadipocyte changes its phenotypic characteristic from a fibroblast-like cell to a fat cell. In fact, gene expression profiling of 3T3-L1 cells during adipogenesis showed that different genes of the cytoskeleton were down-regulated as the first step of cellular differentiation, before any morphological changes (18). The putative functional relationship of ALMS1 with basal bodies of ciliated cells suggests that ALMS1 may be important for the assembly of these structures. Therefore the role of ALMS1 in the fibroblast-like 3T3-L1 could be much more important than in the 3T3-L1 adipocytes and ALMS1 disruption could justify the presence of functional alterations of fibroblast cells including their proliferative, collagen biosynthetic and differentiative properties. It is noteworthy that Alström patients display not only a propensity to accumulate fat at the visceral level, but a strong susceptibility to develop a multi-organ fibrosis (1). Nevertheless, the
functional significance of early Alms1 expression and its impact on adipogenic differentiation remains to be explored. Alms1 may play a role in modulating the adipogenic process and the decline of Alms1 could allow the differentiation and proliferation of adipose cells. The disruption of the gene may thus contribute to the development of obesity in ALMS patients, possibility supported by the observations carried out also in the different animal models of ALMS (4,10,11). However, it seems unlikely that Alms1 could directly influence adipose tissue differentiation acting on the well recognized classic pathway. In fact, we have been unable to induce modulation in Alms1 expression in preadipocytes by treatment with the adipogenic factors present in the AM (including insulin and rosiglitazone), which activates PPARγ, an essential transcription factor for normal adipogenesis (19). These drugs are known to trigger the adipogenic differentiation pathway and to modulate insulin sensitivity. Nonetheless, these acute treatments did not change Alms1 expression in preadipocytes, as it has been described for other adipose-specific genes involved in obesity and insulin resistance (20,21).

ALMS shares some clinical features with BBS (abdominal obesity, insulin resistance and type 2 diabetes) as well as molecular defects at the basal bodies of ciliated cells (5,7,22). These findings suggest that ciliary or vesicular transport deficits may contribute to the pathogenesis of the metabolic abnormalities present in these diseases (6). In particular, an impaired translocation of intracellular membrane vesicles containing GLUT4 to the plasma membrane could explain the appearance of a severe insulin resistance (23).

In order to study the relationships between Alms1 expression and insulin sensitivity in mature fat cells, 3T3-L1 adipocytes were treated with rosiglitazone or with high concentrations of insulin. It is indeed well known that PPARγ activity is essential in regulating adipose tissue insulin sensitivity and glucose homeostasis (19,24-26) leading also to increased expression of GLUT4 in adipose tissue (27). Recent studies demonstrated that PPARγ knockdown attenuates insulin-stimulated glucose uptake in 3T3-L1 adipocytes, providing direct evidence that PPARγ is an important modulator of insulin-stimulated glucose uptake in these cells, involving both GLUT4 translocation and the initial insulin-induced phosphorylation steps (28). It is important to note that in our experiments only 3T3-L1 adipocytes differentiated in the presence of rosiglitazone highlighted an increase in insulin-induced glucose uptake over control, whereas a short-term presence of rosiglitazone highlighted an increase in insulin-stimulated glucose uptake in 3T3-L1 adipocytes, involving both GLUT4 translocation and the initial insulin-induced phosphorylation steps (29,30). Since Alms1 is likely involved in cytoskeleton function, we investigated if 3T3-L1 adipocytes chronically exposed to insulin and then became insulin-resistant could change their Alms1 expression. This was not observed in our experiments.

The fact that most of ALMS1 mutations are in specific regions of the gene together with the identification of several tissue-specific splice variants may explain the inter-individual variability and the complexity of ALMS phenotypes. For this reason, we quantified Alms1 expression using two primers designed in different positions of the mRNA sequence in order to track the regulation of transcripts different in size. The two Alms1 variants putatively present in 3T3-L1 cells only slightly differ in their relative amount and essentially showed the same pattern of expression during the adipogenic differentiation. Thus, they are not differentially regulated during adipogenesis and/or by agents well known to affect fat cell insulin sensitivity.

In conclusion, our results show that fat cell insulin resistance, as obtained by chronic insulin exposure, or the improvement of fat cell insulin action on glucose transport, by chronic rosiglitazone treatment, do not display any direct regulatory effect on Alms1 expression. Moreover, our data suggest a role of Alms1 in the early phase of the adipogenic process from fibroblast like cell to mature adipocyte in 3T3-L1 cellular model.

Acknowledgements

This work was supported by grant 2005060925_002 PRIN (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale) from the MiUR (Ministero dell’Istruzione, dell’Università e della Ricerca) given to R. Vettor. G.B. Collin, J.D. Marshall and J.K. Naggert were supported by National Institutes of Health grant R01 HD036878.

References


