EXPERIMENTAL STUDY

Development of obesity in transgenic rats with low circulating growth hormone levels: involvement of leptin resistance

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Abstract

Background: Human growth hormone (hGH) transgenic (TG) rats have been produced in our laboratory. These TG rats are characterized by low circulating hGH levels, virtually no endogenous rGH secretion, and massive obesity.

Objective: To elucidate how energy balance and leptin sensitivity contributed to the establishment of this obesity.

Design and methods: Food intake, locomotor activity and leptin concentrations in serum and cerebrospinal fluid were measured in TG rats and their non-transgenic littermates (control). The effect of intraperitoneal and intracerebroventricular injection of leptin on food intake and body weight gain was also examined.

Results: An increase in food intake and a decrease in locomotor activity were observed from 4 and 7 weeks of age, respectively, in the transgenic rats compared with control. Serum leptin concentrations of the transgenic rats were more than twice as high as those of control rats and were associated with an increased white adipose tissue mass and *ob* gene expression. Intraperitoneal injection of leptin significantly decreased food intake and body weight gain in control rats, but not in transgenic rats. Leptin concentration in the cerebrospinal fluid of transgenic rats was not different from that of control rats, and intracerebroventricular injection of leptin was similarly effective in reducing food intake and body weight gain as it was in control rats.

Conclusions: These results suggest that the transgenic rats, whose GH secretion is suppressed, develop obesity due to early onset of an increase in food intake and a decrease in locomotor activity with leptin resistance resulting from deteriorating leptin transport from peripheral blood to cerebrospinal fluid.

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Introduction

Growth hormone (GH), a 22 kDa peptide secreted from the pituitary, is not only a hormone which stimulates skeletal bone growth, but also an endocrine regulator of carbohydrate and lipid metabolism (1, 2). In the latter context, it was shown that GH-deficient subjects have an increased body fat mass, which can be reduced by GH therapy (1–4), and that GH had beneficial effects in obese (5) or obese diet-restricted subjects (6). Obesity develops ultimately under conditions in which energy intake and expenditure are unbalanced, but it has yet to be fully elucidated how GH affects these processes.

Leptin, a 16 kDa peptide hormone the gene of which was positionally cloned in 1994 (7), is an adipocyte-derived hormone that decreases food intake and increases energy expenditure, thereby leading to a marked reduction in body weight (8, 9). The leptin receptor has five or more splice variants expressed in a tissue-specific fashion (10, 11). It has been reported that the absence of leptin or a functional leptin receptor causes massive obesity and non-insulin-dependent diabetes mellitus (7, 12, 13), and that leptin levels correlated with specific estimates of body fat (14, 15). In GH-deficient adults, an increased circulating level of leptin is also observed (16–18). In obese individuals, however, their high plasma leptin levels do not induce the anticipated responses, which has led to the concept of leptin resistance (19).

We previously reported the production of a strain of human growth hormone (hGH) transgenic rats that exhibited unique phenotypes. Serum hGH level was relatively low and the endogenous rat GH (rGH) was almost totally suppressed in these transgenic animals. They developed severe obesity and insulin resistance, while their body length was almost normal (20, 21). In

addition, blood glucose, insulin, triglyceride and free fatty acid levels, which are known to be indicators of early diabetes, were elevated. Since the treatment with hGH reduced adipose tissue mass and restored normal weight gain (21), our transgenic rats are regarded as a useful model for analyzing how impaired GH secretory pattern induces obesity.

In the present study, we focused on changes in circulating leptin levels in relation with development of obesity as well as energy balance, i.e. food intake and locomotor activity in these animals. In addition, the effect of intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injection of leptin on food intake and body weight change were also examined to assess how leptin resistance was involved in the development of obesity in these transgenic rats.

Materials and methods

Animals and physiological measurements

Generation of the hGH transgenic rats has been described previously (17). In this experiment, the male transgenic rats (heterozygotes) and their male littermates were used. After being weaned from the mother at 3 weeks of age, they were individually housed with free access to laboratory chow and water. They were kept under constant room temperature ($23 \pm 1\,°C$) and a lighting schedule of 12 h light–12 h dark, lights on at 0500 h). Body weight and food intake over 24 h were recorded once a week from 3 to 15 weeks of age. Locomotor activity was continuously monitored in cages equipped with an infrared counter (Muromachi Kikai, Tokyo, Japan) from 4 to 12 weeks of age. For the measurement of serum leptin concentration, a blood sample was collected from the tail vein every 2 weeks.

Northern hybridization for ob gene

The adipose tissue was obtained from the epididymal fat pad of transgenic or control rats at 12 weeks of age. Total RNA was extracted according to the method described by Louveau *et al.* (22). For the preparation of the rat *ob* cDNA fragment, RT-PCR was performed with the following primers: $5'$-GAGGAAAATGTGCTGAGA-$3'$ as sense primer and $5'$-TGGTGGCCTTTGAAACTTCA-$3'$ as antisense primer corresponding to the nucleotide sequence of rat *ob* cDNA (23). The PCR product was labeled with ^{32}P and used as a probe for Northern hybridization.

Equal masses of total RNA from the adipose tissue were subjected to electrophoresis and photographed after ethidium bromide staining. The RNA was transferred to a nylon membrane (Biodyne B Membrane, Pall BioSupport, Port Washington, NY, USA). After prehybridization, the membranes were hybridized with the specific ^{32}P-labeled probe in Church-phosphate buffer (24). After washing, the membrane was exposed to

X-ray film (Eastman Kodak, Rochester, NY, USA) for 72 h at $-70\,°C$. The pictures were scanned by the scanner (GT-6500, Epson, Tokyo, Japan), and the relative intensity of each band was analyzed using NIH imaging software.

Leptin determination

Thirteen-week-old rats were used for determination of basal leptin concentrations in both the cerebrospinal fluid (CSF) and serum. The rats were anesthetized with pentobarbital sodium and placed in the stereotaxic apparatus (Narishige, Tokyo, Japan). The CSF was withdrawn under gentle suction after introduction of a 27-gauge needle into the cisterna magna. Only CSF samples which were not contaminated with blood, as judged by the absence of an erythrocyte pellet after centrifugation, were used. Blood was obtained by cardiac puncture and the resultant serum samples were used for leptin determinations. After decapitation, epididymal white adipose tissue (WAT) was dissected out and weighed.

Leptin concentration in serum and CSF was determined using a commercial RIA kit (Linco Research Inc., St Charles, MO, USA). All assays were performed in duplicate or triplicate.

Leptin treatment

Transgenic and control rats between 10 and 12 weeks of age were injected i.p. with mouse recombinant leptin (1 mg/rat) or vehicle. For i.c.v. injection, a stainless steel cannula (0.9 mm o.d., 0.6 mm i.d.) was stereotaxically implanted into the third ventricle as previously described (25) using coordinates based on Paxinos & Watson (26). The coordinates were 0 mm lateral, 6.0 mm anterior from the ear bar and 9.5 mm below the endocranium. The cannula was fixed to the skull with anchor screws and dental cement. A sterile stainless steel stylet was inserted into the cannula to prevent formation of clots in the lumen. Five days after surgery, the animals were injected i.c.v. with murine recombinant leptin (Pepro Tech EC Ltd, London, UK), (2 or 20 μg per 10 μl per rat) or vehicle without anesthesia. All injections were given between 1800 and 1900 h. Body weight and food intake were measured 24 h after injection. At the end of the experimental period rats were humanely killed and coronal sections were cut to confirm i.c.v. cannula placement. Only data from animals that showed correct i.c.v. cannula placement were included in the present results.

Statistics

All the data were analyzed using ANOVA followed by Student's *t*-test, the difference was considered significant at $P < 0.05$.

Results

Body weight, locomotor activity and food intake in transgenic and control rats

Changes in body weight, food intake and locomotor activity of hGH transgenic and control rats after weaning are shown in Fig. 1. The body weight of the transgenic rats did not differ from that of the control rats from 3 to 12 weeks of age except at 8 weeks, when control rats were slightly but significantly heavier than transgenic rats. After 12 weeks, transgenic rats were significantly heavier than control rats, and the difference gradually increased with age. Despite no difference in body weight up to week 12, transgenic rats already took significantly more food than control rats by 4 weeks of age. At its maximum (13 weeks old), the food consumption of the transgenic rats was greater by 36% than that of control rats. Locomotor activity was not different between transgenic and control rats younger than 5 weeks old. Unfortunately, records of locomotor activity at 6 weeks old are missing because of computer trouble. Although locomotor activity gradually decreased as the animals grew older, even in the control group, the decrease in locomotor activity was much steeper in transgenic rats. This decrease in locomotor activity in the transgenic rats was mostly due to a decrease in activity during the dark phase, since the activity during the light phase was not significantly different (data not shown).

Leptin levels in serum and CSF

Changes in serum leptin concentrations from 4 to 10 weeks of age are shown in Fig. 2A. Serum leptin concentrations of the transgenic rats were more than twice as high as than those of control rats throughout the experimental period. As shown in Fig. 2B, *ob* gene expression in the adipose tissue of the epididymal fat pad was significantly higher in transgenic than control rats when examined at 12 weeks of age.

Using control and the transgenic rats at 13 weeks of age, basal leptin concentrations were determined in both serum and CSF, together with body weight and epididymal white adipose tissue (WAT) weight (Table 1). Body weight and epididymal WAT weight of the transgenic rats were heavier by 16% and 109% than those of control rats, respectively. Serum leptin
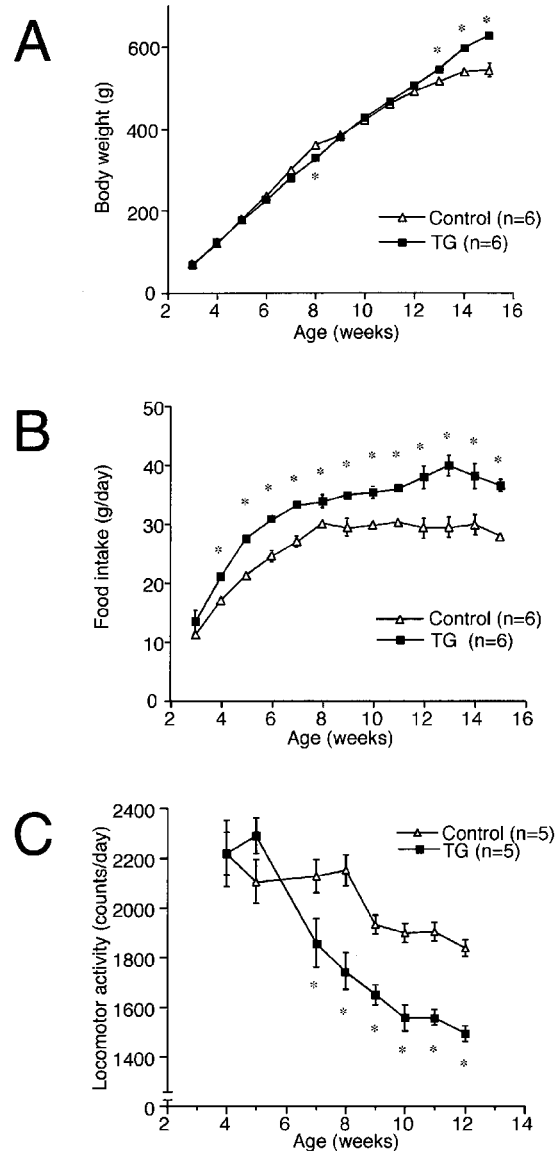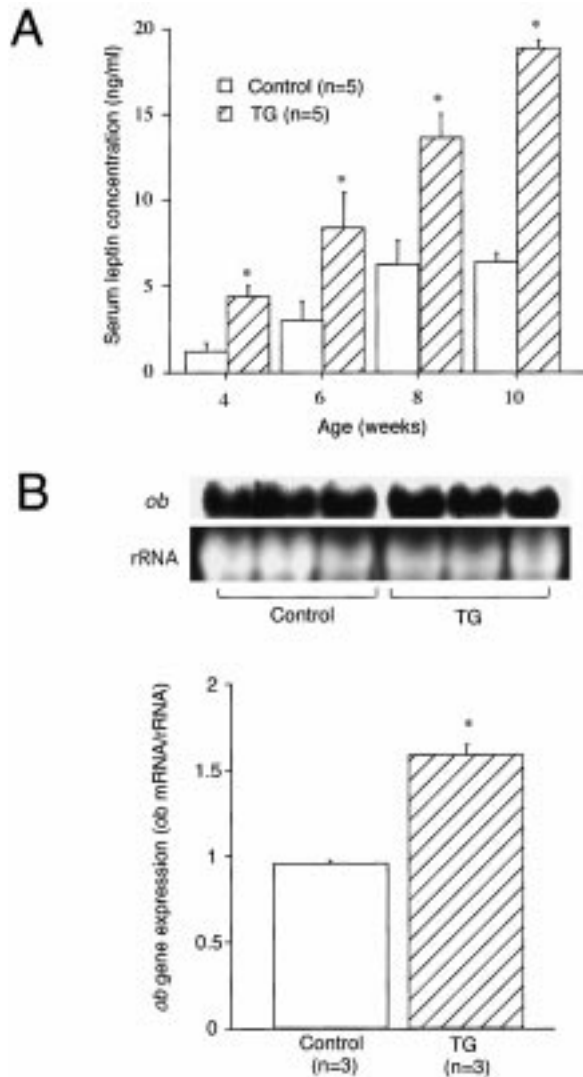

Figure 1 Changes in body weight (A), food intake (B), and locomotor activity (C) in the transgenic and control rats. Symbols and error bars represent means ± s.e.m. *Significantly different (*P*<0.05) from control. TG, transgenic rats.

concentrations in the transgenic rats were about four times higher than those of control rats. On the other hand, there was no difference in leptin concentration in CSF between the control and the transgenic rats.

Table 1. Body weight, adipose tissue weight and leptin concentration in serum and CSF of control and transgenic rats.

| Groups[a]	BW (g)	WAT (g)	WAT/BW (%)	Serum leptin concentration (ng/ml)	CSF leptin concentration (ng/ml)
Control rats (*n* = 4)	544 ± 16.9	10.1 ± 0.64	1.73 ± 0.14	4.81 ± 0.19	0.07 ± 0.04
Trangenic rats (*n* = 5)	630 ± 4.7*	21.1 ± 0.34*	2.84 ± 0.05*	20.63 ± 1.89*	0.06 ± 0.02

Values are means ± s.e.m. [a] Animals were at 13 weeks of age. * Significantly different (*P* < 0.05) from control rats. CSF, cerebrospinal fluid; BW, body weight; WAT, bilateral epidymal white adipose tissue.

Figure 2 (A) Changes in serum leptin concentrations in the transgenic and control rats. Columns and error bars represent means ± s.e.m. *Significantly different (*P*<0.05) from control. TG, transgenic rats. (B) *Ob* gene expression in the epididymal adipose tissue of the transgenic and control rats at 12 weeks of age. Upper panel, Northern blot data; lower panel, densimetrically analyzed data. Columns and error bars represent means ± s.e.m. *Significantly different (*P*<0.05) from control. TG, transgenic rats.

Effect of i.p. or i.c.v. injection of recombinant leptin on food intake and body weight

In control rats treated i.p. with mouse recombinant leptin (1.0 mg/rat), food intake over 24 h was significantly reduced compared with that of rats treated with vehicle (Fig. 3A). On the contrary, no significant changes in food intake were induced in the transgenic rats by the i.p. leptin treatment. In control rats, body weight gain in the leptin-treated group was reduced significantly compared with that in the vehicle-treated group (Fig. 3B). In the transgenic rats, however, no
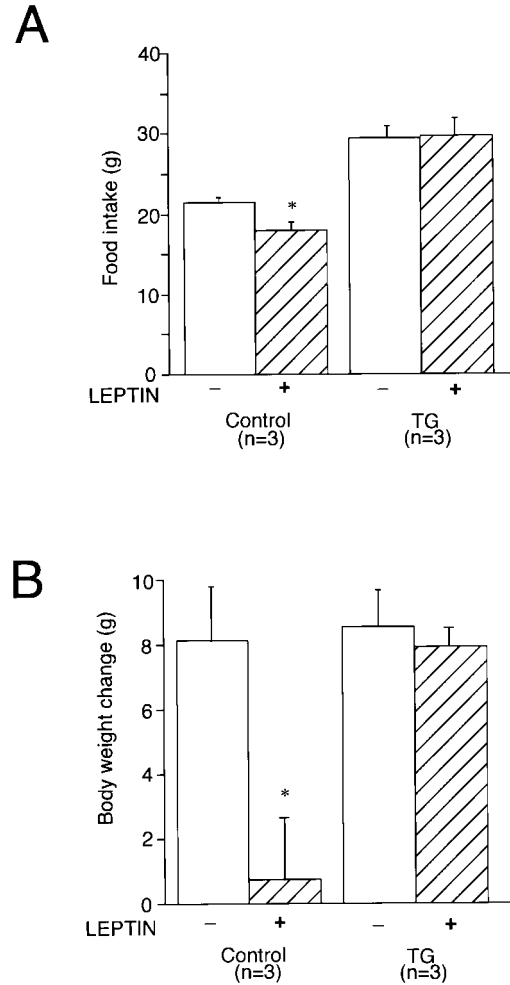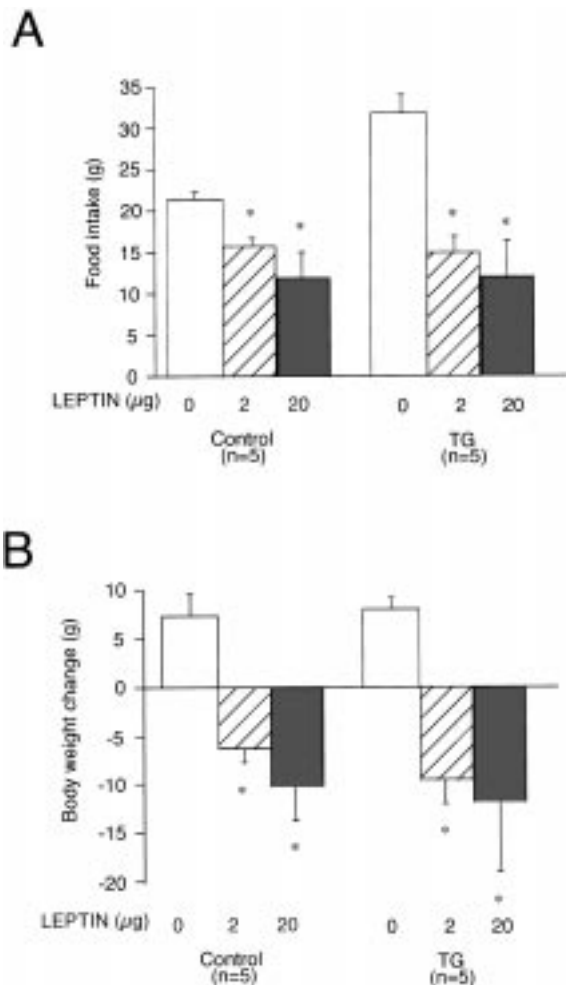
Figure 3 Effect of i.p. leptin injection on food intake (A) and body weight change (B) for 24 h. Columns and error bars represent means ± s.e.m. *Significantly different (*P*<0.05) from control. TG, transgenic rats.

significant differences in body weight gain were noted between leptin- and vehicle-treated animals (Fig. 3B).

In both control and transgenic rats, significant decreases in food intake and body weight gain were induced by i.c.v. leptin treatment in a dose-dependent manner (Fig. 4A and B).

Discussion

In the present study, a significant increase in body weight in hGH transgenic rats compared with control rats was first discernible at 13 weeks of age. This is the age at which onset of hyperlipidemia, hyperglycemia and hyperinsulinemia (21) occurred, thereafter the rats developed severe obesity. However, food intake was already augmented at 4 weeks of age and locomotor activity started to decline from 7 weeks of age, indicating that behavioral changes began much earlier

Figure 4 Effect of i.c.v. leptin injection on food intake (A) and body weight change (B) for 24 h. Columns and error bars represent means ± s.e.m. *Significantly different (P<0.05) from control. TG, transgenic rats.

than apparent metabolic abnormalities as well as abnormal body weight gain. In addition, we previously reported (21) that significant fat deposition in the epididymal fat pad of the transgenic rats was already evident at 4 weeks of age. This is consistent with the present observation that serum leptin levels were much higher in transgenic than control rats at 4 weeks of age. Taken together, the transgenic rats consume more food and accumulate excess energy in fat tissue as early as 4 weeks of age due to impaired GH secretory pattern caused by the transgenic product.

So far, there are no clear experimental observations on the relationship between GH deficiency and leptin, since the existing GH-deficient animals do not develop severe obesity. In the present study, serum leptin levels were found to be much higher in transgenic than in control rats. In addition, *ob* gene expression in the adipose tissue of transgenic rats was enhanced as well.

An increase in serum leptin levels in the transgenic rats may be the result of both increased adipose tissue mass and up-regulated *ob* gene expressions. An elevated circulating leptin level was reported in GH-deficient subjects whose increased leptin levels were reduced by long-term treatment with GH (16–18). This effect of GH on leptin levels is supposed to be indirect because even chronic incubation of isolated adipocytes with neither GH nor insulin-like growth factor-I (IGF-I) affects both leptin gene expression and secretion (27). Since insulin is shown to increase leptin mRNA under both *in vivo* (28, 29) and *in vitro* (30), hyperinsulinemia rather than GH status in the transgenic rats may presumably contribute to the up-regulation of *ob* gene expression.

Leptin is known to have several behavioral and metabolic effects, such as a decrease in food intake and increases in physical activity and energy expenditure (8, 9). In our transgenic rats, however, despite much-elevated leptin levels, enhanced food intake and suppressed locomotor activity were observed. These observations provided experimental evidence that leptin resistance occurred in the transgenic rats. This conclusion was further supported by the results that systemic administration of leptin, which decreased food intake and increased locomotor activity in the control rats, did not affect these parameters in the transgenic rats. Interestingly, however, direct administration of leptin into the brain was similarly effective in both transgenic and control rats, suggesting a dysfunction of transmission of peripheral leptin signals to the central nervous system in the transgenic rats.

The biological actions of leptin are thought to be mediated largely through interactions with its receptors that are expressed in the hypothalamus (31). Because the leptin molecule seems too large to cross the blood–brain barrier, it appears to be transported across the blood–brain barrier by a saturable system (32). Thus, while there is a correlation between plasma and CSF levels of leptin, the relationship is not a linear function, but rather is characterized by a logarithmic relationship between CSF and plasma concentrations of leptin. Recently, this relationship has been postulated as one of the causes of leptin resistance in human (33, 34). In the present study, we found that even though circulating leptin levels were much higher in the transgenic than in the control rats, CSF leptin levels were not different between the two groups. This observation provides experimental evidence that leptin resistance occurs at least partially at the level of transportation of leptin into the brain.

The leptin receptor is a single membrane-spanning protein that resembles gp130, a member of the class I cytokine receptor superfamily (32). The leptin receptor has five or more splice variants expressed in a tissue-specific fashion (10, 11). Wu-Peng *et al.* have reported that the leptin receptor could well be the main candidate for transporting leptin (35). It is thought likely that OB-Ra, a short form of leptin receptor, is the transporter

molecule accounting for uptake into the brain, since it is expressed in the choroid plexus. In this regard, the expression level of OB-Ra in the choroid plexus in the transgenic rats might be lower than the control rats. The early onset of behavioral changes suggests the early onset of leptin resistance, which is presumably one of the primary causes of obesity in the transgenic rat with low circulating levels of GH.

In conclusion, the present study showed that the hGH transgenic rats develop obesity due to early onset of an increase in food intake and a decrease in locomotor activity with leptin resistance, which might result from the suppression of leptin transport from the blood to CSF. The basis for the development of leptin resistance in the transgenic rats could provide important implication for understanding the pathogenesis of fat accumulation in GH-deficient subjects.

Acknowledgements

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