Functional human to mouse adipose tissue xenotransplantation

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Abstract

White adipose tissue (WAT) produces a number of metabolically important factors and, therefore, some inborn errors of metabolism may potentially be corrected by transplantation of normal allogeneic WAT. To explore the ability of human WAT (HuWAT) to compensate for a missing factor and to induce allogeneic immune response, we created leptin-deficient, immunodeficient mice and transplanted them with either 2.5 or 5 ml HuWAT. Recipient mice showed stable levels of human leptin in circulation, reduced body mass gain, and amelioration of hepatic steatosis. Food consumption and plasma insulin levels were reduced only in recipients of 5 ml WAT. Transfer of $2 \times 10^7$ human mononuclear cells to reject WAT as an allograft was ineffective and resulted only in some reduction of circulating leptin and a limited damage to the WAT grafts followed by the loss of human leukocytes. Journal of Endocrinology (2012) 212, 41–47

Introduction

A growing evidence indicates that white adipose tissue (WAT) is an important endocrine organ secreting a number of factors collectively known as adipokines. Some of these factors, such as leptin (Zhang et al. 1994) and adiponectin (Scherer et al. 1995), are unique for adipocytes, whereas others, such as visfatin, apolipoprotein E (ApoE), lipoprotein lipase (LPL), plasminogen activator inhibitor 1 (PAI1), and insulin-like growth factor 1 (IGF1), are also produced by other cell types (Doglio et al. 1987, Sawdew & Loskutoff 1991, Zechner et al. 1991, Fukuara et al. 2005). In humans, congenital isolated leptin deficiency is responsible for severe early onset obesity (Montague et al. 1997, Strobel et al. 1998), while secondary leptin deficiency due to the paucity of WAT is associated with lipodystrophic diabetes (Gold & Steinbach 1967, Seip & Trygstad 1996, Pardini et al. 1998). Deficiencies of other factors have been reported in humans, such as GH-resistant dwarfism due to IGF1 deficiency (Woods et al. 1996, Bonapace et al. 2003), hyperlipoproteinemia caused by mutated ApoE or LPL genes (Zannis & Breslow 1980, Ghiselli et al. 1981, Hayden & Ma 1992), and a bleeding disorder due to PAI1 deficiency (Dieval et al. 1991). These conditions may, in principle, be treated by WAT transplantation (WATTx) for replacement of a missing factor.

Two major approaches may be suggested for WATTx: genetic modification of autologous WAT, including WAT differentiated from WAT-derived stem cells (Zuk et al. 2001), and allogeneic WATTx from normal human donor. In both scenarios, however, it would be important to know whether the graft is recognized by the immune system of the recipient and how graft rejection can be avoided. We have reported that mouse WAT grafts transplanted into leptin-deficient mice may be monitored by reversal of the obese phenotype (Klebanov et al. 2005) and that WAT allografts are acutely rejected unless the host is immunosuppressed (Ablamunits et al. 2007). To study human responses to WAT allograft, we decided to generate a humanized mouse that carries functional human WAT (HuWAT). For that, we bred leptin-deficient (ob/ob), immunodeficient (Rag1 knockout) mice. Our hypothesis was that HuWAT xenograft might also be functionally monitored by the reversal of obese phenotype, whereas a transfer of irrelevant human peripheral blood mononuclear cells (PBMC) would initiate allogeneic immune response to the HuWAT transplant and destroy it with subsequent recurrence of leptin deficiency. Here, we report metabolic and morphologic evidence of long-term survival and function of HuWAT in the mouse and our attempt to reject it as an allograft.

Materials and Methods

Animals

All mouse experiments were approved by the Yale Institutional Animal Care and Use Committee. Leptin-deficient (B6.129S7-Leptob/J), Rag1-deficient (B6.129S7–Rag1<sup>fm1Mom</sup>), and regular C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). They were housed in specific pathogen-free environment and used for breeding or as donors of WAT.
Generation of immunodeficient leptin-deficient mice

Rag1-deficient females were mated with B6.V-Lep\textsuperscript{ob/+} heterozygote males. Resulting heterozygotes for both mutations were intercrossed to produce Rag1\textsuperscript{−/−}Lep\textsuperscript{ob/+} mice, which were identified as IgG negative by ELISA and V-Lep\textsuperscript{ob/+} by PCR (primers: forward, gctatggctggttcttcac; reverse, atcaggtctctgtttta) followed by a restriction enzyme digestion (Hirasawa et al. 1997). These mice were intercrossed and double mutants (Rag1\textsuperscript{−/−}, V-Lep\textsuperscript{ob/ob}) designated here as Rag/ob were identified by the obese phenotype. To render these mice fertile, they were subsequently transplanted with 3–4 ml gonadal WAT from B6 donors and caged as breeding pairs. Progeny of these breeders were all Rag1\textsuperscript{−/−}, V-Lep\textsuperscript{ob/ob} and served as recipients for HuWAT.

HuWAT transplantation

Subcutaneous HuWAT was obtained by liposuction from undislosed donors who underwent this procedure for cosmetic purposes. Each experiment represents a single HuWAT donor so that each mouse within that experiment received equal amount of WAT of the same origin and quality. Samples were irradiated (1000 Rad, X-RAD 320 irradiator, Precision X-Ray, North Branford, CT, USA) to prevent graft vs host disease, and HuWAT (2.5 or 5 ml/mouse) was s.c. injected into multiple points of the dorsal area of recipient mice under isoflurane anesthesia (Forane; Baxter, Deerfield, IL, USA).

HuWAT graft rejection

Normal human leukocytes were obtained from the Central Laboratory Facility, New York Blood Center (Long Island City, NY, USA), and PBMC were separated using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) gradient centrifugation. To reject allogeneic HuWAT, recipient mice were injected with 2×10\textsuperscript{7} PBMC, i.p., 50 days after HuWATTx, and followed for additional 50 days before killing.

Measurements

Body weight was measured weekly. For food consumption, mice were individually caged and food amount in the cage hopper was measured daily for 5 days. To measure plasma leptin and insulin levels, blood samples were taken from the retro-orbital sinus of non-fasting mice on days 50 and 100 after HuWATTx. Quantikine Human Leptin ELISA kit (R&D Systems, Minneapolis, MN, USA) that detects no more than 0.03 ng/ml of a cross-reacting substance in ob/ob mice was used. Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Inc., Downers Grove, IL, USA) was used for measuring insulin in the same samples. Non-fasting glucose was measured from the blood samples of the mice from the tip of the tail and using Easy Check glucose meter (Home Aide Diagnostics, Deerfield Beach, FL, USA). For insulin tolerance test (ITT), mice were fasted for 3 h, injected with Humulin R (Eli Lilly and Company), i.p. (2 U/kg body mass), and blood glucose was measured before and 15, 30, and 60 min after the injection.

Histology

Upon termination of experiments, HuWAT grafts, pancreas, and liver of mice were fixed in 4% formal saline, paraffin embedded, and stained with hematoxylin and eosin (H&E). Morphology was studied using Axioplan 2 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). The degree of hepatic steatosis was evaluated semi-quantitatively by scoring as 0 (no steatosis), 1 (patchy, <50% area), or 2 (severe). Islet size was measured by morphometry using Axiovision 4.8.1 Software (Carl Zeiss).

Flow cytometry

Human leukocytes in circulation were analyzed by flow cytometry (FACSCalibur, BD Immunocytometry Systems, San Jose, CA, USA) using mouse anti-human CD45, CD4, and CD8 antibodies (BD Biosciences, San Diego, CA, USA). Analysis of the data was performed using FlowJo Software (TreeStar, Inc., Ashland, OR, USA).

Data analysis

Data are expressed as mean±s.e.m. To assess the effectiveness of HuWATTx, statistical analysis was performed using GraphPad Prizm Version 5 Software (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA was applied for comparison between the groups. Student’s two-tailed unpaired or paired t-test was applied where applicable and P<0.05 considered significant.

Results

Comparison of Rag/ob and regular ob/ob mice

Rag/ob mice are leptin deficient and hence are overweight, hyperphagic, hyperinsulinemic, and insulin resistant. Figure 1A shows that Rag/ob mice are slightly less overweight than regular ob/ob mice with mean body mass at 3 months of age: 59.0±0.9 (n=13) vs 64.1±1.1 g (n=9) respectively (P=0.009). Despite this, their average food consumption measured at 4 months of age was not different (5.3±0.32 vs 5.6±0.32 g/day per mouse respectively; n=3; not significant (NS); data not shown). Both, Rag/ob and ob/ob mice had similarly elevated non-fasting insulin levels of 21.9±1.5 (n=10) and 19.9±0.9 ng/ml (n=6) respectively (NS; Fig. 1B). Non-fasting blood glucose levels at 3 months were not significantly different (320±41 vs 255±22 mg/dl; NS; Fig. 1C). ITT using 2 U/kg body mass of Humalog revealed
transplantation, blood samples were taken from the mice for determination of human leptin in circulation. As shown in Fig. 1A, all transplanted mice had detectable levels of human leptin, which varied from one experiment to another even within the same experimental set, probably because each individual experiment represented one HuWAT donor. Mean plasma leptin levels in Experiments 1 through 4 were 125 ± 13.5 (n = 6), 58 ± 9.1 (n = 6), 38 ± 3.4 (n = 6), and 194 ± 17.4 (n = 10) pg/ml respectively. In Experiments 5 and 6, plasma leptin levels were 599 ± 6.3 (n = 5) and 250 ± 15.4 (n = 6) pg/ml respectively. In non-transplanted (non-Tx) Rag/ob mice (n = 15), we detected immunoreactive ‘leptin’ at the level of 24 pg/ml in only one animal; the remaining 14 animals had leptin levels below the detection limit of our assay (15-60 pg/ml) and were considered zeros.

Leptin-deficient mice are obese, hyperphagic, and hyperinsulimic due to insulin resistance (Coleman 1978). Transplantation of normal mouse WAT results in the reversal of this phenotype (Klebanov et al. 2005). We, therefore, measured non-fasting plasma insulin as an indicator of physiological efficiency of human leptin in the recipients of HuWAT. Figure 1F shows that, in non-Tx mice (n = 10), insulin levels were 21.9 ± 4.7 ng/ml. Transplantation of 2.5 ml HuWAT was not sufficient to treat hyperinsulinemia, and insulin levels remained high in these mice (20.8 ± 4.0 ng/ml; n = 12). However, transplantation of 5 ml HuWAT reduced insulin almost tenfold (2.4 ± 0.3 ng/ml; n = 14; P < 0.0001). In agreement with reduction of non-fasting insulin, mice transplanted with 5 ml WAT (ATTx 5.0) had an improved ITT (Fig. 1D), suggesting an increased sensitivity to insulin.

To evaluate the effects of HuWAT on body weight of the recipient mice, we compared body mass changes at the end of an 8-week post-transplant observation period. Figure 1G shows that non-Tx Rag/ob mice gained 17.5 ± 0.2 g (n = 12), i.e. significantly more than mice that received 2.5 ml HuWAT (11 ± 1.6 g; n = 28; P < 0.05). This effect on body mass gain was even more striking in the group that received 5 ml HuWAT, in which the recipients actually lost an average of 2.0 ± 1.1 g (n = 13; P < 0.001). As expected from the effects on body mass, HuWATTx was associated with reduced food consumption (Fig. 1H). Non-Tx mice consumed 5.3 ± 0.17 g food daily (n = 5). Mice that received 2.5 ml HuWAT consumed 4.9 ± 0.17 g (n = 6; NS), whereas recipients of 5 ml HuWAT consumed 4.6 ± 0.08 g daily (n = 6; P = 0.017 vs non-Tx; ANOVA).

The persistent presence of human leptin in circulation and the amelioration of leptin-regulated parameters, namely food consumption, body weight gain, and insulin levels, strongly suggest that transplanted HuWAT was functionally active in immunedeficient mice in a long term.

**Morphologic evidence of HuWAT graft function**

Upon termination of each experiment, we could easily identify HuWAT as bright yellow mass, which was harder and much more yellow than normal mouse s.c. WAT. Histological

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**Metabolic evidence of HuWAT graft function**

In the first set of experiments, we transplanted 2.5 ml (Experiments 1–4) and in the second set 5 ml (Experiments 5 and 6) HuWAT into Rag/ob mice. On day 50 after transplantation, blood samples were taken from the mice for determination of human leptin in circulation. As shown in Fig. 1A, all transplanted mice had detectable levels of human leptin, which varied from one experiment to another even within the same experimental set, probably because each individual experiment represented one HuWAT donor. Mean plasma leptin levels in Experiments 1 through 4 were 125 ± 13.5 (n = 6), 58 ± 9.1 (n = 6), 38 ± 3.4 (n = 6), and 194 ± 17.4 (n = 10) pg/ml respectively. In Experiments 5 and 6, plasma leptin levels were 599 ± 6.3 (n = 5) and 250 ± 15.4 (n = 6) pg/ml respectively. In non-transplanted (non-Tx) Rag/ob mice (n = 15), we detected immunoreactive ‘leptin’ at the level of 24 pg/ml in only one animal; the remaining 14 animals had leptin levels below the detection limit of our assay (15-60 pg/ml) and were considered zeros.

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**Morphologic evidence of HuWAT graft function**

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evaluation of the HuWAT grafts usually showed normal adipocytes surrounded by a fibrotic capsule, mild fibrosis, and numerous small blood vessels penetrating the graft (Fig. 2A).

Leptin-deficient ob mice are characterized by lipid accumulation in hepatocytes, i.e. hepatic steatosis. Immuno-deficient Rag/ob mice also develop severe hepatic steatosis (Fig. 2D, an example of score 2). HuWATTx resulted in a reduction of steatosis in most of the recipient mice (Fig. 2E, an example of score 1), and even in a complete normalization of liver morphology in some (Fig. 2F, an example of score 0). We scored liver sections in a blinded fashion and compared the severity of hepatic steatosis in HuWAT recipients to that in non-Tx animals. Figure 3A shows that, in non-Tx animals, mean steatosis score was 1.6 ± 0.15 (n = 11), i.e. higher than in the recipients of 2.5 ml HuWAT (0.87 ± 0.13; n = 16; P < 0.01), and much higher than in the recipients of 5 ml HuWAT (0.55 ± 0.16; n = 11; P < 0.001, ANOVA). Liver steatosis was completely resolved in three out of 16 (18.7%) and in five of 11 (45.5%) recipients of 2.5 and 5 ml HuWAT respectively.

Insulin resistance due to leptin deficiency is associated with hypertrophy of pancreatic islets. To test whether HuWATTx is capable of reducing islet size and total islet mass, we performed islet morphometry on H&E-stained pancreatic sections and compared islet sizes and total islet area between the mouse groups. Mean islet area in non-Tx group (n = 7) was 26 360 ± 2169 μm² (range 16 476–32 419 μm²); it was not different from that in recipients of 2.5 ml (n = 6) or 5 ml (n = 12) HuWAT, 22 968 ± 1920 (range 14 344–26 753) and 24 000 ± 2369 (range 13 267–43 379) μm² respectively.

There was, however, a trend toward a reduction of the total islet area in HuAT-recipient mice (Fig. 3B). In non-Tx animals, the total islet area was 1.45 ± 0.24 mm² (n = 7; range 0.63–2.32 mm²). In the recipients of 2.5 ml (n = 6) and 5 ml (n = 12) HuWAT, the total islet area was 0.96 ± 0.13 (range 0.62–1.39) and 0.89 ± 0.15 (range 0.28–1.72) mm² respectively (NS).

These data suggest that, although leptin levels achieved were not sufficient to significantly reduce islet size, human leptin in circulation was associated with a considerable alleviation of hepatic steatosis in the recipients of HuWAT, arguing in favor of physiological significance of HuWATTx.

Rejection of HuWAT allograft

Before testing whether HuWAT transplanted into the mouse can be rejected as an allograft, we assessed the stability of HuWAT grafts over time. We took blood samples from the recipient mice on days 50 and 100 after transplantation and compared leptin levels. Figure 4A shows that there was no change in circulating leptin levels over this period of time (162 ± 26.2 and 150 ± 24.8 ng/ml on days 50 and 100 respectively; n = 12; NS), suggesting that HuWAT graft was functionally stable in the long term.

Recipient mice with established grafts (day 50 post transplant) were then injected with allogeneic PBMC from irrelevant healthy human donors. Mice were observed for additional 50 days, blood samples were taken for measuring leptin levels, and mice were then killed for histology. Figure 4B–D show data from Experiments 4–6 respectively. PBMC injection slightly reduced circulating leptin levels in Experiments 4 (from 194 ± 17.4 to 156 ± 11.3 (n = 10; 19.6% reduction) and 5 (from 506 ± 75.4 to 434 ± 64.9 ng/ml (n = 7; 14.2% reduction)) and had no effect on leptin levels in Experiment 6.

In agreement with a very modest reduction in leptin levels observed on day 50 post-PBMC, body weight dynamic in HuWAT recipients treated with PBMC was very similar to that of mice that did not get PBMC: animals in Experiment 4 gained 3.7 ± 0.55 g; in Experiment 5 lost 0.3 ± 0.46 g; and in Experiment 6 gained 0.42 ± 0.37 g. This was much less than...
the body weight gain of non-Tx animals, which was 10.3 ± 2.0 g (P < 0.001 by ANOVA).

Histological evaluation on day 50 after injection of PBMC revealed mostly well-preserved grafts with some areas of damage as judged by fusion of adipocytes into large cysts and accumulation of small lipid droplets in the stroma (Fig. 2B and C). Grafts that were infiltrated by mononuclear cells were seen in only two recipients (Fig. 2C). All grafts remained well vascularized.

To understand the reason for incomplete rejection of HuWAT by allogeneic PBMC, we analyzed mouse spleens and blood for the presence of human lymphocytes by flow cytometry on day 50 after PBMC injection. Surprisingly, no human CD45<sup>+</sup> cells were found in the recipient mice, suggesting that the cells did not survive in the Rag/ob environment (data not shown).

### Discussion

Recent recognition of WAT as an important endocrine organ stimulated studies on its transplantation (Tran & Kahn 2010). In mouse models of isolated leptin deficiency and lipodystrophy, congenic WATTx restored metabolic abnormalities (Gavrilova et al. 2000, Klebanov et al. 2005). Clinical WATTx, however, have been limited to esthetic and reconstructive surgeries. In addition to limited applicability of WATTx as a replacement therapy for treatment of rare diseases such as isolated or lipodystrophic leptin deficiency, deficiencies of ApoE, IGF1, and some other factors, s.c. WAT has a potential to correct symptoms associated with central obesity, such as insulin resistance (Tran et al. 2008). In contrast to other transplantation approaches, where donor organs and tissues are the major limiting factor, allogeneic s.c. WAT is widely available as a by-product of liposuction. In addition, WATTx is technically very simple. However, as long as allogeneic donor tissue is considered, graft rejection will be an obstacle to this treatment, and conventional immune suppression may outweigh the benefits of this approach.

To study the mechanisms involved in rejection of allogeneic HuWAT, we created a humanized mouse that carried HuWAT grafts. Our data demonstrate that all mice transplanted with HuWAT had clearly detectable human leptin in circulation, although plasma levels of this adipokine varied among HuWAT donors (Fig. 1A). This could be due to different degrees of engraftment, or reflect intrinsic interindividual differences in the leptin-producing capacity of HuWAT. Human leptin remained stable at least 100 days after transplantation (Fig. 4A).

Although generally low, leptin levels in HuWAT recipients were physiologically significant, as evidenced by their effect on body weight gain, food consumption, insulin levels, and liver steatosis (Figs 1B–D, 2D–E, and 3A). The effects on food uptake and insulin levels were seen only in the group that received larger volumes of HuWAT. It is interesting that mouse liver appeared to be very sensitive to leptin: steatosis was improved even in the recipients of 2.5 ml HuWAT. Unfortunately, unlike body mass and circulating hormone measurements, evaluation of steatosis cannot be used for continuous monitoring of the graft function. Taken together, our data show that HuWAT engrafts and is functional for a long term in immunodeficient ob/ob mice. Although HuWATTx in immunodeficient mice has been reported previously (Yi et al. 2006, Mojallal et al. 2009, Hamed et al. 2010, Ko et al. 2011), to our knowledge, our report is the first that demonstrates HuWAT graft function.

The goal of this study was to establish a model that would allow, by simple metabolic monitoring, to assess HuWAT rejection by unrelated, human leukocyte antigens-mismatched PBMC. Surprisingly, human PBMC damaged only some of the HuWAT grafts, and the damage was generally not severe. After PBMC injection, leptin levels remained either unaffected (in one out of three cases) or only moderately decreased (in the other two cases). Human PBMC were not found in mouse circulation or spleen 50 days after injection,
suggested that, although capable of initial allogeneic recognition of HuWAT, PBMC were unable to propagate and survive sufficiently. The reason for this is unclear and may be due to low leptin levels, since leptin is known to be important for the immune system (Lam & Lu 2007, Fernández-Riejos et al. 2010). Another possibility is depletion of human PBMC by mouse NK cells: our recipient mice are on C57BL/6 background known to have relatively high NK activity compared with NOD strain (Poulton et al. 2001). This may be overcome by breeding an inactivated allele of common cyclotrope receptor γ chain: human PBMC readily expand in NOD.scid mice that carry this defect (V Ablamunits & K C Herold, unpublished observations; King et al. 2009, Pino et al. 2010). Alternatively, leptin deficiency may be bred onto Rag1-deficient, perforin-deficient NOD mice, which have been reported to lack NK cytotoxicity and allow for a better engraftment of human T cells (Shultz et al. 2003). If, indeed, high mouse NK activity is responsible for the loss of human PBMC, it is noteworthy that HuWAT grafts appear to be resistant to killing by NK cells. In addition, it has been demonstrated that signal regulatory protein α on NOD, but not on B6 phagocytes, can interact with ubiquitously expressed human CD47 with subsequent inhibition of phagocytes; this accounts for a better human hematopoietic stem cell engraftment in NOD.scid recipients (Takena et al. 2007). Again, if B6 phagocytes’ inability to interact with human CD47 is responsible for the loss of human lymphocytes, i.e. by phagocytosis, it is interesting that HuWAT is not susceptible to this mechanism.

This study utilized only HuWAT samples from s.c. sites and provided a proof of principle that human adipokines might be monitored in the mouse. However, humanized mouse that carries HuWAT grafts may also be used to compare biological effects of various HuWAT depots (Vohl et al. 2004, Tran et al. 2008) on metabolism, as well as their responses to therapies. Furthermore, immunodeficient mice that lack factors other than leptin (i.e. ApoE and IGF1) can be used to reveal the potential of WAT to restore these factors to physiological levels in vitro.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

V A did the experiments, analyzed the data, and wrote the manuscript. S K did the experiments, analyzed the data, and wrote the manuscript. S Y G provided HuWAT and wrote the manuscript. K C H provided laboratory space and equipment and wrote the manuscript.

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