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Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation

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Abstract

Obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that they might represent an important source of inflammation. We have provided *in vitro* evidence that saturated fatty acids, which are released from hypertrophied adipocytes via the macrophage-induced adipocyte lipolysis, serve as a naturally occurring ligand for Toll-like receptor 4 (TLR4) to induce the inflammatory changes in macrophages. Here we show the attenuation of adipose tissue inflammation in C3H/HeJ mice carrying a functional mutation in the TLR4 gene relative to control C3H/HeN mice during a 16-week high-fat diet. We also find that adiponectin mRNA expression is significantly reduced by co-culture of hypertrophied 3T3-L1 adipocytes and C3H/HeN peritoneal macrophages, which is reversed, when co-cultured with C3H/HeJ peritoneal macrophages. This study provides *in vivo* evidence that TLR4 plays a role in obesity-related adipose tissue inflammation and thus helps to identify the therapeutic targets that may reduce obesity-induced inflammation and the metabolic syndrome.

Keywords: Adipocytes; C3H/HeJ mice; Fatty acids; Inflammation; Macrophages; Obesity; Toll-like receptor 4 (TLR4); Tumor necrosis factor-α (TNFα)

The metabolic syndrome is a combination of visceral fat obesity, impaired glucose metabolism, atherogenic dyslipidemia, and blood pressure elevation, which all increase independently a risk of atherosclerotic diseases. There is considerable evidence that visceral fat obesity is a key etiological factor in the metabolic syndrome [1,2]. A large number of studies have demonstrated that imbalance between pro-inflammatory and anti-inflammatory adipocytokines in obese adipose tissue plays an important role in the development of obesity-related sequelae [3,4]. Thus, obesity may be viewed as a metabolic as well as a chronic low-grade inflammatory disease.

Previous studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that they might represent an important source of inflammation in the adipose tissue [5,6]. We have recently developed an *in vitro* co-culture system composed of adipocytes and macrophages and suggested that a paracrine loop involving saturated fatty acids and tumor necrosis factor-α (TNFα) derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes; i.e., marked up-regulation of pro-inflammatory adipocytokines such as monocyte chemoattractant protein-1 (MCP-1) and TNFα and down-regulation of anti-inflammatory adiponectin [7]. These findings have led us to speculate that macrophages, when infiltrated, are able to induce the release of saturated fatty acids from adipocytes via lipolysis, which, in turn,

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may serve as a pro-inflammatory adipocytokine locally in the adipose tissue.

Free fatty acids represent an important energy source mobilized from triglycerides stored in the adipose tissue, particularly during periods of starvation, but recent evidence has suggested the pathophysiologic roles other than the supply of nutrients in times of fasting or increased energy demand [7–11]. There are a couple of reports demonstrating that exogenous administration of saturated fatty acids exerts the pro-inflammatory effects in certain cell types through the activation of one member of the Toll-like receptor (TLR) gene family or TLR4, an essential receptor for the recognition of lipopolysaccharide (LPS) [12,13].

The C3H/HeJ mice have a defect in LPS responsiveness caused by a missense mutation in the cytoplasmic domain of TLR4 [14,15] and thus have been widely used to investigate the role of TLR4 in the regulation of innate immunity. Using peritoneal macrophages obtained from C3H/HeJ mice, we have recently demonstrated that saturated fatty acids, which are released from adipocytes via lipolysis, is a naturally occurring ligand for TLR4 to induce nuclear factor-κB (NF-κB) activation [13]. Importantly, co-culture of hypertrophied 3T3-L1 adipocytes with C3H/HeJ peritoneal macrophages has resulted in marked inhibition of pro-inflammatory cytokine production and adipocyte lipolysis relative to that with the control C3H/HeN peritoneal macrophages [13]. These observations strongly suggest that the TLR4/NF-κB pathway plays a role in obesity-related adipose tissue inflammation and insulin resistance. It is, therefore, interesting to know whether C3H/HeJ mice respond to high-fat diet differently from control C3H/HeN mice. Here we show the attenuation of adipose tissue inflammation in C3H/HeJ mice relative to control C3H/HeN mice during a high-fat diet. We also find that adiponectin mRNA expression is significantly reduced by co-culture of hypertrophied 3T3-L1 adipocytes and C3H/HeN peritoneal macrophages, which is reversed, when co-cultured with C3H/HeJ peritoneal macrophages. This study provides both *in vivo* and *in vitro* evidence that TLR4 plays a role in obesity-induced adipose tissue inflammation, thereby suggesting that antagonism of TLR4 may offer a novel therapeutic strategy to prevent or treat obesity-induced inflammation and thus the metabolic syndrome associated with excess adiposity.

Materials and methods

Animals. Six-week-old male C3H/HeJ mice which have defective LPS signaling due to a missense mutation in the TLR4 gene and control C3H/HeN mice were purchased from CLEA Japan (Tokyo, Japan). The animals were housed in individual cages in a temperature-, humidity-, and light-controlled room (12 h light and 12 h dark cycle) and allowed free access to water and standard chow (Oriental MF; 362 kcal/100 g, 5.4% energy as fat) (Oriental Yeast, Co., Ltd., Tokyo, Japan), when otherwise noted. In the high-fat feeding experiments, mice were given free access to water and either the standard chow or high-fat diet (D12492; 556 kcal/100 g, 60% energy as fat; Research Diets, New Brunswick, NJ) for 16 weeks. At the end of the experiments, mice were sacrificed after a 1-h fast under intraperitoneal pentobarbital anesthesia (30 mg/kg). All animal experiments were conducted in accordance to the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0060026).

Histological analysis. The epididymal white adipose tissue (WAT) was fixed with neutral-buffered formalin and embedded in paraffin. The presence of F4/80-positive macrophages in the epididymal WAT was detected immunohistochemically using the rat monoclonal anti-mouse F4/80 antibody [16]. The number of F4/80-positive cells was counted in more than 10 μm^2 area of each section and expressed as the mean number/μm^2.

Analysis of blood parameters. Blood glucose levels were measured by the blood glucose test meter (Glutest PRO R; Sanwa-Kagaku, Nagoya, Japan). An enzymatic assay kit was used to measure serum free fatty acids (Wako Pure Chemical, Osaka, Japan). Serum insulin and adiponectin concentrations were determined by the respective enzyme-linked immunosorbent assay kits (insulin: Morinaga, Tokyo, Japan; adiponectin: Otsuka Pharmaceutical, Tokyo, Japan).

Co-culture experiments. Peritoneal macrophages were prepared from C3H/HeJ and C3H/HeN mice at 8–10 weeks of age [13] and 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (BioWest, Miami, FL). The 3T3-L1 preadipocytes were differentiated into lipid-laden mature adipocytes and cultured up to 21 days after the induction of differentiation to be used as hypertrophied 3T3-L1 adipocytes with larger lipid droplets [7]. Co-culture of 3T3-L1 adipocytes and peritoneal macrophages was performed as described [7,13]. In brief, serum-starved hypertrophied 3T3-L1 adipocytes were cultured in a 35-mm dish and 1.0×10^5 cells of peritoneal macrophages were plated onto 3T3-L1 adipocytes (Fig. 4A). The cells were cultured for 24 h with contact each other and harvested. As a control, adipocytes and macrophages, the numbers of which were equal to those in the co-culture, were cultured separately and mixed after harvest.

Quantitative real-time PCR. Total RNA was extracted from the epididymal WAT and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and quantitative real-time PCR was performed with an ABI Prism 7000 Sequence Detection System using PCR Master Mix Reagent (Applied Biosystems, Foster City, CA). Primers used to detect MCP-1, TNFα, adiponectin, F4/80, and 36B4 mRNAs are described elsewhere [7]. Levels of mRNA were normalized to those of 36B4 mRNA.

Statistical analysis. Data were expressed as means ± SE. Statistical analysis was performed using analysis of variance followed by Scheffe's test. $P < 0.05$ was considered to be statistically significant.

Results and discussion

Animal studies

We examined the effect of high-fat diet on the metabolic phenotypes of C3H/HeJ mice. Body weight gain was indistinguishable between C3H/HeJ and C3H/HeN mice during the 12-week standard diet and thereafter C3H/HeJ mice weighed slightly more than C3H/HeN mice (Fig. 1A). There was no marked difference in body weight between C3H/HeJ and C3H/HeN mice during a high-fat diet, although C3H/HeJ mice weighed more than C3H/HeN mice in response to 6- to 10-week high-fat diet. The weights of the epididymal and mesenteric WATs were similarly increased in both C3H/HeJ and C3H/HeN mice fed high-fat diet for 16 weeks ($P < 0.01$ vs. those fed standard diet, Fig. 1B). In this study, there was no significant difference in liver weight between C3H/HeJ and C3H/HeN mice on either a standard or a high-fat diet for 16 weeks (Fig. 1B).

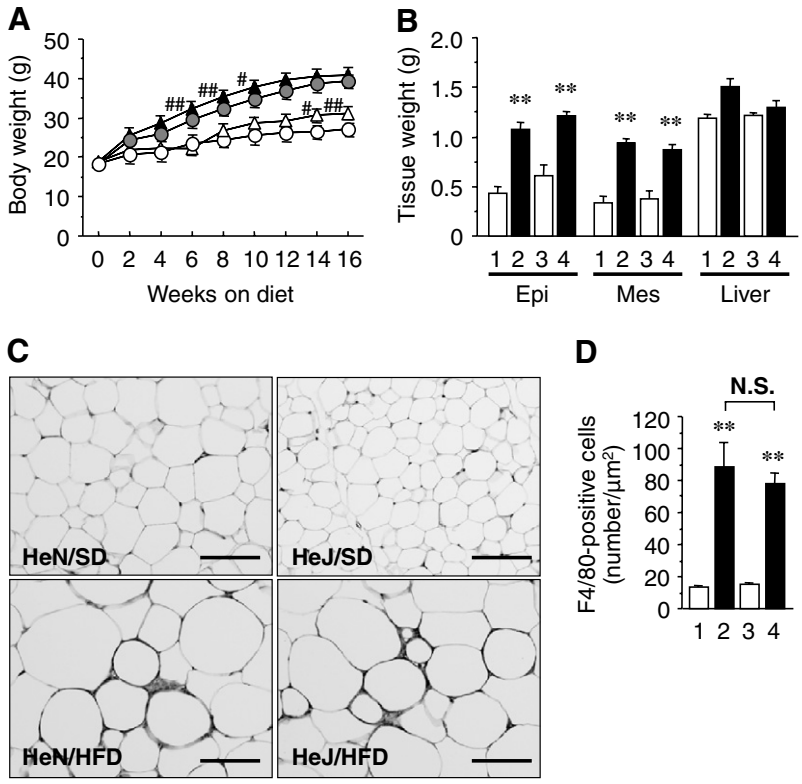

Fig. 1. Body weight, adipose tissue and liver weight, and adipose histology in TLR4-mutant C3H/HeJ and wild-type C3H/HeN mice during a 16-week high-fat diet. (A) Growth curve of C3H/HeJ (HeJ) and C3H/HeN (HeN) mice on either a standard diet (SD) or a high-fat diet (HFD). Open circles, HeN/SD ($n = 12$); gray circles, HeN/HFD ($n = 18$); open triangles, HeJ/SD ($n = 12$); closed triangles, HeJ/HFD ($n = 18$). (B) Weight of the epididymal (Epi) white adipose tissue (WAT), mesenteric (Mes) WAT, and liver in C3H/HeJ and C3H/HeN mice. Lane 1, HeN/SD; lane 2, HeN/HFD; lane 3, HeJ/SD; lane 4, HeJ/HFD. (C) F4/80 immunostaining of the epididymal WAT from C3H/HeJ and C3H/HeN mice. Original magnification, 200×. Scale bars, 100 μm. (D) Cell count of F4/80-positive cells in the epididymal WAT from C3H/HeJ and C3H/HeN mice. N.S., not significant. $^{**}P < 0.01$ vs. the SD group of each genotype; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. C3H/HeN on the respective diets.

Histological examination revealed no appreciable difference in interstitial cells stained positively for F4/80, a marker of activated macrophages, in the adipose tissue between C3H/HeJ and C3H/HeN mice fed high-fat diet (Fig. 1C and D). These observations indicate that the TLR4 mutation does not affect macrophage infiltration in the adipose tissue during a 16-week high-fat diet.

In this study, there was no significant difference in MCP-1 and F4/80 mRNA expression in the epididymal WAT between C3H/HeJ and C3H/HeN mice during the 16-week high-fat diet (Fig. 2). Interestingly, TNFα mRNA expression was significantly reduced, whereas adiponectin mRNA expression was significantly increased in the epididymal WAT from C3H/HeJ mice relative to C3H/HeN mice ($P < 0.05$). These observations, taken together, indicate that the extent of inflammatory changes in the adipose tissue is attenuated in C3H/HeJ mice relative to C3H/HeN mice during the high-fat diet.

Blood glucose and serum insulin concentrations were significantly increased in C3H/HeN mice fed high-fat diet relative to those fed standard diet ($P < 0.05$, Fig. 3). In C3H/HeJ mice, however, there was no significant difference in blood glucose and serum insulin concentrations irrespective of the diets, suggesting the preservation of insulin

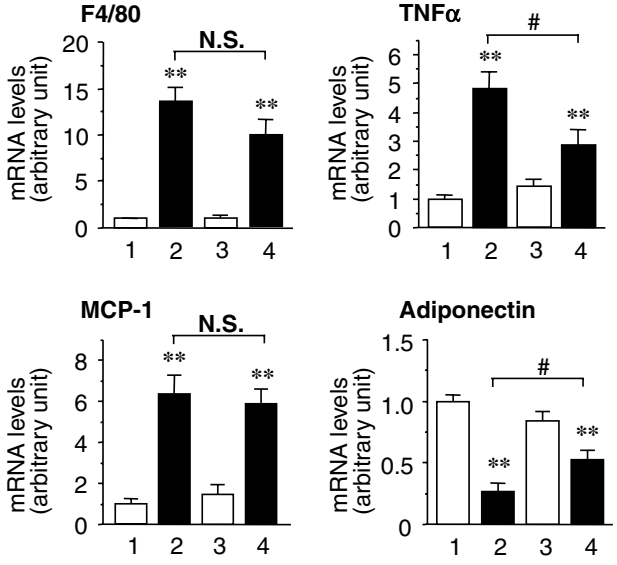

Fig. 2. Adipose mRNA expression in C3H/HeJ and C3H/HeN mice. F4/80, TNFα, MCP-1, and adiponectin mRNA levels in the epididymal WAT. Lane 1, HeN/SD ($n = 12$); lane 2, HeN/HFD ($n = 18$); lane 3, HeJ/SD ($n = 12$); lane 4, HeJ/HFD ($n = 18$). N.S., not significant. $^{**}P < 0.01$ vs. the SD group of each genotype; $^{\#}P < 0.05$ between the indicated groups.

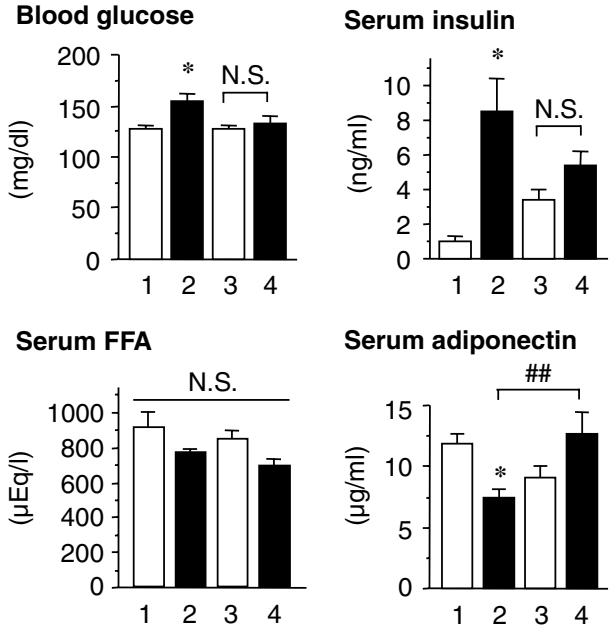

Fig. 3. Blood parameters of C3H/HeJ and C3H/HeN mice. Blood glucose and serum insulin, free fatty acids (FFAs), and adiponectin concentrations of C3H/HeJ and C3H/HeN mice. Lane 1, HeN/SD ($n = 8$–12); lane 2, HeN/HFD ($n = 12$–18); lane 3, HeJ/SD ($n = 8$–12); lane 4, HeJ/HFD ($n = 12$–18). N.S., not significant. $^{*}P < 0.05$ vs. HeN/SD; $^{\#\#}P < 0.01$ between the indicated groups.

sensitivity in C3H/HeJ mice fed high-fat diet. We also examined serum concentrations of free fatty acids and adiponectin which are known to play an important role in glucose metabolism [8,9,17,18]. Serum free fatty acids tended to be reduced in C3H/HeJ mice relative to C3H/HeN, although there was no statistical significance (Fig. 3). Serum adiponectin concentrations were significantly reduced in C3H/HeN mice fed high-fat diet relative to those fed standard diet ($P < 0.05$, Fig. 3), which were reversed in C3H/HeJ mice fed high-fat diet ($P < 0.01$ vs. C3H/HeN mice). The data on serum adiponectin concentrations are roughly parallel to those on adiponectin mRNA expression in the epididymal WATs from C3H/HeJ and C3H/HeN mice (Fig. 2). Given the anti-diabetic effect of adiponectin, it is likely that increased adiponectin concentrations is related to the improvement of blood glucose and serum insulin concentrations in C3H/HeJ mice than in C3H/HeN mice during the high-fat diet.

Cell culture studies

Recently, we have provided *in vitro* evidence that co-culture of hypertrophied 3T3-L1 adipocytes with C3H/HeJ peritoneal macrophages results in the marked attenuation of pro-inflammatory cytokine production, i.e., MCP-1 and TNFα, and adipocyte lipolysis relative to that with control C3H/HeN macrophages [13]. In this study, we observed that adiponectin mRNA expression is significantly reduced by co-culture of hypertrophied 3T3-L1 adipocytes and C3H/HeN peritoneal macrophages ($P < 0.01$, Fig. 4B). On the other hand, adiponectin mRNA expression was significantly preserved by co-culture of hypertrophied 3T3-L1 adipocytes and C3H/HeJ peritoneal macrophages ($P < 0.01$, Fig. 4B), which is consistent with the data on adiponectin mRNA expression in the epididymal WAT of obese C3H/HeN (Fig. 2). These observations, taken together, suggest that TLR4 plays a critical role in obesity-induced adipose tissue inflammation both *in vivo* and *in vitro*.

Since TLR4 is expressed in macrophages more abundantly than in adipocytes, we have speculated that the inflammatory changes induced by the interaction between adipocytes and macrophages are largely mediated through the activation of TLR4 in macrophages [13]. Indeed, we found that co-culture induced decrease in adiponectin mRNA expression is reversed, when TLR4 is mutated only in macrophages (Fig. 4), indicating that the functional alterations in macrophages *per se* affect the production of adiponectin, which is expressed exclusively in adipocytes. We speculate that saturated fatty acids, which are released from adipocytes, activate TLR4 in macrophages, thereby reducing adiponectin production in adipocytes. In this regard, Song et al. reported that TLR4 mRNA expression is increased in the adipose tissue, mainly in adipocytes rather than the remaining stromal vascular fraction of genetically obese *db*/*db* mice [19]. The authors showed that stearate is capable of activating TLR4 in differentiated 3T3-L1 adipocytes, though palmitate does not [19]. The functional significance of TLR4 in adipocytes must await further investigation.

During the preparation of this manuscript, Shi et al. reported that C57BL/6 mice deficient in TLR4 have increased obesity but are partially protected against high fat diet-induced insulin resistance, possibly due to reduced inflammatory changes in the liver and adipose tissue [20]. The above phenotypes described by Shi et al. are similar to those in this study, despite the differences in mouse strain and gender, TLR4 deficiency or mutation, and duration of high-fat diet, suggesting a critical role of TLR4 in

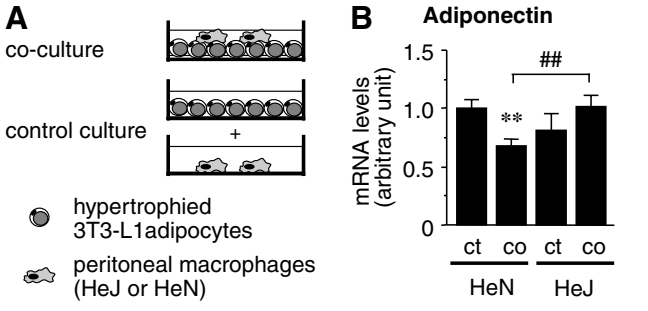

Fig. 4. Effect of co-culture of adipocytes and peritoneal macrophages from C3H/HeJ and C3H/HeN mice on adiponectin mRNA expression. (A) Illustration of the co-culture system composed of hypertrophied 3T3-L1 adipocytes and peritoneal macrophages from C3H/HeJ and C3H/HeN mice. (B) Effect of co-culture on adiponectin mRNA expression. ct, control culture; co, co-culture. $^{**}P < 0.01$ vs. ct/HeN; $^{\#\#}P < 0.01$ between the indicated groups. $n = 6$.

obesity-induced adipose tissue inflammation and systemic glucose metabolism.

In conclusion, this study demonstrates the attenuation of adipose tissue inflammation in C3H/HeJ mice relative to control C3H/HeN mice during a high-fat diet, thereby suggesting that TLR4 plays a role in obesity-induced adipose tissue inflammation *in vivo*. The data of this study also suggest that antagonism of TLR4 offers a novel therapeutic strategy to prevent or treat obesity-induced inflammation and thus the metabolic syndrome associated with excess adiposity.

Acknowledgments

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