Role of Gas-6 in Adipogenesis and Nutritionally Induced Adipose Tissue Development in Mice

Erik Maquoi, Gabor Vörös, Peter Carmeliet, DéSiré Collen, H. Roger Lijnen

Objective—A potential role of growth arrest-specific gene 6 (Gas-6) in energy storage in adipose tissue was investigated in murine models of obesity. Gas-6 is a ligand for the Axl, C-Mer, and Sky family of tyrosine kinase receptors.

Methods and Results—Whereas Gas-6, C-Mer, and Sky were expressed in mature murine adipocytes, the expression of Axl was restricted to the stromal-vascular fraction, which includes pre-adipocytes. During the in vitro conversion of adipogenic 3T3-F442A cells into mature adipocytes, the expression of Gas-6 increased in undifferentiated confluent pre-adipocytes during a transient phase of growth arrest. On treatment of these cells with an adipogenic medium, Gas-6 expression decreased sharply, coinciding with expression of early adipocytes markers. This modulation was not observed in the nonadipogenic 3T3-C2 cells. The Gas-6 mRNA level was transiently downregulated during nutritionally induced expansion of adipose tissues in vivo. When kept on a standard diet, no significant difference in either total body weight or weight of gonadal or subcutaneous fat pads was observed between Gas-6-deficient and wild-type mice. On exposure to a high-fat diet, however, Gas-6-deficient mice had significantly less fat mass than their wild-type counterparts.


Key Words: adipogenesis ■ adipose tissue ■ Gas-6 ■ high-fat diet ■ obesity

Obesity and its related diseases such as noninsulin-dependent diabetes mellitus, cardiovascular disease, atherosclerosis, and hypertension are main causes of increased morbidity and mortality in Western societies. Development of obesity arises from increased size of individual adipose cells caused by lipid accumulation, and from enhanced number of adipocytes arising from differentiation of adipose precursor cells to mature adipocytes under the appropriate nutritional and hormonal stimuli.1

The growth arrest-specific gene 6 (Gas-6) is a secreted vitamin K-dependent protein, which binds the receptor tyrosine kinases Axl (also called Ark, Ufo), Sky (also called Rse, Tyro3, Dtk), and C-Mer (also called Eyk, Nyk) via its carboxy-terminal globular domain.2 Gas-6 was originally identified as a gene of which the expression in fibroblasts increased by serum starvation and contact inhibition, and was therefore implicated in reversible cell growth arrest.3 Gas-6 has also been implicated in survival, proliferation, cell adhesion, and hemostasis. Recently, it was shown that deficiency of Gas-6 prevented venous and arterial thrombosis and protected against fatal thromboembolism.4

Fibroblast-like cell lines such as the closely related 3T3-L1 and 3T3-F442A cell lines are well-characterized pre-adipocyte models capable of differentiating into mature adipocytes after appropriate hormonal stimulation.5 On reaching confluence, these cells become contact-inhibited and growth-arrested. After hormonal induction of differentiation, these confluent cells re-enter the cell cycle and undergo a phase of mitotic clonal expansion, which is essential to complete terminal differentiation into mature adipocytes.6 Gas-6 expression was previously reported during the clonal expansion of postconfluent 3T3-L1 pre-adipocytes.6

The biological role of Gas-6 in adipose tissue development has, however, remained unclear. In this study, we report a novel critical role for Gas-6 in promoting development of adipose tissues in diet-induced obese mice.

Methods

Diet Model

Wild-type mice (100% C57Bl/6 genetic background) and Gas-6-deficient (Gas-6−/−) and wild-type (Gas-6+/+) mice with the same genetic background (Swiss:129SV, 50:50), were fed a standard fat diet (SFD) or a high-fat diet (HFD) ad libitum during 12 to 15 weeks, as described.7 Mice were from Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium. Intra-abdominal (gonadal [GON]) and inguinal subcutaneous (SC) fat pads were removed and weighted.
Adipose Tissue Dissociation
Adipose tissue dissected from C57Bl/6 mice were used to separate mature adipocytes from stromal-vascular (S-V) cells by collagenase treatment as described.8

Differentiation of 3T3-F442A and 3T3-C2 Cells
The 3T3-F442A pre-adipocytes and 3T3-C2 fibroblasts were grown in basal medium: DMEM supplemented with 10% fetal bovine serum (FBS). Forty-eight hours after confluence, the cells were treated for 3 days with basal medium supplemented with 17 nM insulin, 100 nM dexamethasone, 100 μM methylisobutylxanthine, and 2 nM triiodothyronine. Cultures were then switched to a basal medium supplemented with 17 nM insulin and 2 nM triiodothyronine.

Growth arrest of 3T3-F442A pre-adipocytes was induced by switching the cells to DMEM supplemented with 1% FBS during 24 or 48 hours.

Results
Adipose Tissue Expression of Gas-6 and Axl, C-Mer, and Sky Receptors in Mice with Diet-Induced Obesity
At the start of the diet (5 weeks of age), the body weight of the wild-type C57Bl/6 mice in the SFD and HFD groups was comparable. C57Bl/6 mice kept on HFD for 2, 5, or 15 weeks had a significantly higher total body weight than age-matched mice on SFD (26.4±0.6 grams versus 21.1±0.7 grams after 2 weeks, 32.9±0.9 grams versus 25.4±0.4 grams after 5 weeks and 46.3±1.8 grams versus 30.0±0.6 grams after 15 weeks; all P<0.001). In agreement with our previous observations,7 the weight of the SC and GON adipose tissues as well as the average adipocyte size was significantly increased in mice fed a HFD (not shown).

In GON adipose tissue from mice on SFD, a transient 2-fold upregulation of Gas-6 mRNA level was observed at 5 weeks (Figure 1A). In contrast, Gas-6 level in GON samples of mice on HFD was relatively constant. A similar but less extensive modulation was observed in SC adipose tissues (Figure 1B).

Axl mRNA level in GON adipose tissue was stable during the 15 weeks of diet (Figure 1C). In the SC tissues of mice on SFD, Axl expression peaked at 5 weeks and decreased after 15 weeks. On HFD, Axl expression was maximal after 15 weeks (Figure 1D).

The expression of C-Mer did not show marked modulations in the adipose tissues (Figure 1E and 1F), except in the SC samples of mice fed HFD during 15 weeks, in which a 4-fold increase was observed.

Sky expression in GON adipose tissues was downregulated 2-fold after 2 weeks and remained low at later time points, irrespective of the diet used (Figure 1G). In the SC adipose tissues, Sky mRNA level was transiently upregulated at 5 weeks (Figure 1H). However, because of the high variability in the quantification of Sky mRNA level in the SC samples after 5 weeks of diet, this upregulation was not statistically significant but revealed a trend.

Cellular Localization of Gas-6, Axl, C-Mer, and Sky mRNAs in Adipose Tissue
To identify the cellular source of Gas-6 and its 3 receptors, GON and SC fat depots were isolated from C57Bl/6 mice kept for 5 weeks on HFD and dissociated to separate mature adipocytes from the S-V cells. Adipose-specific leptin mRNA was present in the 2 adipose fractions and was absent in the corresponding S-V fractions. Conversely, tissue inhibitor of matrix metalloproteinase-2 and von Willebrand factor (a marker of endothelial cells) mRNAs were not detected in the adipose fractions, in agreement with previous studies.8 Gas-6 and 2 of its receptors (C-Mer and Sky) were detected in both mature adipocytes and S-V cells. In contrast, the expression of Axl was nearly exclusively restricted to the S-V fraction. This expression pattern was similar in both GON and SC fat depots (Figure I, available online at see http://atvb.ahajournals.org).

Diet-Induced Obesity in Gas-6–Deficient Mice
To determine the contribution of Gas-6 in the development of obesity, we investigated the impact of a HFD on Gas-6−/− mice. In accordance with the expression profile observed in the C57Bl/6 mice, Gas-6 expression was detected by semi-quantitative reverse-transcription polymerase chain reaction in GON and SC adipose tissues of Gas-6−/− mice but not in those of Gas-6+/+ mice (Figure 2A).

At the start of the diet (5 weeks of age), the body weight of the Gas-6+/+ and Gas-6−/− mice was comparable (28±0.8 and 26±0.6 grams). When Gas-6+/+ or Gas-6−/− mice were kept on SFD for 12 weeks, the body weight (34±1.0 versus...
Figure 2. Effect of Gas-6 deficiency on adipose tissue development. A, Expression of Gas-6 mRNA and 28S rRNA in subcutaneous (SC) and gonadal (Gon) adipose tissues of wild-type (+/+) or Gas-6-deficient (−/−) mice was analyzed by semi-quantitative reverse-transcription quantitative polymerase chain reaction. Kidney (Kid) was used as a positive control for Gas-6 expression. B, Body weight of Gas-6−/− (white circles) and Gas-6+/+ (black circles) mice kept on HFD during 12 weeks. C and D, Weight of different tissues obtained from Gas-6−/− (white bars) and Gas-6+/+ (black bars) mice kept for 12 weeks on SFD (O) or HFD (D). * P<0.001 vs Gas-6−/−.

33±0.9 grams) and the weight of the SC or the GON adipose tissue was not different. The weight of other organs, including lung, kidney, heart (not shown), and liver was not different from the Gas-6+/+ group. The only noticeable difference was a smaller spleen in Gas-6−/− mice (Figure 2C).

The weight gain with time on the HFD was identical in both genotypes, resulting in comparable body weights at the end of the 12-week diet (Figure 2B). However, the weight of the isolated SC or GON fat pads was significantly lower in the Gas-6−/− group (Figure 2D). In SC and GON fat pads of Gas-6−/− and Gas-6+/+ mice kept on HFD, the mean diameter of the adipocytes was comparable (56±2.7 versus 53±2.1 μm for SC adipose tissue, and 80±2.1 versus 76±2.2 μm for GON adipose tissue). Body fat, calculated on the basis of GON adipose tissue weight, amounts to 4.4±0.18% or 3.4±0.25% (P=0.011) for Gas-6−/− or Gas-6+/+ mice. The weight of other organs, including lung, kidney, heart (not shown), and liver was not different from the Gas-6+/+ group, but the spleen weight was lower in Gas-6−/− mice (63±2.1 versus 80±4.5 mg; P<0.005) (Figure 2D).

The average food intake measured during the HFD period was comparable for both genotypes (3.8±0.1 or 4.4±0.3 grams per mouse per 24 hours for Gas-6−/− or Gas-6+/+ mice), and the average physical activity was not significantly different (6800±450 or 6400±530 cycles/12 hour for Gas-6−/− or Gas-6+/+ mice).

To evaluate adipose tissue-related angiogenesis, SC or GON adipose tissue of Gas-6−/− and Gas-6+/+ mice was stained with an endothelial cell specific lectin; this revealed comparable stained areas, blood vessel density, and size (not shown).

Figure 3. Relationship between Gas-6 mRNA level (A and B) and growth rate (C and D) during in vitro adipogenesis in 3T3-F442A (A and C) and 3T3-C2 (B and D) cells. Forty-eight hours after reaching confluence, the differentiation of pre-adipocytes was induced by sequentially treating the cells with an induction (black arrow) and a differentiation (white arrow) medium as described in Methods. At different stages of the differentiation process, cells were counted and Gas-6 mRNA levels were analyzed by quantitative real-time polymerase chain reaction and normalized to 18S rRNA. Arrowhead indicates time when the cells reached confluence.

Plasma hematologic parameters including red blood cells, platelet counts, hemoglobin, and hematocrit levels of Gas-6−/− and Gas-6+/+ mice were comparable after 12 weeks of HFD (not shown), whereas white blood cells were higher (2.7±0.2×10⁹/L versus 1.3±0.1×10⁹/L P<0.005) in the Gas-6−/− mice. Plasma plasminogen activator inhibitor-1 antigen levels were ~2- to 3-fold enhanced as a result of the HFD, but were comparable in both groups. Triglyceride and total cholesterol levels were also comparable for both genotypes, whereas glucose levels were somewhat reduced in the Gas-6−/− animals (P=0.05). Circulating plasma leptin levels were drastically enhanced as a result of the HFD in Gas-6−/− or Gas-6+/+ mice on SFD, but were comparable for both genotypes (Table I, available online at http://atvb.ahajournals.org).

Modulation of Gas-6 Expression During In Vitro Adipogenesis

We used 3T3-F442A pre-adipocytes as an in vitro model of adipogenesis. This process was monitored by measuring the mRNA level of several markers of differentiation including pref-1, Id-2, PPARγ, GPDH, and aP2. Although the level of pref-1 and Id-2 mRNAs, 2 negative regulators of adipogenesis, were rapidly downregulated as the cells reached confluence, the level of PPARγ, GPDH, and aP2, 3 positive markers of adipogenesis, sharply increased on exposure to the induction medium (Figure II, available online at http://atvb.ahajournals.org; not shown for aP2).

In this model, Gas-6 mRNA level increased progressively (Figure 3A) as proliferating cells reached confluence. On treatment with the adipogenic medium, Gas-6 mRNA de-
clined sharply and reached the level observed in preconfluent cultures (day 1). Gas-6 expression is known to be increased in contact-inhibited fibroblasts and is therefore implicated in reversible growth arrest. It has been shown that adipose conversion of 3T3-F442A cells begins when the cells have grown to confluence and reach a brief phase of quiescence. It is therefore tempting to speculate that the peak of Gas-6 expression observed at day 5 (Figure 3A) coincides with a phase of growth arrest. To assess this hypothesis, 3T3-F442A cells were counted at regular intervals before and after confluence. Figure 3C shows that after reaching confluence (day 3), the cells stop proliferating. On day 7, ≈48 hours after the induction of differentiation, cells transiently resume proliferation and undergo a limited number of divisions that selectively increases the number of adipose cells. After this clonal expansion, the cells entered in a quiescent state. These observations strongly support the concept that in 3T3-F442A cells, the upregulation of Gas-6 is associated with the transient phase of growth arrest that precedes the phase of clonal expansion.

To determine whether the previous observations were specific to adipocyte differentiation, similar experiments were performed with 3T3-C2 cells. Like the 3T3-F442A cells, the 3T3-C2 cells were also isolated as clonal derivatives from murine 3T3 cells, but they differentiate to mature adipocytes with a very low frequency. Quantification of Gas-6 mRNA level in 3T3-C2 cells reveals that Gas-6 expression did not increase when these cells reached confluence (day 3). Furthermore, Gas-6 expression showed a strong and rapid increase by day 6 (24 hours after induction of differentiation) and remained elevated until the end of the experiment (Figure 3B). To ascertain that the downregulation of Gas-6 expression observed in 3T3-F442A cells 24 hours after the induction of differentiation did not occur later in the 3T3-C2 cells, the differentiation experiment was prolong until day 13. However, Gas-6 mRNA level did not decrease but rather increased slightly during this extended period. The growth rate of the 3T3-C2 cells also differed fundamentally from that of 3T3-F442A cells. The 3T3-C2 line was growing exponentially until day 7 before reaching a stationary phase (Figure 3D). Interestingly, this stationary phase coincides precisely with the highest Gas-6 mRNA level (compare Figure 3B and 3D), thus further emphasizing the close association between Gas-6 expression and growth arrest in these cell lines.

**Gas-6 Expression During Serum Stimulation of 3T3-F442A Pre-Adipocytes**

We subsequently determined if the peak of Gas-6 expression was specific to the transient phase of growth arrest that precedes the differentiation in 3T3-F442A cells, or if it occurred solely as a result of quiescent postconfluent cells having been stimulated to re-enter the cell cycle. This was tested by causing cell cycle arrest by 24-hour serum restriction of logarithmically dividing cells and subsequently allowing cell cycle re-entry by serum stimulation. Logarithmically dividing 3T3-F442A cells (normally cultured basal medium supplemented with 10% FBS) were subjected to serum restriction by treatment with medium supplemented with only 1% FBS for 48 hours. This treatment has been previously shown to cause cell cycle arrest in pre-adipocytes. Cells were released from cell cycle arrest by treating serum-restricted cultures with basal medium supplemented with 10% FBS. Gas-6 mRNA levels and cell numbers were quantified in 3T3-F442A cells that were proliferating in basal medium (A and B), serum-restricted for 48 hours (C and D), or serum restimulated after a 24-hour serum restriction (E and F). Gas-6 mRNA levels were analyzed by real-time quantitative polymerase chain reaction and normalized to 18S rRNA.

Figure 4. Effect of serum restriction/stimulation on growth rate and Gas-6 expression in 3T3-F442A pre-adipocytes. Gas-6 mRNA levels and cell numbers were quantified in 3T3-F442A cells that were proliferating in basal medium (A and B), serum-restricted for 48 hours (C and D), or serum restimulated after a 24-hour serum restriction (E and F). Gas-6 mRNA levels were analyzed by real-time quantitative polymerase chain reaction and normalized to 18S rRNA.
Gas-6 has been implicated in several cellular functions including reversible cell growth arrest, survival, proliferation, apoptosis, and cell adhesion. These various functions may be tissue-specific. Gas-6 has been shown to be a potentiating growth factor for many cell types, including fibroblasts and mesangial cells. Despite the potential implication of Gas-6 in these different functions, it was reported that Gas-6-deficient mice develop normally, survive, and are apparently healthy.

Because it was recently reported that Gas-6 mRNA is preferentially expressed during the clonal expansion of post-confluent 3T3-L1 pre-adipocytes, we have in the present study investigated a potential role of Gas-6 in the development of adipose tissue. Our results demonstrate for the first time the expression of Gas-6 and its 3 receptors (Axl, Sky, C-Mer) in murine adipose tissues. Gas-6, C-Mer, and Sky mRNAs were detected in mature adipocytes as well as in the S-V cell fraction, which includes different cell types such as pre-adipocytes, endothelial cells, and fibroblasts. In contrast, the expression of the Axl receptor, the major receptor for Gas-6, was restricted to the S-V fraction. Thus, in the adipose tissues, Gas-6 may act in an autocrine and/or paracrine manner.

When wild-type C57Bl/6 mice were exposed to a HFD during 15 weeks, they gained more weight and had enlarged fat pads when compared with mice kept on SFD. The analysis of the expression profile of Gas-6 during the development of HFD-induced obesity revealed that Gas-6 mRNA level was transiently upregulated in mice kept on SFD. In contrast, such a transient upregulation was not observed in mice exposed to a HFD. In agreement with our observations, a DNA microarray analysis of genes differentially expressed in the GON adipose tissues of wild-type C57Bl/6 and genetically obese C57Bl/6J ob/ob mice revealed a 2-fold downregulation of Gas-6 expression in the obese animals (http://www.biochem.wisc.edu/attie/supplemental_data/). Collectively, these different observations support an association between the obesity-driven expansion of the adipose tissues and the downregulation of Gas-6 expression.

To clarify the potential implication of Gas-6 in the HFD-induced expansion of the adipose tissues, mice with inactivation of the Gas-6 gene and wild-type controls were kept on a HFD for 12 weeks, and total body weight, weight, composition and cellularity of SC and GON adipose tissue, and plasma metabolic parameters were monitored, using a previously described model. Because in mice important genetic differences in the metabolic response to fat have been reported, we have used wild-type and Gas-6-deficient mice with the same genetic background (Swiss:129SV, 50:50). In accordance with previously published data, Gas-6-deficient mice kept on regular diet (SFD) have no obvious phenotype. Their size, total body weight, and the weight of their major organs and adipose tissues were comparable, suggesting that Gas-6 was not mandatory for the normal development of the adipose tissues. When exposed to a HFD, wild-type and Gas-6-/- mice displayed similar food intake and physical activity; nevertheless, Gas-6-/- animals exhibited a significant decrease in fat deposition when compared with their wild-type counterparts. The lower adipose accumulation was not accompanied by a significant decrease in the overall body weight. A similar discrepancy between the fluctuation of adiposity and total body weight has also been reported in other models such as SPARC-/- mice. The lower level of glucose measured in Gas-6-/- mice on HFD may be related with the lower fat mass. The reduction of GON and SC fat pads cannot be attributed to a decrease in the average size of Gas-6-/- adipocytes. Moreover, the overall morphology of the fat pads of these mice was not altered as suggested by their similar vascularization and fibrillar collagen content (not shown). These observations suggest that the decrease in overall mass of the fat pads in the absence of Gas-6 may be attributable to a decrease in the number of mature adipocytes per fat pad. Adult adipose tissue is a complex cellular milieu that is composed of a variety of cells, including proliferative pre-adipocytes and terminally differentiated, nonproliferative mature adipocytes. In response to an obesity-induced stimulus, adipose tissue increases in mass by adipocyte hypertrophy and hyperplasia. Modifications in adipocyte number are mostly governed by changes in pre-adipocytes replication, cell (both pre-adipocytes and adipocytes) depletion by apoptosis and pre-adipocytes maturation by adipogenesis.

To uncover the contribution of Gas-6 to adipogenesis, we have examined the expression of Gas-6 mRNA during the in vitro differentiation of 3T3-F442A pre-adipocytes. Our data revealed that the expression of Gas-6 was maximal in undifferentiated confluent pre-adipocytes during a transient phase of growth arrest that precedes the phase of clonal expansion. When the differentiation of these confluent cells was initiated by the addition of the adipogenic medium, a sharp decrease of Gas-6 expression was observed, coincident with the upregulation of several markers of adipogenesis including PPARγ, GPDH, and aP2. In 3T3-C2 cells, a cell line that differentiates to mature adipocytes with a very low frequency, Gas-6 expression was elevated in confluent growth-arrested cells, but the addition of the adipogenic medium failed to induce its downregulation. A recent profiling by DNA microarray analysis of changes in gene expression that occur during the differentiation of another adipogenic cell line, the 3T3-L1 cells, revealed a transient upregulation of Gas-6 expression (http://www.personal.umich.edu/~macdouga/MacDougalLab.html), thus confirming our observations in the 3T3-F442A cells. It is worth noting that the overexpression of Wnt-1 in the 3T3-L1 cells completely blocked adipocyte differentiation in response to adipogenic medium. Interestingly, the expression of Gas-6 in Wnt-1-overexpressing cells was not upregulated. The
signal pathway of Wnt involves a complex network of proteins, among which cytosolic β-catenin plays a central role. In the absence of Wnt, β-catenin is degraded in proteosomes. In the presence of Wnt, degradation of β-catenin is blocked, thereby allowing the activation of Wnt target genes.27 Gas6 has been previously shown to increase β-catenin stability in contact-inhibited cells,28 suggesting that the transient increase of Gas6 expression observed in this study in 3T3-F442A pre-adipocytes during the phase of growth arrest might contribute to maintain pre-adipocytes in an undifferentiated state. Interestingly, the addition of the adipogenic medium decreased Gas6 expression and concomitantly upregulated PPARγ, the activation of which has been shown to downregulate β-catenin.10 In that respect, it is worth noting that the treatment of 3T3-C2 cells with the adipogenic medium failed to downregulate Gas6 expression and to induce adipogenesis. These data support the concept that the downregulation of Gas6 expression during adipogenesis may contribute to downregulate β-catenin, thereby reducing the antiadipogenic effect of Wnt signaling and maintaining adipocytes in the differentiated state.

Collectively, these different observations suggest that Gas-6 is involved in the reversible growth arrest of confluent pre-adipocytes and that the downregulation of its expression is associated with adipogenesis.

Our in vitro and in vivo data support the concept that in the adipose tissue of mice kept on SFD, the increased expression of Gas-6 is associated with the accumulation of quiescent growth-arrested committed pre-adipocytes. The presence of Gas-6 in these quiescent cells might allow them to respond to adipogenic stimuli (induced on the exposure to a HFD), which promote their proliferation and/or differentiation into mature adipocytes as suggested by the lower fat deposition observed in Gas-6−/− mice kept on HFD.

Altogether, these observations suggest that Gas-6 belongs to a class of molecules that are redundant for normal homeostasis, but critical for stress responses. Not surprisingly, inactivation or malfunctioning of such genes does not necessarily cause life-threatening developmental defects but may significantly modulate the severity of disease-related phenotypes such as thrombosis4 and obesity.

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References

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Figure I. Cellular localization of mRNAs in stromal-vascular (S.V.) and mature adipocyte (Adip.) fractions isolated from gonadal (GN) or subcutaneous (SC) adipose tissues dissected from C57Bl/6 mice kept for 5 weeks on HFD. The expression level of murine leptin, TIMP-2, von Willebrand factor (vWF), Gas-6, Axl, C-Mer, Sky mRNAs and 28S rRNA were determined by semi-quantitative RT-PCR.
Figure II. Expression of differentiation markers during *in vitro* adipogenesis. Forty height hours after reaching confluence, the differentiation of 3T3-F442A preadipocytes was induced by sequentially treating the cells with an induction (*filled arrow*) and a differentiation (*open arrow*) medium as described in Methods. Total RNA was extracted from the cells at different stages of differentiation and pref-1 (*A*), Id-2 (*B*), PPAR-γ (*C*) and GPDH (*D*) mRNA levels were analyzed by quantitative real time PCR and normalized to 18S rRNA.
Diet model

Five week old male wild-type mice of about 20 g (100% C57Bl/6 genetic background) were kept in micro-isolator cages on a 12 h day/night cycle and fed water and a standard fat diet (SFD) or a high fat diet (HFD) ad libitum, as described previously. C57Bl/6 mice after 2, 5 or 15 weeks on SFD or HFD (n=7 in each group) were fasted overnight and anesthetized by i.p. injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected from the retroorbital sinus with addition of trisodium citrate (final concentration 0.01 mol/L). Intra-abdominal (gonadal, GON) and inguinal subcutaneous (SC) fat pads were removed and the wet weight determined. Other organs, including the lung, kidney, liver, spleen and heart were also removed and weighed.

Gas-6-deficient (Gas-6\(^{-/-}\)) and wild-type (Gas-6\(^{+/+}\)) mice with the same genetic background (Swiss:129SV, 50:50) were obtained and genotyped as described elsewhere. Five week old male Gas-6\(^{-/-}\) and Gas-6\(^{+/+}\) mice were kept on SFD or HFD during 12 weeks. Food intake and physical activity were measured as described previously.

All animal experiments were approved by the local ethical committee and were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Haemostasis.

Haematologic and metabolic parameters

White blood cells, red blood cells, platelets, haemoglobin and haematocrit levels were determined using standard laboratory assays. PAI-1 antigen levels were measured with a specific home-made ELISA; leptin concentrations in the plasma were determined with a commercially available ELISA (R&D Systems). Blood glucose concentrations were measured
using Glucocard strips (Menarini Diagnostics), and total triglyceride and cholesterol levels were evaluated using routine clinical assays.

**Morphometric and immunohistochemical analysis**

The mean diameter of adipocytes was determined by computer-assisted image analysis as described previously.\(^1\) Blood vessels were visualized using the biotinylated Bandeiraea (Griffonia) Simplicifolia BSI lectin (Sigma-Aldrich).\(^4\) Nine to twelve sections were analyzed per animal and then averaged.

**Adipose tissue dissociation**

Pooled GON or SC fat pads dissected from C57Bl/6 mice kept for 5 weeks on HFD were used to separate mature adipocytes from stromal-vascular (S-V) cells by collagenase treatment as described previously.\(^5\)

**Culture and differentiation of 3T3-F442A and 3T3-C2 cells**

3T3-F442A preadipocytes and 3T3-C2 fibroblasts (a kind gift of Prof. P. Djian)\(^6\) were routinely grown in basal medium: DMEM containing 1 g glucose/L (Gibco, Merelbeke, Belgium) supplemented with biotin (8 mg/ml) and 10% (v/v) FBS (Gibco). To induce differentiation, 3T3-F442A and 3T3-C2 cells were grown to confluence in basal medium. Forty height hours after confluence, cultures were treated for 3 days with an induction medium: basal medium supplemented with 17 nM insulin, 100 nM dexamethasone, 100 \(\mu\)M methylisobutylxanthine and 2 nM triiodothyronine (T\(_3\)). Cultures were then switched to a differentiation medium (basal medium supplemented with 17 nM insulin and 2 nM T\(_3\)) for 7 days (induction and differentiation media were renewed every 2 days).
To investigate the influence of growth arrest on Gas-6 expression, 3T3-F442A preadipocytes were plated at a density of 29x10³ cells/cm² in basal medium containing 10% FBS. After 24 hours, the subconfluent cells were switched to basal medium supplemented with 1% FBS (serum deprivation) during 24 or 48 hours. Growth resumption of cultures maintained during 24 hours in 1% serum was induced by replacing this medium with complete basal medium (10% FBS) and the cells were then incubated for a further 24 hours. Control cultures were maintained during 48 hours in basal medium containing 10% FBS.

RNA extraction and reverse transcription-PCR (RT-PCR)

DNA-free total RNAs were extracted from frozen adipose tissues (GON and SC) or isolated cells by using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. RNA concentrations were measured using the RiboGreen RNA quantification kit (Molecular Probes).

Murine Gas-6, Axl, Sky, C-Mer, adipocyte fatty-acid binding protein (aP2), preadipocyte factor-1 (Pref-1), glycerophosphate dehydrogenase (GPDH), peroxisome proliferator-activated receptor (PPARγ), inhibitor-of-differentiation-2 (Id-2), leptin, tissue inhibitor of matrix metalloprotease (TIMP)-2 and von Willebrand factor (vWF) mRNA levels were quantified by real-time quantitative PCR as described previously⁷ or by semi-quantitative RT-PCR using the GeneAmp Thermostable RNA PCR kit (Applied Biosystems) as described.⁵ The sequences of the primers and probes (Eurogentec, Seraing, Belgium) used in this study are shown in Table I. Samples were normalized using the housekeeping gene 18S rRNA (for real-time quantitative PCR) or 28S rRNA (for semi-quantitative RT-PCR) as described previously.⁵
Table I: Sequences of the primers and probes used for the real time quantitative PCR and semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Probes (FAM-labeled)</th>
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<td>Gas-6</td>
<td>CGAGTCTTCTTCA</td>
<td>GCACTCTTGATATCG</td>
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Statistical analysis

Data are expressed as mean ± SEM. Statistical significance between groups is evaluated using non-parametric t-testing. Values of p < 0.05 are considered statistically significant.

References


**Table I.** Metabolic and haematologic parameters of *Gas-6*<sup>+/−</sup> and *Gas-6*<sup>−/−</sup> mice after 12 weeks of HFD.

<table>
<thead>
<tr>
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<th><em>Gas-6</em>&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th><em>Gas-6</em>&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>84 ± 8.2</td>
<td>66 ± 4.0*</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>160 ± 13</td>
<td>180 ± 14</td>
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<td>Cholesterol (mg/dl)</td>
<td>160 ± 12</td>
<td>170 ± 7.4</td>
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<td>Leptin (ng/ml)</td>
<td>30 ± 6.8</td>
<td>28 ± 3.8</td>
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<td>PAI-1 (ng/ml)</td>
<td>5.4 ± 0.4</td>
<td>4.8 ± 0.7</td>
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</table>

Data are mean ± SEM of 10 experiments; * p < 0.005 and versus *Gas-6*<sup>+/−</sup>