Association Between Body Weight at Weaning and Remodeling in the Subcutaneous Adipose Tissue of Obese Adult Mice With Undernourishment In Utero

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Abstract

Rapid growth in infancy considerably increases the risk of obesity and metabolic disorders in adulthood especially among neonates born small. To investigate the mechanism involved, we developed an animal model of undernourishment in utero by maternal caloric restriction, in which the Z scores of body weight at weaning (19.5 days) positively correlated with parameters of obesity, metabolic disorders, and remodeling of subcutaneous adipose tissue, such as numbers of macrophages in adipose tissue, the ratio of inflammatory M1 to anti-inflammatory M2 macrophages, estimated by gene expression of specific antigens, and the relative ratio of small adipocytes less than 30 μ m in diameter, on a high-fat diet at 17 weeks of age. To our knowledge, this is the first report of a possible connection between infantile body weight and adipose tissue remodeling in obesity after undernourishment in utero.

Keywords

obesity, pregnancy, developmental origins of health and disease (DOHaD), low birth weight, high-fat diet, macrophage

Introduction

Obesity has increased in developed as well as developing countries and is now a worldwide public health issue.¹ As obesity is closely associated with reduced glucose tolerance, dyslipidemia, and hypertension, the concept of metabolic syndrome has been proposed.^{2,3} Epidemiological evidence suggests that intrauterine undernutrition is closely associated with obesity in adulthood.⁴⁻⁶

Eriksson et al, reported that a high body mass index (BMI) at the age of 7 years is a risk factor for adult obesity if born small.⁷ Ong et al demonstrated by systematic reviews that rapid infancy weight gain is consistently associated with increased subsequent obesity risk.⁸ As for the critical window of early weight gain, some suggested an importance of the first 6 months⁹ and others the first week of life.¹⁰ Thus, the critical window of early weight gain remains controversial concerning the risk of obesity in later life. Nevertheless, increasing epidemiological evidence supports the concept that a high growth rate after birth considerably increases the risk of adult obesity especially in neonates born small.

Numerous animal models have been proposed for adult obesity associated with insults in utero, such as maternal caloric restriction (CR),¹¹⁻¹⁶ maternal protein restriction,¹⁷⁻²⁰ uterine artery ligation,²¹ hemiovariectomy prior to pregnancy,²²

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In the present study, we hypothesized that rapid infantile growth of small newborns accelerates adipose tissue remodeling in obesity and worsens obesity-related metabolic disorders. The study's first aim was to develop a mouse model in which body weight at weaning (19.5 days) parallels the development of obesity on a high-fat diet (HFD; 9-17 weeks) when exposed to undernourishment in utero by maternal CR. The second aim of the study was to investigate the association between body

We modified a previously reported animal model of maternal CR^{11,12,36-38} and successfully developed a new model in which the Z scores of body weight at weaning (19.5 days) positively correlated with parameters of obesity on an HFD at 17 weeks of age in CR, but not in AD, pups. In CR pups, the Z scores of body weight at weaning (19.5 days) positively correlated with those of macrophage detection rates, the inflammatory M1/anti-inflammatory M2 macrophage ratio, estimated by gene expression of specific markers, and the ratio of small adipocytes, in subcutaneous adipose tissue, and blood glucose and total cholesterol levels on an HFD at 17 weeks.

Mouse Model of Undernutrition in Utero

The procedures used are illustrated in Figures 1 and 2. We modified a previously reported animal model of maternal CR,^{11,12,36–38} so that body weight at weaning (19.5 days) positively correlates with the development of obesity on an HFD at 17 weeks. In brief, pregnant C57Bl/6 mice were purchased at 7.5 days postcoitum (dpc) from Japan SLC, Inc (Hamamatsu, Japan) and housed individually with free access to water during 12-h/12-h light/dark cycles. Regular chow (formula number D06121301, Research Diets Inc, New

Figure 2. Schematic illustration of experimental procedures in pups. Four cohort groups of pups, groups A, B, C, and D, were classified at weaning (19.5 days). AD indicates ad libitum; CR, caloric restriction.

Brunswick, New Jersey) was grinded into a fine powder and placed in a feeding basket (SN-950; Shinano Manufacturing, Co, Ltd, Tokyo, Japan). The use of powdered chow resulted in a 10% increase in ad libitum calorie intake compared with that of solid chow of identical content (data not shown). Dams were divided into 2 groups at 11.5 dpc. One group was fed ad libitum with the powdered regular chow (AD). The daily caloric intake of the other group (CR) was restricted to 70% of the calories consumed by AD dams, based on the data of the previous day, from 11.5 dpc to the day before delivery of the pups (17.5 dpc). In the morning of 18.5 dpc, 2.5 g of powdered standard diet was supplied to CR dams, followed by 3.5 g of extra food in the evening of 18.5 dpc, just before the night of parturition, to prevent them from eating their own pups. Both AD and CR dams were fed ad libitum after delivery, but the powdered chow was changed to sticks 2.5 days after delivery.

The same mothers nursed pups, and the number of pups was adjusted to 8 per litter at 5.5 days of age (days). Gender was not taken into consideration in the adjustment of litter size. At 19.5 days, AD and CR pups were weaned and divided into 2 groups, that is, individual body weight above (UP) and below (LO) the mean body weight of the group at weaning, resulting in groups of A (LO of AD), B (UP of AD), C (LO of CR), and D (UP of CR). Rodent Lab Diet EQ 5L37 (Japan SLC, Inc, Hamamatsu, Japan) was supplied after weaning. An HFD containing 60% lipid (formula number D12492, Research Diets Inc) was supplied to all the pups from 9 to 17 weeks. The BMI of pups was calculated using the formula $\text{weight}/(\text{height} \times \text{length between the tip of nose and the base of tail})^2$ and expressed as g/cm^2 . Changes in dam and litter weights as well as caloric intake are summarized in Figure 4.

We started the strict identification of each pup with an ear tag at weaning (19.5 days) because of technical difficulty

Table 1. Numbers of Dams and Pups Used in the Experiments.

		Number of Dams		Number of Pups	
		AD	CR	AD	CR
19.5 Days	Body weight	8	7	31	26
	Adipose tissue weight	8	7	30	26
	Adipocyte diameter	8	7	8	7
	Blood glucose level	8	7	31	26
8 Weeks	Body weight	11	9	30	37
	Adipose tissue weight	11	9	30	37
	Adipocyte diameter	8	7	8	7
	Blood glucose level	11	9	30	37
17 Weeks	Total cholesterol, triglyceride, and HDL levels	11	9	30	37
	Body weight	10	10	20	30
	Adipose tissue weight	10	10	18	27
	F4/80-positive cell/field	10	10	20	29
	Adipocyte diameter	10	10	17	29
	mRNA expression	10	10	18	21
	Blood glucose level	10	10	18	26
Total cholesterol, triglyceride, and HDL levels		10	10	18	26

Abbreviations: AD indicates ad libitum; CR, caloric restriction; HDL, high-density lipoprotein; mRNA, messenger RNA.

during earlier periods and used the *Z* scores of body weight at weaning as a parameter of early growth after birth in the present study. However, even before weaning, we weighed the pups repeatedly and obtained rates (percentage increase/day; %/d) by assessing the weight of each pup from the order of the weight in a litter from 12.5 to 19.5 days of age.

Only male pups were used for the subsequent experiments except for the data on fetal body weight. Female pups were not studied because body weight gain on an HFD was more prominent in males than in females among AD (Figure 4D) as well as CR (Figure 4E) offspring and of the limited animal care space available. After establishment of the animal model, the experiment was repeated 3 times and representative data were addressed. Numbers of pups and dams used in each study are summarized in Table 1.

Festing recommended that researchers use the number of dams in developmental animal studies.³⁹ However, we considered heterogeneous infantile growth, including the difference among a litter, important to an animal model in which a variety of changes in body weight at weaning (19.5 days) affect the development of adult obesity; therefore, we used the number of pups, not dams, as the basic unit of each cohort in the present study.

All experimental procedures were conducted in accordance with the standards of humane animal care and approved by the Animal Research Committee, Hamamatsu University School of Medicine (H20-014).

Tissue Sampling

Sampling points are illustrated in Figure 2. In an independent experimental cohort, all pups were delivered by uterine section at 18.5 dpc and weighed. At 19.5 days, corresponding to the time of weaning, the pups were weighed and decapitated, and samples of blood and subcutaneous adipose tissue around the entire trunk were collected. At 8 weeks of age, before the HFD, all the pups

of the independent experimental cohort were decapitated and samples of blood, subcutaneous adipose tissue around the waist (left lower quadrant of the entire trunk), left epididymal adipose tissue, and left retroperitoneal adipose tissue were collected. The sampling was systematically carried out by a trained technician blinded to the study as illustrated in Figure 3. At 17 weeks of age on an HFD, a similar sampling was carried out. To measure adipose tissue weight, we used a GX-600 (A&D Company, Limited, Tokyo, Japan) with a minimum weighing value of 0.001 g and repeatability (standard deviation [SD]) of 0.001 g. Some of the adipose tissue was fixed in 10% formaldehyde (0.1 mol/L sodium cacodylate buffer, pH 7.4) and embedded in paraffin for morphological analysis. Tissue orientation was not taken into consideration when embedding the tissue. The remaining tissue was snap frozen using liquid nitrogen in blocks and stored at -80°C for messenger RNA (mRNA) extraction.

Blood Sampling and Measurement

In each cohort, immediately after the decapitation of pups, blood glucose levels were measured with ACC-CHEK Compact Plus (Roche Diagnostics Japan, Tokyo, Japan) and blood samples were collected with heparin-coated glass tubes and centrifuged at 1200g for 15 minutes at 4°C . The plasma thus obtained was aliquoted and stored at -30°C until assayed. Total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels were measured using FUJI DRI-CHEM 3500 (FUJIFILM Holdings Co, Tokyo, Japan).

Histological Assessment of Adipose Tissue

The adipose tissue blocks embedded in paraffin were cut into 3 μm sections. Hematoxylin and eosin (HE) staining was carried out. Adipocyte cell diameters were measured according to the reports of Zhang et al⁴⁰ and Tchoukalova et al,⁴¹ with

Figure 3. Systematic sampling of subcutaneous adipose tissue around the waist (left-side lower quadrant of the entire trunk; A, B), left epididymal adipose tissue (C, D), and left retroperitoneal adipose tissue (E, F) from a pup at 8 weeks.

modifications as follows. In each section, 4 microscopic fields ($0.6 \text{ mm}^2 \times 4$) were arbitrarily selected and digital images were recorded. Then, diameters of 375 to 415 adipocytes per pup were assessed by tracing the outline of each adipocyte, and the cellular diameter was estimated by assuming a circular form using Scion Image software (Scion Corp, Fredric, Maryland), according to the report of Tchoukalova et al.⁴¹ In each pup, the adipocytes were classified by estimated diameters and expressed as a percentage. In other sections, the antigen was retrieved in a water bath (95°C for 40 minutes), and the endogenous peroxidase activity was quenched by incubation in methanol with H_2O_2 (3%) for 10 minutes, as described previously.⁴² A macrophage-specific F4/80 rat monoclonal antibody⁴³ was applied to the sections as described.²⁷ Detection was performed with a polymer detection kit (ChemMate EnVision; Dako Japan, Tokyo, Japan), according to the manufacturer's instructions, followed by a reaction with 3,3'-diaminobenzidine and counterstaining with hematoxylin. In each section, 4

microscopic fields ($0.6 \text{ mm}^2 \times 4$) were arbitrarily selected and the positive cells were counted.

Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis of the Adipose Tissue

Total RNA from the subcutaneous adipose tissue was extracted as described.¹¹ Gene expression of CD11c, CD163, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNF- α), leptin, Toll-like receptor 4, interleukin 6 (IL-6), and IL-10 was determined by quantitative reverse transcription–polymerase chain reaction (RT-PCR) using high-capacity RNA to complementary DNA (cDNA) Master Mix (catalog number 4390777; Applied Biosystems, Foster City, California) and SYBR Green PCR Master Mix (catalog number 4309115; Applied Biosystems), according to the manufacturer's recommendations. 18S ribosomal mRNA expression was used as an internal control. The primers used are listed in Table 2.

Statistics

Data are expressed as means $\pm$ SDs. The statistical significance of differences between 2 mean values was assessed using Student *t* test or the Mann-Whitney *U* test, as appropriate. The statistical significance of differences among 4 mean values was assessed with an analysis of variance followed by Scheffe *F* test. The Z scores were calculated by using the formula $\{(\text{data} - \text{mean of the population}) / (\text{SD of the population})\}$.⁴⁴ The Spearman rank correlation coefficient was calculated between the Z scores of 2 parameters. A *P* value of less than .05 was regarded as statistically significant.

Results

Body Weight Changes of Dams and Pups With Intrauterine Undernutrition

Figure 4A and B shows representative maternal caloric intake and body weight changes during CR. Mean fetal body weight several hours before delivery at 18.5 dpc was significantly lower in CR fetuses (1.00 ± 0.08 [SD] g, $n = 81$) than AD fetuses (1.12 ± 0.08 , $n = 113$; Figure 4C). The growth of male pups, especially on an HFD, was much more prominent than that of female pups among AD (Figure 4D) and CR (Figure 4E) offspring; therefore, we used only male pups after weaning. By contrast, the mean body weights of CR pups were similar to those of AD pups at 19.5 days, 8 weeks, and 17 weeks of age (Figure 5A). The mean body weight of group D pups (UP of CR) on an HFD at 17 weeks was significantly higher than that of group C pups (CR and LO; Figure 5C), but there was no significant difference between groups B (UP of AD) and A (LO of AD) pups (Figure 5B). The Z scores positively correlated with those of body weight at weaning (19.5 days) in CR, but not in AD, pups (Figure 5D, E). The BMI of group D pups (UP of CR) on an HFD at 17 weeks was significantly higher than that of the other groups (Table 3).

Figure 4. Maternal caloric intake (A), body weight changes in dams (B) and fetuses at 18.5 dpc (C), and longitudinal changes in body weight in male and female AD (D) and CR (E) pups. Dots and columns indicate means of the data. Error bars indicate standard deviation (SD). White squares and black circles indicate AD and CR groups, respectively (A, B, C). White triangles and circles indicate male and female, respectively (D). Black triangles and circles indicate male and female, respectively (E). White arrows indicate the period on an HFD (D, E). ###*P* < .01 AD versus CR. ####*P* < .001 AD versus CR. †*P* < .05 versus female. ‡*P* < .001 versus female. CR indicates caloric restriction; HFD, high-fat diet.

Table 2. Primers Used in Quantitative RT-PCR.

	Forward Primer	Reverse Primer
CD11c	5'-CTGGATAGCCTTCTTCTGCTG-3'	5'-GCACACTGTGTCCGAAGTC-3'
CD163	5'-GGGTCATTACAGAGGCACACTG-3'	5'-CTGGCTGTCCTGTCAAGGCT-3'
Leptin	5'-TTTCACACACGCAGTCGGTATC-3'	5'-GTCTGGTCCATCTTGGACAA-3'
MCP-1	5'-CCACTCACCTGCTGCTACTCAT-3'	5'-TGGTGATCCTCTTGTAGCTCTCC-3'
TNF-α	5'-ACCCTCACACTCAGATCATCTTC-3'	5'-TGGTGGTTTGCTACGACGT-3'
IL-6	5'-ACAACCACGGCCTTCCCTACTT-3'	5'-CACGATTTCCCAGAGAACATGTG-3'
IL-10	5'-GATGCCCCAGGCAGAGAA-3'	5'-CACCCAGGGAATTCAAATGC-3'
TLR-4	5'-AGAAATTCCTGCAGTGGGTCA-3'	5'-TCTCTACAGGTGTTGCACATGTCA-3'
18S	5'-GGGAGCCTGAGAAACGGC-3'	5'-GGGTCGGGAGTGGGTAATTTT-3'

Abbreviations: IL, interleukin; MCP, monocyte chemoattractant protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; RT-PCR, reverse transcription–polymerase chain reaction.

Estimated Growth Rate During Lactation and Body Weight at Weaning (19.5 Days)

Z scores of the estimated growth rate (%/d) before weaning, from 12.5 to 19.5 days of age, significantly correlated with those of body weight at weaning in both the AD ($r = .58, P < .01$) and CR ($r = .73, P < .001$) groups, when we started the exact identification of each pup with an ear tag.

Adipose Tissue Weight Changes

At 19.5 days of age, the mean weight of subcutaneous adipose tissue was significantly higher in the UP than LO pups among both AD (group B vs group A) and CR (group D vs group C) offspring (Table 4A). The mean weights of all (ie, subcutaneous, retroperitoneal, and epididymal) adipose tissues in group B (UP of AD) pups were similar to those in group A

Figure 5. Comparison between AD and CR (A), group A (LO of AD) and group B (UP of AD) (B), and group C (LO of CR) and group D (UP of CR) (C), at 19.5 days, 8 weeks, and 17 weeks of age, and correlation of the Z scores between body weight on an HFD at 17 weeks and body weight at weaning at 19.5 days in AD (D) and CR (E) pups. Dots and columns indicate means of the data. Error bars indicate standard deviation (SD). Light gray and black columns indicate AD and CR pups, respectively (A). White and black columns indicate LO and UP pups, respectively, among AD (B) and CR (C) offspring. White squares, black squares, white circles, and black circles indicate group A (LO of AD), group B (UP of AD), group C (LO of CR), and group D (UP of CR), respectively (D, E). * $P < .05$. CR indicates caloric restriction; HFD, high-fat diet; LO, body weight below the mean body weight; UP, body weight above the mean body weight.

Table 3. Body Mass Index.

	Body Mass Index (BMI; g/cm ²)				Correlation of Z Scores in Comparison With Body Weight at 19.5 days	
	Group A LO of AD	Group B UP of AD	Group C LO of CR	Group D UP of CR	AD	CR
8 Weeks	0.33 ± 0.02 ^a (n = 10)	0.33 ± 0.03 ^a (n = 10)	0.34 ± 0.02 (n = 19)	0.37 ± 0.03 (n = 11)	$r = .53^b$ (n = 20)	$r = .55^c$ (n = 29)
17 Weeks	0.45 ± 0.04 ^a (n = 10)	0.46 ± 0.04 ^a (n = 10)	0.44 ± 0.04 ^a (n = 19)	0.52 ± 0.03 (n = 11)	NSC (n = 20)	$r = .80^d$ (n = 29)

Abbreviations: AD indicates ad libitum; CR, caloric restriction; LO, body weight below the mean body weight; NSC, no statistical correlation; UP, body weight above the mean body weight.

^a $P < .05$ versus group D.

^b $P < .01$ in correlation of Z scores.

^c $P < .01$ in correlation of Z scores.

^d $P < .001$ in correlation of Z scores.

Table 4. Weight of Adipose Tissue at 19.5 Days (A), 8 Weeks (B), and 17 Weeks (C).

		Tissue Weight (g)				Correlation of Z Scores in Comparison With Body Weight at 19.5 d	
		Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	AD	CR
(A)		n = 14	n = 16	n = 10	n = 16	–	–
19.5 d	Total subcutaneous adipose tissue	0.14 ± 0.03 ^{a,b}	0.17 ± 0.04 ^a	0.09 ± 0.04 ^c	0.15 ± 0.03	–	–
(B)		n = 17	n = 13	n = 12	n = 25	n = 30	n = 37
8 wks	Left subcutaneous adipose tissue	0.11 ± 0.02 ^c	0.10 ± 0.01 ^c	0.11 ± 0.02 ^c	0.13 ± 0.02	NSC	r = .437 ^d
	Left epididymal adipose tissue	0.14 ± 0.02 ^a	0.13 ± 0.03	0.11 ± 0.02 ^c	0.15 ± 0.02	NSC	r = .583 ^e
	Left retroperitoneal adipose tissue	0.04 ± 0.01 ^a	0.03 ± 0.01	0.02 ± 0.01 ^c	0.04 ± 0.01	NSC	r = .540 ^d
(C)		n = 9	n = 9	n = 16	n = 11	n = 18	n = 27
17 wks	Left subcutaneous adipose tissue	0.75 ± 0.12	0.86 ± 0.09 ^a	0.67 ± 0.17 ^c	0.92 ± 0.09	NSC	r = .633 ^e
	Left epididymal adipose tissue	1.16 ± 0.21	1.22 ± 0.20	1.08 ± 0.15	1.20 ± 0.12	NSC	NSC
	Left retroperitoneal adipose tissue	0.57 ± 0.17	0.66 ± 0.13	0.57 ± 0.17 ^c	0.68 ± 0.11	NSC	r = .598 ^d

Abbreviations: AD, ad libitum; CR, caloric restriction; LO, body weight below the mean body weight; NSC, no statistical correlation; UP, body weight above the mean body weight.

^a $P < .05$ versus group C.

^b $P < .05$ versus group B.

^c $P < .05$ versus group D.

^d $P < .01$ in correlation of Z scores.

^e $P < .001$ in correlation of Z scores.

(LO of AD) at 8 and 17 weeks of age (Table 4B, C). By contrast, the mean weights of subcutaneous and retroperitoneal adipose tissues were significantly higher in group D (UP of CR) than in group C (LO of CR), respectively, at 8 and 17 weeks of age (Table 4B, C). Z scores positively correlated with those of body weight at weaning (19.5 days) in CR, but not in AD, pups (Table 4B, C).

Macrophage Infiltration in the Subcutaneous Adipose Tissue

Crown-like structures were detected in the subcutaneous adipose tissue of both AD and CR pups on an HFD at 17 weeks of age (Figure 6A), but not at 19.5 days or 8 weeks of age (data not shown). Most of these crown-like structures were immunohistochemically F4/80 positive, corresponding to infiltrated macrophages (Figure 6A). The mean number of F4/80-positive cells was significantly higher in group D (UP of CR) than in group C (LO of CR) at 17 weeks, but there was no significant difference between groups B (UP of AD) and A (LO of AD; Table 5A). The Z scores positively correlated with those of body weight at weaning (19.5 days) in CR, but not in AD, pups (Table 5A).

The ratio of CD11c mRNA expression (inflammatory M1 macrophage-specific) to CD163 mRNA expression (anti-inflammatory M2 macrophage-specific) was significantly higher in group D (UP of CR) than in group C (LO of CR) on an HFD at 17 weeks of age, but there was no significant difference between groups B (UP of AD) and A (LO of AD; Table 5B). The Z scores positively correlated with those of body weight at weaning (19.5 days) in CR, but not in AD, offspring (Table 5B).

Distribution of Adipocyte Size

Figure 6B to H shows representative HE staining of the subcutaneous adipose tissue from relatively heavy pups at 19.5 days (Figure 6B, C), 8 weeks (Figure 6D, E), and 17 weeks of age (Figure 6F, G, H). The patterns of distribution of adipocyte size in CR pups were similar to those in AD pups at 19.5 days (Figure 7A) and 8 weeks of age (Figure 7B). By contrast, the rates of small adipocytes (ie, less than 30 μ m, 30–40 μ m, and 40–50 μ m in diameter) were significantly higher in CR than in AD pups at 17 weeks of age (Figure 7C). The rate of small adipocytes, less than 30 μ m, was significantly higher in group D (UP of CR) than in group C (LO of CR), but there was no significant difference between groups B (UP of AD) and A (LO of AD; Table 6). The Z scores positively correlated with those of body weight at weaning (19.5 days) in CR, but not in AD, pups (Table 6).

Gene Expression of Leptin and Inflammatory Cytokines

The Z score of leptin gene expression was significantly correlated with that of subcutaneous adipose tissue weight on an HFD at 17 weeks of age in both AD and CR pups (Table 7). By contrast, the Z scores of *MCP-1* and *TNF- α* gene expressions were significantly correlated with those of subcutaneous adipose tissue weight at 17 weeks of age in CR, but not in AD, pups (Table 7). However, for the gene expression of leptin, *MCP-1*, *TNF- α* , *TRL-4*, *IL-6*, and *IL-10*, there was no significant difference among the 4 groups at 17 weeks of age (Table 8). The Z scores did not correlate with those of body weight at weaning in the AD or CR group at 17 weeks of age (Table 8).

Figure 6. Immunostaining of F4/80 in the crown-like structure observed in subcutaneous adipose tissue from relatively heavy CR pups at 17 weeks of age (A) and representative HE staining of subcutaneous adipose tissue from group B (UP of AD; B, D, F), group D (UP of CR; C, E, G), and group C (LO of CR; H) offspring at 19.5 days (B, C), 8 weeks (D, E), and 17 weeks of age (F, G, H), respectively. Black bars indicate 50 μ m. CR indicates caloric restriction; HE, hematoxylin and eosin; HFD, high-fat diet; LO, body weight below the mean body weight; UP, body weight above the mean body weight.

Blood Glucose Levels and Lipid Profiles

Both mean glucose and total cholesterol levels were significantly higher in group D (UP of CR) than that in group C (LO of CR), but there was no significant difference between groups B (UP of AD) and A (LO of AD; Table 9A, B). The Z scores of glucose and total cholesterol levels at 17 weeks of age positively correlated with those of body weight at

weaning (19.5 days) in CR, but not in AD, pups (Figure 7D, E and Table 9A, B).

Discussion

A systematic review of the literature showed that rapid weight gain in infancy is consistently associated with an increased

Table 5. Mean Number of F4/80 Positive Cells/Field (A) and Gene Expression of CD11c and CD163 (B).

(A)	Mean Number of F4/80 Positive Cells/Field				Correlation of Z Scores in Comparison With Body Weight at 19.5 d	
	Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	AD	CR
17 wks	n = 10 0.75 ± 0.53	n = 10 0.70 ± 0.60	n = 18 0.28 ± 0.24 ^a	n = 11 1.14 ± 0.78	n = 20 NSC	n = 29 r = .388 ^b
(B)	CD11c mRNA/CD163 mRNA (ratio of arbitrarily units)				Correlation of Z scores in comparison with body weight at 19.5 d	
	Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	AD	CR
17 wks	n = 9 0.99 ± 0.55	n = 9 0.97 ± 0.45	n = 14 0.77 ± 0.47 ^a	n = 7 1.39 ± 0.44	n = 18 NSC	n = 21 r = .674 ^c

Abbreviations: AD, ad libitum; CR, caloric restriction; LO, body weight below the mean body weight; mRNA, messenger RNA; NSC, no statistical correlation; UP, body weight above the mean body weight.

^a $P < .05$ versus group D.

^b $P < .05$ in correlation of Z scores.

^c $P < .001$ in correlation of Z scores.

subsequent risk of obesity,⁸ especially in neonates who were born small.⁷ In the present study, we successfully developed an animal model in which the Z scores of body weight at weaning (19.5 days) positively correlated with parameters of obesity as well as dysregulation of glucose and lipid metabolism on an HFD at 17 weeks of age in CR pups, while the correlation was not observed in AD pups (Figures 5 and 7 and Tables 3-8). Of note, the mean body weight at 17 weeks of age was significantly higher in group D (UP of CR) than that in group C (LO of CR; Figure 5C) but did not differ between groups B (UP of AD) and A (LO of AD; Figure 5B). Similar observation was observed in the BMI at 17 weeks (Table 3). Moreover, the difference in mean body weight between groups D (UP of CR) and C (LO of CR) was more prominent at 17 weeks of age than 19.5 days or 8 weeks of age in the CR pups (Figure 5C). That is to say that an HFD exaggerated both the amelioration and deterioration of obesity in CR pups in comparison with AD pups. Indeed, the Z scores of body weight at 17 weeks of age positively correlated with those at weaning (19.5 days) in CR, but not in AD, offspring (Figure 5D, E). Thus, this animal model supports, at least partly, the human evidence that rapid infantile weight gain is a risk factor for adult obesity especially if born small.

There is a limitation of this animal model; we started the exact identification of each pup with an ear tag at weaning (19.5 days) because of technical difficulty during earlier periods and used Z scores of body weight at weaning as a parameter of early growth after birth. Since birth weight as well as infantile growth could affect the body weight of pups at weaning, it does not represent the actual growth rate after birth. It was preferable to use real growth rate (%/d), not real body weight at weaning, for evaluating growth of pups during lactation period, if available. However, we weighed the pups repeatedly before

weaning and estimated the changes in the weight of each pup from the order of the weight in a litter. The Z scores of the estimated growth rate (%/d) from 12.5 to 19.5 days of age in the pups were significantly correlated with those of body weight at weaning in both the AD ($r = .58$, $P < .01$) and CR ($r = .73$, $P < .001$) groups. Indeed, the Z scores of the estimated growth rate from 12.5 to 19.5 days of age in the pups showed a similar correlation with most of the parameters of adult obesity examined in the present study (K. Y. Kohmura and H. Itoh, unpublished data, September 18, 2012). Moreover, the body weight of CR pups was similar to that of AD pups at weaning (19.5 days; Figure 5A), although the body weight of CR fetuses was significantly lower than that of AD fetuses several hours before delivery at 18.5 dpc (Figure 4C). This discrepancy indicates an important contribution of infantile growth to body weight at weaning. Therefore, we believe that the present animal model could be used to investigate the effect of infantile growth patterns on the risk of adult obesity on an HFD. It should be also noted that we used only male pups after weaning due to limited space for animal care, in consideration that the growth of male pups, especially on an HFD, was much more prominent than the female pups (Figure 4D, E). As for adipose tissue weight, the subcutaneous adipose at 19.5 days (Table 4A) and epididymal and retroperitoneal adipose tissue at 8 weeks (Table 4B) of group A (LO of AD) were heavier than those of group C (LO of CR), respectively. It was suggested that undernourishment in utero was associated with the light weight of fat pads in group C (LO of CR), although these tendencies were not observed on an HFD (Table 4C). We did not compare the quality of milk between AD and CR dams.

An increase in the weight of adipose tissue is an essential pathological change of obesity. At weaning (19.5 days), the

Figure 7. Profiles of adipocyte size at 19.5 days (A), 8 weeks (B), and 17 weeks of age on an HFD (C). Correlation of Z scores between blood glucose levels on an HFD at 17 weeks of age and body weight at weaning (19.5 days) in AD (D) and CR (E) pups. Light gray and black columns indicate AD and CR pups, respectively (A-C). White squares, black squares, white circles, and black circles indicate group A (LO of AD), group B (UP of AD), group C (LO of CR), and group D (UP of CR), respectively (D, E). # $P < .05$ AD versus CR. ### $P < .01$ AD versus CR, #### $P < .001$ AD versus CR. CR indicates caloric restriction; HFD, high-fat diet; LO, body weight below the mean body weight; UP, body weight above the mean body weight.

mean weight of subcutaneous adipose tissue was significantly higher in the UP than in the LO pups among both AD (group B vs group A) and CR (group D vs group C) offspring (Table 4A). However, in AD pups, there was no significant difference in the mean weights of adipose tissue between group B (UP of AD) and group A (LO of AD) at 8 (Table 4B) and 17 weeks of age (Table 4C), indicating that fat deposits developed on an HFD independent of body weight at weaning if the pups had been normally nourished in utero. By contrast, at 8 and 17 weeks of age, the mean weights of subcutaneous and retroperitoneal, but not epididymal (17 weeks), adipose tissues were

consistently higher in group D (UP of CR) than in group C (LO of CR) pups (Table 4B, C). Indeed, the Z scores positively correlated with those of body weight at weaning (Table 4B, C). Given these phenotypical characteristics, we supposed that rapid infantile growth following undernourishment in utero could induce an acceleration of fat deposition in adult subcutaneous and retroperitoneal adipose tissue on an HFD. Then, we focused on adipose tissue remodeling in the fat pad.

In this animal model, acceleration of adipose tissue remodeling at 17 weeks was observed in proportion to the development of obesity in both AD and CR pups. Interestingly,

Table 6. Ratio of Small Adipocyte Diameter.

Adipocyte Diameter	Ratio of Adipocyte Diameter, at 17 wks (%)				Correlation of Z Scores in Comparison With Body Weight at 19.5 d	
	Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	AD	CR
	n = 8	n = 9	n = 18	n = 11	n = 17	n = 29
Less than 30 μ m	0.39 $\pm$ 0.47	0.10 $\pm$ 0.18 ^a	0.30 $\pm$ 0.41 ^a	1.01 $\pm$ 0.99	NSC	$r = .411^b$
30-40 μ m	0.95 $\pm$ 0.7 ^a	1.04 $\pm$ 1.20 ^a	2.46 $\pm$ 2.17	3.66 $\pm$ 1.70	NSC	NSC
40-50 μ m	3.75 $\pm$ 1.69	2.77 $\pm$ 0.78	5.63 $\pm$ 3.97	5.72 $\pm$ 3.20	NSC	NSC

Abbreviations: AD, ad libitum; CR, caloric restriction; LO, body weight below the mean body weight; NSC, no statistical correlation; UP, body weight above the mean body weight.

^a $P < .05$ versus group D.

^b $P < .05$ in correlation of Z scores.

Table 7. Gene Expression of Leptin and Inflammatory Cytokines.

	Correlation of Z-Scores in Comparison With Subcutaneous Adipose Tissue Weight at 17 wks	
	AD	CR
17 wks	n = 15	n = 21
Leptin	$r = .497^a$	$r = .422^a$
MCP-1	NSC	$r = .609^a$
TNF- α	NSC	$r = .417^a$

Abbreviations: AD, ad libitum; CR, caloric restriction; MCP, monocyte chemoattractant protein; NSC, no statistical correlation; TNF, tumor necrosis factor.

^a $P < .05$ in correlation of Z scores.

it manifested only on an HFD, because we detected F4/80-positive crown-like structures, corresponding to macrophages, in the adipose tissue at 17 weeks of age, but not before the HFD at 19.5 days or 8 weeks of age (Figure 6) and there was no difference in the distribution of adipocyte size between AD and CR pups at weaning (19.5 days) or 8 weeks (Figure 7A, B). It was plausible that the HFD induced the infiltration of macrophages into adipose tissue in both AD and CR adult mice, because an excess supply of nutrients was reported to lead to adipocyte hypertrophy, followed by cell death, which could act as a stimulus for immune cell infiltration into adipose tissue.^{45,46} However, there is a difference between AD and CR pups concerning the association of adipose remodeling on an HFD at 17 weeks with body weight at weaning (19.5 days).

In CR pups, the acceleration of adipose tissue remodeling paralleled the body weight change at weaning (19.5 days) as follows. The mean number of F4/80-positive cells per field was significantly higher in group D (UP of CR) than in group C (LO of CR) pups (Table 5A), and the Z scores positively correlated with those of body weight at weaning (19.5 days) in CR offspring (Table 5A). The endocrinal and metabolic milieu of obesity switch the phenotype of macrophages from type M2, noninflammatory resident cells, to type M1, inflammatory cells (lipid-engorged foam cells), expressing dendritic cell markers such as CD11c.^{47,48} In the present study, we estimated

this phenotypical change from the ratio of mRNA expression of M1 macrophage-specific CD11c to M2-specific CD163, as an estimated ratio of M1/M2 macrophages. The ratio was significantly higher in group D (UP of CR) than in group C (LO of CR), and the Z scores positively correlated with those of body weight at weaning (19.5 days) in CR pups on an HFD at 17 weeks of age (Table 5B). As for adipocyte size distribution, a considerable increase in small adipocytes, supposedly due to adipogenesis or lipolysis, was reported in adipose tissue remodeling,^{32,33} although the enlargement of adipocytes is a major morphological change in the fat pads of obese individuals. A significant increase was observed in the ratio of small adipocytes, less than 50 μ m, at 17 weeks of age in CR pups in comparison with AD pups on an HFD (Figure 7C). Particularly in small adipocytes less than 30 μ m, the ratio was significantly higher in group D (UP of CR) than in group C (LO of CR) pups (Table 6). The Z score positively correlated with that of body weight at weaning (19.5 days) in CR pups (Table 6).

By contrast, in AD pups, the degree of adipose tissue remodeling at 17 weeks was independent of the pattern of body weight at weaning (19.5 days), assessed by the numbers of F4/80-positive cells (Table 5A), an estimated ratio of M1/M2 macrophages (Table 5B), and adipocyte size distribution (Table 6).

The secretions from M1 macrophages, such as TNF- α , along with those from hypertrophoid adipocytes, such as MCP-1 and leptin, are involved in the worsening of obesity and detrimental metabolic changes.⁴⁷ In this animal model, the Z score of leptin mRNA expression positively correlated with adipose tissue weight on an HFD at 17 weeks in both AD and CR pups (Table 7). By contrast, the Z scores of MCP-1 and TNF- α positively correlated with adipose tissue weight on an HFD at 17 weeks in CR, but not in AD, pups (Table 7), suggesting these inflammatory cytokines to be involved in the accumulation of fat only when the mice experienced undernourishment in utero. Interestingly, the Z score of their mRNA did not correlate with that of body weight at weaning (19.5 days; Table 8), suggesting infantile growth to be indirectly associated with

Table 8. Gene Expression of Inflammatory Cytokines and Body Weight at 19.5 Days.

	mRNA Expression/18S RNA Expression				Correlation of Z Scores in Comparison With Body Weight at 19.5 d	
	Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	AD	CR
17 wks	n = 9	n = 9	n = 14	n = 7	n = 18	n = 21
Leptin	0.89 ± 0.23	0.90 ± 0.17	0.94 ± 0.16	0.83 ± 0.19	NSC	NSC
MCP-1	0.98 ± 0.09	1.03 ± 0.30	0.97 ± 0.33	1.05 ± 0.27	NSC	NSC
TNF- α	1.01 ± 0.35	1.07 ± 0.40	0.96 ± 0.43	1.18 ± 0.23	NSC	NSC
TLR-4	1.22 ± 0.25	1.21 ± 0.24	1.28 ± 0.25	1.09 ± 0.14	NSC	NSC
IL-6	0.82 ± 0.54	1.01 ± 0.46	0.95 ± 0.42	0.85 ± 0.32	NSC	NSC
IL-10	1.52 ± 0.89	1.97 ± 0.85	1.78 ± 0.95	1.67 ± 0.49	NSC	NSC

Abbreviations: AD, ad libitum; CR, caloric restriction; IL, interleukin; LO, body weight below the mean body weight; MCP, monocyte chemoattractant protein; NSC, no statistical correlation; TLR, Toll-like receptor; TNF, tumor necrosis factor; UP, body weight above the mean body weight.

^a $P < .05$ in correlation of Z scores.

Table 9. Blood Glucose Levels (A), and Total Cholesterol Levels (B), and Other Lipid Profiles (C) at 19.5 Days, 8 Weeks, or 17 Weeks.

			Group A, LO of AD	Group B, UP of AD	Group C, LO of CR	Group D, UP of CR	Correlation of Z Scores in Comparison With Body Weight at 19.5 d	
							AD	CR
(A) Blood glucose levels, mg/dL	19.5 d	179 ± 25 (n = 14)	184 ± 18 (n = 17)	172 ± 33 (n = 12)	184 ± 56 (n = 17)	NSC (n = 31)	NSC (n = 29)	
	8 wks	182 ± 34 (n = 17)	207 ± 37 (n = 13)	201 ± 30 (n = 12)	200 ± 29 (n = 25)	NSC (n = 30)	NSC (n = 37)	
	17 wks	199 ± 31 (n = 9)	218 ± 50 (n = 9)	181 ± 31 ^a (n = 16)	237 ± 48 (n = 10)	NSC (n = 18)	$r = .688^b$ (n = 26)	
(B) Total cholesterol levels, mg/dL	8 wks	93 ± 24 (n = 17)	93 ± 20 (n = 13)	90 ± 7 (n = 12)	93 ± 21 (n = 25)	NSC (n = 30)	NSC (n = 37)	
	17 wks	394 ± 121 (n = 9)	391 ± 119 (n = 9)	270 ± 88 ^a (n = 16)	364 ± 70 (n = 10)	NSC (n = 18)	$r = .480^c$ (n = 26)	
(C) Triglyceride, mg/dL	17 wks	140 ± 44 (n = 9)	190 ± 100 (n = 9)	178 ± 37 (n = 16)	185 ± 34 (n = 10)	NSC (n = 18)	NSC (n = 26)	
HDL cholesterol, mg/dL	17 wks	238 ± 66 (n = 9)	234 ± 70 (n = 9)	200 ± 63 (n = 16)	230 ± 43 (n = 10)	NSC (n = 18)	NSC (n = 26)	

Abbreviations: AD, ad libitum; CR, caloric restriction; HDL, high-density lipoprotein; LO, body weight below the mean body weight; NSC, no statistical correlation; UP, body weight above the mean body weight.

^a $P < .05$ versus group D.

^b $P < .001$ in correlation of Z scores.

^c $P < .05$ in correlation of Z scores.

gene expression. Some inflammatory cytokines did not show this association (Table 8). However, we detected significant changes in MCP-1 and TNF- α in the present study (Table 7). Tumor necrosis factor- α plays a central role in the interaction between activated macrophages and hypertrophoid adipocytes,²⁸⁻³¹ and MCP-1 is one of the main contributors inducing chemotaxis of macrophages.²⁸⁻³¹ It was suggested that inflammatory responses were associated with, at least partly, the remodeling of adipose tissue observed in this animal model, although further investigation is necessary to draw an overall picture of the associated inflammatory network.

Adipose tissue remodeling is reported to induce insulin resistance³⁴ and/or disorders of lipid metabolism.³⁵ Indeed, a positive correlation of Z scores was observed between blood glucose and total cholesterol levels on an HFD at 17 weeks of age and body weight at weaning (19.5 days) in CR, but not in AD, pups (Table 9A, B, Figure 7D, E). The present findings suggested that intrauterine undernourishment lets subcutaneous adipose tissue keep

the information of an infantile growth pattern and that the change to an HFD induces its remodeling being causatively associated with metabolic disorders of glucose and lipids. The effect of intrauterine undernourishment on other organs is now under investigation in our research groups.

One major hypothesis regarding the developmental origins of obesity is the acquisition of a “thrifty phenotype” during undernourishment in utero, as a “predictive adaptive response,” followed by an unexpected encounter with era of excessive eating after birth, which causes a “mismatch” and obesity and associated metabolic disorders.⁴⁹⁻⁵¹ However, this does not fully explain why rapid infantile growth accelerates the development of obesity in adulthood. The present study has demonstrated that rapid growth in infancy could induce remodeling in the adipose tissue and be involved, at least partly, in the deterioration of metabolic disorders, which may provide a clue as to how infantile growth patterns modify the phenotype already acquired in utero. It was speculated that there might be at least 3 hits by

environmental changes, that is intrauterine undernutrition, infantile rapid growth, and excessive eating in later life, for the acceleration of adipose tissue remodeling in adult obesity. Since there are considerable species differences between humans and mice, human studies are necessary before the application of this hypothesis to people. Further study is ongoing.

In the present study, we longitudinally assessed the morphology of subcutaneous adipose tissue, for a greater impact of visceral fat on adipose tissue remodeling in obesity, rather than total body fat.⁵² The reasons were that we could not completely isolate visceral adipose tissue around the intestine from the pancreas for technical reasons, that body weight at weaning (19.5 days) correlated with subcutaneous and retroperitoneal, but not epididymal, adipose tissue (17 weeks; Table 4C), that HE staining of retroperitoneal adipose tissue showed incidental contamination by a small amount of brown adipose tissue presumably located around the spine (data not shown), which may be inappropriate for the analysis of gene expression, and that we could obtain subcutaneous, but not retroperitoneal or epididymal, adipose tissue at weaning (19.5 days), when the initial assessment of infantile growth started. The contribution of visceral adipose tissue is our next aim of the study.

In summary, we developed a mouse animal model of maternal CR, in which the Z scores of body weight of the offspring at weaning (19.5 days) positively correlated with various parameters of obesity on an HFD in adulthood. Using this model, we first revealed a possible effect of rapid infantile growth on adipose tissue remodeling, associated with metabolic disorders. As far as we know, this is the first report that rapid infantile growth could accelerate adipose tissue remodeling and a pathological transformation from a metabolically healthy obese state into a metabolically morbidly obese state in later life.

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