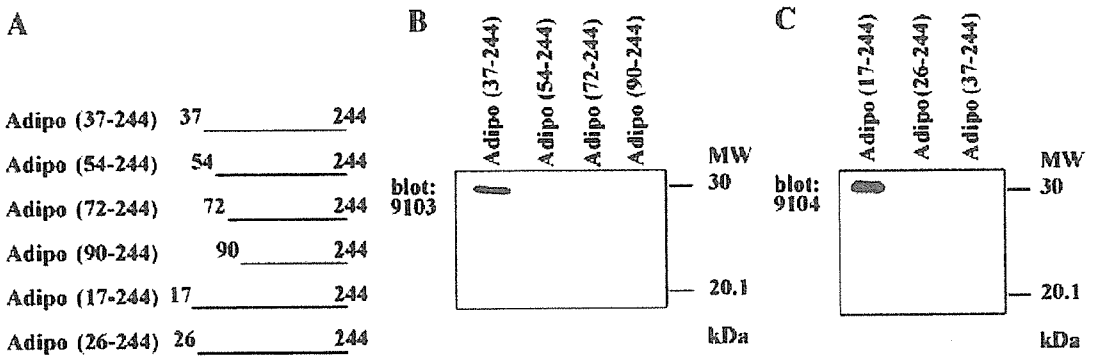

Fig. 2. Identification of the ANOC 9103- and ANOC 9104-recognition sites. A: Amino acid residues of human adiponectin with gradual deletions between AA 37 and 90 or between AA 17 and 37 are schematically shown. B, C: Each lysate (derived from 0.03 μl of bacterial culture) containing the indicated truncated form of human adiponectin was prepared as described in the Materials and Methods, and subjected to Western blot analysis with ANOC 9103 (B) or ANOC 9104 (C). The binding of Abs was detected with horse radish peroxidase-labeled anti-mouse Ig, followed by the enhanced chemiluminescence detection system. Similar results were observed in three independent experiments.

Taken together our results for epitope mapping, ANOC 9103 recognizes AA 47–53 of human adiponectin, in addition, ANOC 9104 recognizes AA 17–25 of human adiponectin. Another anti-human adiponectin Ab, ANOC 9132 bound to AA 107–244 of human adiponectin (data not shown), suggesting that it recognizes the globular domain. The Ab-recognition sites were confirmed with an ELISA technique. Fragment-1 (DQETTTQGPGVLLPLPKGACTGW-MA) represents AA 17–41, and fragment-2 (ACTGWMAGIPGHPGHNGAPGRDGRD) represents AA 35–59 of human adiponectin, respectively. Each fragment was coated on plates, and the binding of ANOC 9103 or ANOC 9104 Ab to the fragments was detected by biotinylated anti-mouse IgG, followed by the avidin-biotin-alkaline phosphatase complex reaction. As shown in Figure 3, ANOC 9103 bound to fragment-2, but not to fragment-1. Conversely, ANOC 9104 bound to fragment-1, but not to fragment-2. In either ELISA, ANOC 9132 did not bind to fragment-1 or fragment-2. Therefore, ANOC 9103 specifically recognizes AA 47–53 of human adiponectin, which correspond to the starting portion of the collagen-like region, in addition, ANOC 9104 recognizes AA 17–25 of human adiponectin, which correspond to the hypervariable region.

Recognition of Cells by the Fragments Corresponding to the 9103- and 9104-Epitopes

The binding capacity of FITC-labeled fragment-1 and fragment-2 to several types of cells was evaluated with flow cytometry analysis (Table II). Both fragment-1 and fragment-2 bound to the cell surface of MS-5 stromal cells. Fragment-2 significantly bound to a THP-1 monocytic leukemia line, while the binding of fragment-1 was detected faintly. Interestingly, the binding of both fragments was enhanced when differentiation of THP-1 cells into macrophages were induced by PMA. HUVEC and a C2C12 myoblast line showed higher reactivity with fragment-2 than with fragment-1. Their recognition was influenced neither by the activation state of HUVEC nor by the differentiation state of C2C12 cells. In the case of a 293 T embryonic line, a MKN45 gastric cancer line, a Nalm6 B-cell leukemia line, an ONHL-1 B-lymphoma line, and a Ba/F3 pro-B cell line, the binding of fragment-2, but not fragment-1 was detected. Therefore, fragment-1 binds to

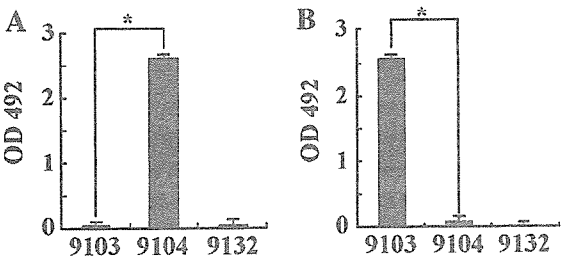

Fig. 3. Recognition of adiponectin fragments by ANOC 9103 and ANOC 9104. Five microgram per milliliter of either fragment-1 corresponding to AA 17–41 of human adiponectin (A) or fragment-2 corresponding to AA 35–59 of human adiponectin (B) was coated on 96-well microtiter plates overnight. The binding of ANOC 9103, ANOC 9104, and ANOC 9132 (2 μg/ml) to each fragment was evaluated with ELISA, and expressed as OD492. The results represent mean ± SD of triplicate samples. Similar results were obtained in two independent experiments. *$P < 0.01$ by Student' t-test.

TABLE II. Binding Capacity of Adiponectin Fragments to Several Types of Cells

Cells	Origin	Δ Mean fluorescence[d]	
		Fragment-1	Fragment-2
MS-5	Bone marrow stroma	1.62	1.69
THP-1	Monocytic leukemia	1.98	3.59
THP-1 with PMA[a]		4.68	8.29
C2C12	Myoblast	1.01	6.46
C2C12 with horse serum[b]		1.16	6.66
293T	Embryonic	0.26	5.51
MKN45	Gastric cancer	0.50	4.06
Ba/F3	Pro-B lymphocyte	0.32	1.51
Nalm-6	B-cell leukemia	0.22	2.57
ONLH-1	B-lymphoma	0.53	5.35
HUVEC	Umbilical vein endothelial cells	2.10	4.82
HUVEC with IL-1 β[c]		1.58	4.52

[a]Differentiation of THP-1 cells into macrophages was induced by the stimulation with PMA (50 ng/ml) for 24 h.
[b]Differentiation of C2C12 cells into myocytes was induced by exchanging medium.
[c]Activation of HUVEC was induced by the stimulation with IL-1 β (10 ng/ml) for 24 h.
[d]Δ Mean fluorescence was calculated as [fluorescence intensity of the staining with FITC-fargment-1 or FITC-fragment-2)−(fluorescence intensity of the control staining]. Data are shown as mean in at least two independent staining results.

the restricted type of cells, in contrast, fragment-2 to a variety of cells.

Fragment-1 Inhibits Adiponectin-Induced Cox-2 Gene Expression and Prostanoid Production In Vitro

As we reported previously, adiponectin inhibited not only the production of B-lymphocytes but also the formation of fat cells in long-term bone marrow cultures [Yokota et al., 2002, 2003]. These inhibitory effects of adiponectin were mainly mediated through the induction of Cox-2 in stromal cells. Because ANOC 9103 blocked adiponectin-induced growth inhibition of B-lymphocytes on MS-5 stromal cells, we analyzed influences of fragment-1 and fragment-2 on adiponectin functions in this culture system. As shown in Figure 4A, the treatment of MS-5 cells with adiponectin induced Cox-2 gene expression approximately threefold when the induction was evaluated with Real-time PCR. Both ANOC 9103 and ANOC 9104 blocked this adiponectin function, while ANOC 9132, anti-globular adiponectin Ab did not (Fig. 4A,C). Notably, the adiponectin function was also blocked by the addition of fragment-1, but not fragment-2 (Fig. 4B,C: 41.9 ± 14.9 % inhibition with fragment-1 and 1.7 ± 8.6 % inhibition with fragment-2), and the inhibition by fragment-1 was dependent on its concentration (Fig. 4D). Expression of Cox-2 mediates the synthesis of prostanoids including PGE_2 [Goetzl et al., 1995]. We next evaluated the inhibitory effects of fragment-1 and fragment-2 on the synthesis of PGE_2 induced by adiponectin. When the concentrations of PGE_2 in culture supernatants were analyzed with ELISA, MS-5 cells treated with adiponectin began to produce significant levels of PGE_2 (Fig. 4E: 96 pg/ml in Experiment 1 and 126 pg/ml in Experiment 2). Fragment-1 significantly inhibited PGE_2 secretion induced by adiponectin, but fragment-2 did not (<1 pg/ml with fragment-1, 150 pg/ml with fragment-2 in Experiment 1 as well as 8.5 pg/ml with fragment-1, 154 pg/ml with fragment-2 in Experiment 2). Therefore, the addition of fragment-1, which contains the epitope of ANOC 9104, reverses adiponectin-induced Cox-2 gene expression and PGE_2 production in MS-5 stromal cells.

Adiponectin Lacking its N-Terminal Region Inhibits LPS-Induced TNF-α Secretion In Vivo

To examine physiological roles of N-terminal domain of adiponectin including the ANOC 9104-recognition site, we prepared adenovirus producing murine adiponectin lacking the N-terminal domain (Ad-delAdipo) as well as full-length of murine adiponectin (Ad-fAdipo). The product of Ad-delAdipo was composed of AA 40–247 of murine adiponectin whose deleted N-terminal region corresponds to the human sequence of fragment-1. Adenovirus producing β-gal (Ad-βgal) was used as a negative control. Each protein produced by adenovirus was monitored with Western blot analysis (Fig. 5).

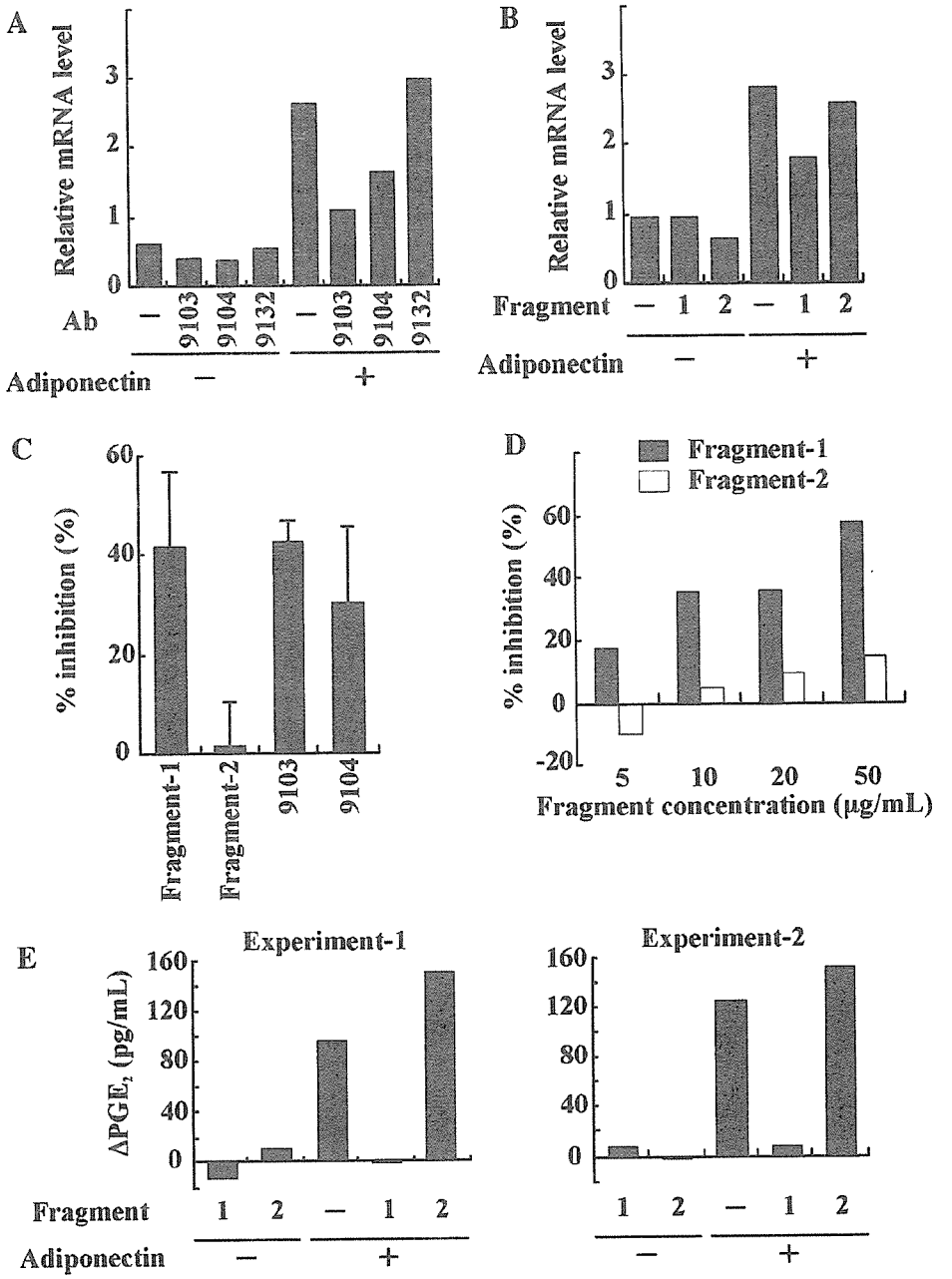

Fig. 4. Effects of adiponectin fragments on adiponectin-induced Cox-2 gene expression and prostanoid production in MS-5 cells. **A–C**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of the indicated Abs (30 μg/ml) or fragments (10 μg/ml) for 4 h. Total RNAs were prepared with TRIzol extraction, and Cox-2 mRNA levels were measured by real-time quantitative RT-PCR. The mRNA levels of Cox-2 were divided by those of β-actin, a standard control gene, and normalized. The relative mRNA expressions of Cox-2 in the presence of anti-adiponectin Abs (A) or adiponectin fragments (B) are shown. The results represent mean of duplicate samples. Data are representative of four independent experiments. The percentage inhibition was calculated as [1 – (mRNA level with Abs or fragments)/(mRNA levels with control] × 100, and the data are shown as mean ± SD percentages of inhibition in four independent experiments (C). **D**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of the indicated concentrations of adiponectin fragments for 4 h. The percentage inhibition by fragment-1 (closed column) or by fragment-2 (open column) is shown. The results represent mean of duplicate samples. Data are representative of three independent experiments. **E**: MS-5 cells in a confluent condition were preincubated for 24 h in α-MEM medium, and then exposed to recombinant human adiponectin (10 μg/ml) in the presence of fragment-1 or fragment-2 (10 μg/ml) for 24 h. Supernatant was then collected from each culture, and subjected to ELISA for PGE_2. The increase of PGE2 from control cultures without adiponectin or fragments is shown from two independent experiments.

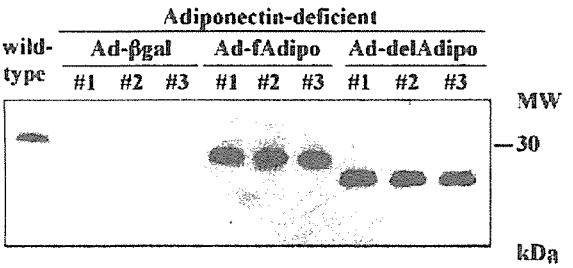

Fig. 5. Adiponectin proteins produced by adenovirus. Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, plasma samples were obtained. Ten microliters of diluted plasma sample (dilute 500-fold with PBS for adenovirus-injected mice and 50-fold with PBS for wild-type mice) was subjected to Western blot analysis with anti-murine adiponectin Ab. The binding of Ab was detected with horse radish peroxidase-labeled anti-rabbit Ig, followed by the enhanced chemiluminescence detection system.

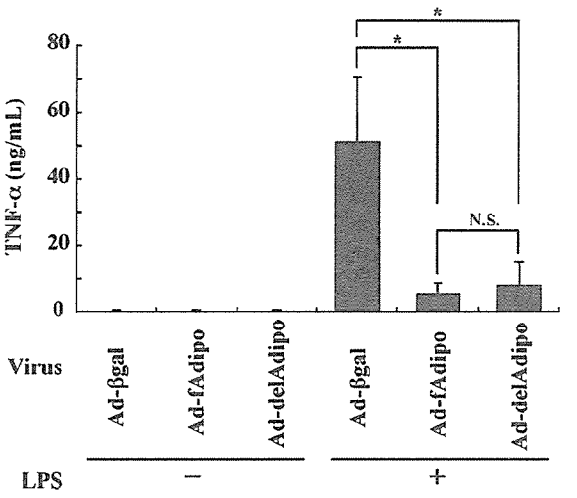

Fig. 6. In vivo effects of murine adiponectin lacking its N-terminal region on TNF-α production after LPS-injection. Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, LPS (1 μg/head) was injected intraperitoneally, and blood samples were obtained after 1 h of the LPS-injection. Serum TNF-α levels were measured with ELISA. The data indicate mean ± SD from 10 mice of each group. $^*P < 0.01$ and N.S. not significant by Student' *t*-test.

Adiponectin-deficient mice display high expression of TNF-α mRNA in adipose tissue and high concentrations of TNF-α in plasma [Maeda et al., 2002]. Moreover, the supplementation of plasma adiponectin decreases TNF-α concentration in adiponectin-deficient mice [Maeda et al., 2002]. We measured serum concentration of TNF-α of adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal after LPS-injection. Although serum TNF-α levels of mice treated with Ad-βgal were significantly elevated, those of Ad-fAdipo- as well as Ad-delAdipo-treated mice were not elevated (Fig. 6). Therefore, N-terminal region of adiponectin is not required for the inhibition of LPS-induced TNF-α secretion in vivo.

Adiponectin Lacking its N-Terminal Region Shows Reduced Activities to Inhibit Collagen-Induced Platelet Aggregation

We recently found that adiponectin inhibited collagen-induced platelet aggregation (Kato, unpublished observation). We used PPP obtained from whole blood of adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal as a source of adiponectin. At a low concentration of collagen (2.5 μg/ml), full-length adiponectin greatly inhibited platelet aggregation, but the inhibitory effect of N-terminal-truncated adiponectin was limited (Fig. 7A). Figure 7B summarizes the results of five independent experiments, and the inhibition of platelet aggregation by full-length adiponectin was always greater than that by N-terminal-truncated adiponectin. Therefore, N-terminal region of adiponectin plays a role in the inhibition of platelet aggregation.

Adiponectin Lacking its N-Terminal Region Shows Reduced Activities to Inhibit CDAA-Defined Diet-Induced Hepatic Steatosis In Vivo

Xu and his colleagues reported that administration of mice with recombinant adiponectin alleviated steatohepatitis induced by chronic consumption of high-fat ethanol-containing food [Xu et al., 2003]. We employed a CDAA-defined diet model experiment to induce hepatic steatosis without alcohol consumption [Koteish and Diehl, 2001; Jin et al., 2005]. The hepatic steatosis was evaluated after 14 days of CDAA-defined diet in adiponectin-deficient mice treated with Ad-fAdipo, Ad-delAdipo, or Ad-βgal. As shown in Figure 8A, the accumulation of fat in liver was greatly reduced by full-length adiponectin, and partly by N-terminal-truncated adiponectin. When the degree of fat accumulation in liver was evaluated with the area of red spots in the liver sections, mean percentages of the steatosis areas were calculated as 59.1 ± 16.3% for mice expressing β-gal, 29.7 ± 7.1 % for mice expressing full-length adiponectin, and 43.0 ± 9.4 % for mice expressing N-terminal-truncated adiponectin, respectively (Fig. 8B).

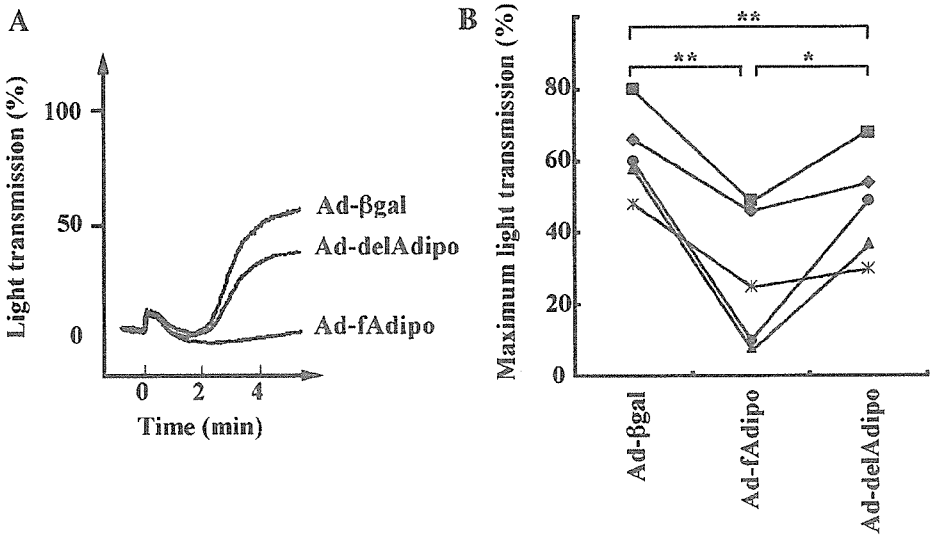

Fig. 7. Effects of murine adiponectin lacking its N-terminal region on platelet aggregation induced by collagen. A: Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head) was injected into tail veins of adiponectin-deficient mice. On day 5, PPP was obtained from the adenovirus-injected mice as a source of adiponectin. PRP was obtained from adiponectin-deficient mice without adevovirus-injection as a source of platelets. The PPP and PRP were mixed at a concentration of 3.0×10^8/ml of platelets. Platelet aggregation was initiated by the addition of 2.5 μg/ml of collagen under stirring condition and monitored by aggregometer. B: PPP from Ad-fAdipo-, Ad-delAdipo-, or Ad-βgal-injected adiponectin-deficient mice and PRP from adiponectin-deficient mice without adevovirus-injection were mixed at a concentration of 3.0×10^8/ml of platelets. Platelet aggregation was initiated by the addition of 2.5 μg/ml of collagen under stirring condition and monitored by aggregometer. $^{**}P < 0.01$ and $^{*}P < 0.05$ by Student' *t*-test.

Therefore, N-terminal region of adiponectin plays a role in the alleviation of diet-induced accumulation of fat in liver.

DISCUSSION

In a series of our experiments, anti-human adiponectin Abs, ANOC 9103 or ANOC 9104, have blocked several biological activities of adiponectin in vitro. Adiponectin inhibits the uptake of acetylated LDL by human monocyte-derived macrophages in a dose dependent manner [Ouchi et al., 2001]. Adiponectin inhibits the colony formation of granulocyte-macrophage-colony forming units [Yokota et al., 2000]. These adiponectin functions were abrogated by the addition of ANOC 9104. Adiponectin-induced Cox-2 gene expression and PGE_2 production in stromal cells were significantly blocked by both ANOC 9103 and ANOC 9104 as shown in Figure 4. Moreover, adiponectin-binding to cells was partially inhibited by ANOC 9103 and ANOC 9104 (unpublished observation). Their blocking capacities have suggested that ANOC 9103 and ANOC 9104 may recognize important functional regions on adiponectin molecule. In the present study, we identified the ANOC 9103- and ANOC 9104-recognition sites with an epitope mapping based on the ability to bind to the deleted adiponectin mutants. ANOC 9103 recognizes AA 47–53 of human adiponectin, which correspond to the starting portion of the collagen-like region, and ANOC 9104 recognizes AA 17–25, which correspond to the hypervariable domain. Structurally, the adiponectin protein in its most basic form is a homotrimer of 30 kDa subunits [Tsao et al., 2003]. The trimer exhibits a "ball-and-stick" structure where the globular domain forms the ball and the collagen-like domain forms the stick [Tsao et al., 2003]. There is also a small ball-like structure, which represents the N-terminal region of adiponectin upstream of the collagen-like domain, on the other side of the stick [Tsao et al., 2003]. The trimers are connected into a larger multimer by disulfide bonds, and the hexamer shows two trimers lying adjacent to each other in parallel head-to head fashion [Tsao et al., 2003]. The high molecular weight of adiponectin shows a "bouquet-like" high order structure [Shapiro and Scherer, 1998]. Taken together with our results of epitope mapping, both ANOC 9103 and ANOC 9104 recognize the N-terminal region of

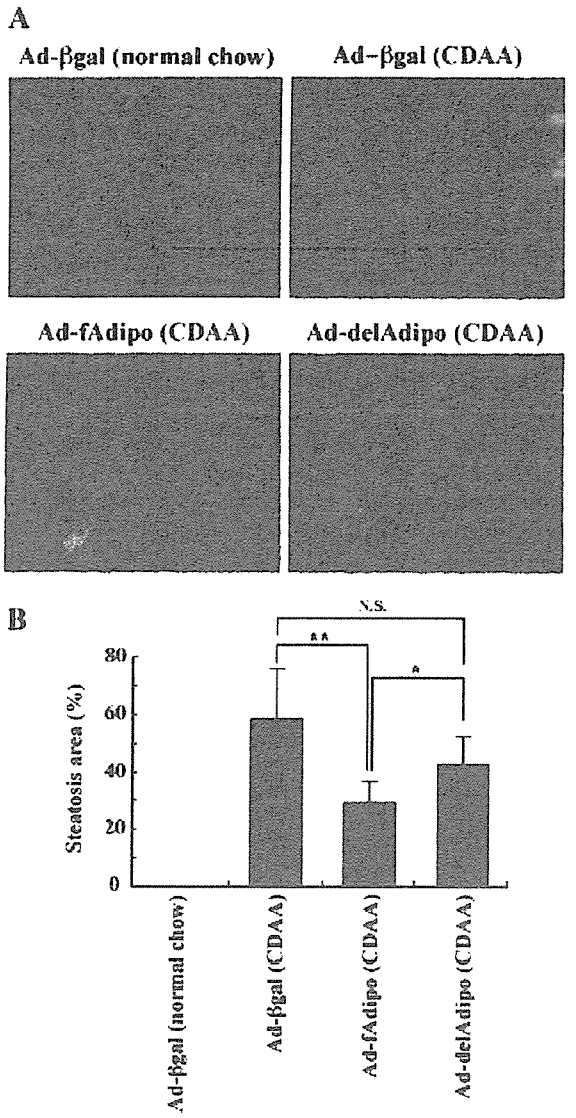

Fig. 8. In vivo effects of murine adiponectin lacking its N-terminal region on CDAA-defined diet-induced hepatic steatosis. After adiponectin-deficient mice were injected with Ad-fAdipo, Ad-delAdipo, or Ad-βgal (1×10^8 plaque-forming units/head), the mice were fed a CDAA-defined diet for 2 weeks. On day 14, their liver samples were collected and subjected to histological analysis with Oil Red O staining. A series of representative photomicrographs (at magnitude ×4) of the liver sections from each adenovirus-injected mouse are shown (**A**). The red spotted areas, Oil Red O-positive areas, were quantified with an image analyzing computer software, and expressed as the percentage of the total area of the specimen (**B**). The data indicate mean ± SD from five mice of each group. $^{**}P < 0.01$, $^{*}P < 0.05$, and N.S. not significant by Student' *t*-test.

adiponectin before the beginning of the collagen-like domain, which corresponds to the small ball in the trimer as well as the root of the rigid stick of a bouquet-like structure in high molecular weight form of adiponectin.

A peptide fragment-1 (AA 17–41), which corresponds to the epitope of 9104, can bind to the surface of several types of cells such as a MS-5 stromal cell line. It is noteworthy that the treatment of MS-5 cells with fragment-1 inhibited adiponectin-induced Cox-2 gene expression and PGE_2 production. These facts are likely to suggest that the N-terminal region of adiponectin including the hypervariable region is a functional domain to induce Cox-2 gene expression and PGE_2 production and that MS-5 cells may express some receptors, which recognize the N-terminal region of adiponectin. Both ANOC 9103 and ANOC 9104 could inhibit the stimulatory effect of adiponectin on Cox-2 gene expression. However, only fragment-1 showed the same effect. These results between Abs and fragments were not consistent. One possibility is that ANOC 9103 can block the binding of adiponectin to the receptor even if ANOC 9103 recognizes near the receptor-binding site because the ANOC 9103-recognition site exists by the ANOC 9104-recognition site and because antibodies are larger than peptide fragments. AdipoR1 is a high-affinity receptor for globular adiponectin as well as a low-affinity receptor for full-length adiponectin, and is abundantly expressed in skeletal muscle [Yamauchi et al., 2003a]. AdipoR2 is an intermediate-affinity receptor for full-length and globular adiponectin, and is mainly expressed in liver [Yamauchi et al., 2003a]. Both AdipoR1 and AdipoR2 mediate the increment of AMP kinase and peroxisomal proliferator-activated receptor (PPAR) activities, resulting in the increased fatty-acid oxidation and glucose uptake, which accounts for the increased insulin sensitivity [Yamauchi et al., 2003a]. Moreover, transgenic mice expressing globular adiponectin significantly upregulated insulin sensitivity [Combs et al., 2004]. Thus, the globular domain of adiponectin is thought to be required for the binding to AdipoR1 and AdipoR2. On the other hand, the collagen-like domain has been known to be important for multimerization of adiponectin, which determines its affinity to the receptors [Pajvani et al., 2003; Tsao et al., 2003; Waki et al., 2003]. Our results suggesting some physiological roles of the N-terminal region of adiponectin including the hypervariable region are very exciting because there is little information about this region.

Platelets initially adhere to the injured vascular surface and/or the exposed subendotherial

materials [Fuster et al., 1992]. The activated platelets then aggregate to each other, followed by next steps of thrombogenesis such as the hemostatic plug formation and the pathologic thrombus formation. Thus, our result that adiponectin inhibits the homotypic aggregation of platelets is one of the important mechanisms how adiponectin displays anti-thrombogenic activities in vivo. On the other hand, Xu and his colleagues reported that circulating adiponectin levels decreased by chronic consumption of high-fat ethanol-containing food and that administration of those mice with recombinant adiponectin dramatically reduced hepatomegaly and steatohepatitis [Xu et al., 2003]. Our results also show that replenishment of adiponectin with an adenovirus expression system improves CDAA-defined diet-induced hepatic steatosis in adiponectin-deficient mice. Thus, adiponectin alleviates both alcoholic and non-alcoholic hepatic steatosis in vivo. Interestingly, our constructed truncated-form of adiponectin lacking its N-terminal region showed less activity to inhibit the collagen-induced platelet aggregation and the diet-induced accumulation of fat in liver than full-length of adiponectin. It is possible that unknown receptors, which recognize the N-terminal region of adiponectin may be expressed on the surface of hepatocytes and platelets, and that they may attribute to some adiponectin functions in vivo. However, the interpretation about our in vivo experiments is complicated. Recent studies have revealed that the recognition of AdipoR1 and AdipoR2 as well as the binding to cytokines is dependent on state of multimerization of adiponectin [Pajvani et al., 2003; Tsao et al., 2003; Waki et al., 2003]. T-cadherin, a novel adiponectin-receptor recognizes only hexameric or high molecular weight forms of adiponectin [Hug et al., 2004]. Thus, multimerization of adiponectin seems to determine its affinity to the receptors. Recently, a cysteine residue lying at the N-terminal region of adiponectin was shown to play an essential role in assembling high molecular weight form of adiponectin [Pajvani et al., 2003; Tsao et al., 2003]. The delAdipo proteins lack the cysteine residue because it lies at the Ab-recognition sites. Thus, N-terminal-truncated adiponectn may loose the ability to assemble high molecular weight complex. Indeed, our Western blot analysis under the non-reducing condition showed that the N-terminal-truncated adiponectn proteins were detected predominantly as low molecular weight forms (data not shown). Thus, the possibility also exists that the impaired activities of N-terminal-truncated adiponectin may result from the failure of multimerization. We do not know either is the case because molecular mechanisms for these in vivo adiponectin functions are unclear. Further analysis will clarify in vivo roles of N-terminal region of adiponectin.

Recent studies have suggested the role of adipose tissue in the development of a systemic inflammatory state, which contributes to obesity-associated vasculopathy and cardiovascular risk [Berg and Scherer, 2005]. Although the mechanisms of adiponectin to exhibit anti-atherogenic effects are largely unknown, adiponectin seems to regulate low-grade inflammation in vivo. Indeed, an inverse relationship was observed between adiponectin and C-reactive protein in plasma of patients with coronary artery diseases [Ouchi et al., 2003]. In the present study, we showed that N-terminal region of adiponectin is important to mediate signals for Cox-2 induction. In an animal model of carrageenin-induced pleurisy, Cox-2 is pro-inflammatory during early phase of inflammation, but aids resolution of inflammation at the later phase by generating an alternative set of anti-inflammatory prostaglandins [Gilroy et al., 1999]. In addition, several clinical trials have suggested that Cox-2 inhibitors may lead to increased cardiovascular events [Mukherjee et al., 2001]. Thus, one possible mechanism how adiponectin exhibits anti-atherogenic effects may be to keep base-line expression of Cox-2 in vivo, because adiponectin is abundant. Our results also propose the existence of a possible receptor, which recognizes the N-terminal region of adiponectin and mediates signals for Cox-2 induction in MS-5 cells. In this situation, our designated fragment-1 will be a powerful tool to isolate the novel adiponectin receptor with an expression cloning based on the binding capacity. Further analysis will facilitate the understanding of molecular mechanisms of adiponectin and the designing of novel strategies to treat patients with vascular-dysfunctional and/or chronic inflammatory diseases.

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BASIC RESEARCH

Adiponectin deficiency exacerbates lipopolysaccharide/D-galactosamine-induced liver injury in mice

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Abstract

AIM: To examine the effects of adiponectin on the functions of Kupffer cells, key modulators of lipopolysaccharide (LPS) -induced liver injury.

METHODS: D-galactosamine (GalN) and LPS were injected intraperitoneally into adiponectin-/- mice and wild type mice. Kupffer cells, isolated from Sprague-Dawley rats, were preincubated with or without adiponectin, and then treated with LPS.

RESULTS: In knockout mice, GalN/LPS injection significantly lowered the survival rate, significantly raised the plasma levels of alanine transaminase and tumor necrosis factor-α (TNF-α) and significantly reduced IL-10 levels compared with wild type mice. TNF-α gene expression in the liver was which higher and those of IL-10 were lower in knockout mice than in wild type mice. In cultured adiponectin-pre-treated Kupffer cells, LPS significantly lowered TNF-α levels and raised IL-10 levels in the culture media and their respective gene expression levels, compared with Kupffer cells without adiponectin-pre-treatment.

CONCLUSION: Adiponectin supresses TNF-α production and induces IL-10 production by Kupffer cells in response to LPS stimulation, and a lack of adiponectin enhances LPS-induced liver injury.

Key words: Adiponectin; Lipopolysaccharide; Kupffer cell; TNF-alpha; IL10

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INTRODUCTION

Obesity is currently a serious medical problem world wide. It is an independent risk factor of non-alcoholic steatohepatitis (NASH) and alcoholic liver injury[1,2]. The pathogenesis of these diseases involves lipopolysaccharide (LPS)[3-5]. The complex of LPS and LPS-binding protein (LBP) activates Kupffer cells to secrete tumor necrosis factor (TNF)-α, which plays an important role in liver injury[6,7]. The control of the response of Kupffer cells to LPS is thought to be critical in the prevention of LPS-induced liver injury. Indeed, the selective inhibition of Kupffer cells by the administration of either gadolinium chloride or methyl palminate results in the abrogation of liver injury with an inhibition in TNF-α secretion[8]. Interleukin (IL)-10, which is secreted by Kupffer cells in the liver after LPS stimulation[9], has strong anti-inflammatory effects in the liver, and prevents liver fibrosis[10,11]. Interferon (IFN)-γ activates the signal transducer and activator of transcription (STAT-1) in hepatocytes and promotes apoptosis in hepatocytes after administration of D-Galactosamine (GalN)/LPS[12]. In GalN/LPS induced liver injury, the modulation of the production of these cytokines from Kupffer cells is important.

Adiponectin, a 30 ku adipocyte complement-related protein (Acrp30), is an adipocyte-specific plasma protein (normal level, 5-30 mg/L), and is paradoxically decreased in obesity and in cases of alcoholic fatty liver[13,14]. Adiponectin contains two types of receptors, AdipoR1 and AdipoR2. Whereas AdipoR1 is expressed ubiquitously and abundantly in skeletal muscle, AdipoR2 is most abundantly expressed in the liver and is an intermediate-affinity receptor for full-length and globular adiponectin[15]. Adiponectin improves the insulin sensitivity, and has anti-

atherogenic and anti-inflammatory effects. In the liver, it has anti-fibrogenic effects and regulates hepatic stellate cells[16].

We recently reported that adiponectin inhibits the phagocytosis of human macrophages and LPS-induced TNF-α release[17], and induces IL-10 gene expression in human macrophages[18]. We hypothesized that adiponectin has anti-inflammatory effects on Kupffer cells, key modulators of LPS induced liver injury, by regulating the release of cytokines. However, the effects of adiponectin on LPS-induced liver injury and Kupffer cells remained poorly understood. In the present study, to clarify the effects of adiponectin on LPS-induced liver injury, we investigated the effects of adiponectin on GalN/LPS-induced liver injury using adiponectin knockout mice. We also examined the effects of adiponectin on cytokine production from Kupffer cells in primary cultures.

MATERIALS AND METHODS

All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. Recombinant mouse full-length adiponectin was prepared as described previously[16]. The method of disruption of the mouse adiponectin gene was described previously[19].

Murine model of acute hepatitis

To clarify the role of adiponectin in LPS-induced liver injury, experiments were conducted using adiponectin-/- mice and wild-type (WT) control C57B6 mice (28-32 g body mass; 10-12 wk old)[19]. Each mouse was simultaneously injected intraperitoneally with 700 mg/kg of GalN and 10 μg/kg of LPS. The doses of GalN and LPS were determined in previous studies and our preliminary study[12]. LPS from *E. coli* O55:B5 and D-galactosamine (GalN) were purchased from Sigma (St. Louis, MO). Groups of 10 mice were treated to determine the survival curve. Groups of 6 mice were treated to measure the plasma levels of alanine aminotransferase (ALT), TNF-α, IL-10, and IFN-γ. Groups of 6 mice were sacrificed as follows: before, 0.5, 1, and 4 h after the administration to measure the gene expressions of TNF-α IL-10, IFN-γ in the whole liver and IFN-γ in the spleen, assessed by means of real-time polymerase chain reaction (PCR).

LPS stimulation on Kupffer cells

Male Sprague-Dawley (SD) rats (10 wk old, $n = 6$) were anesthetized with pentobarbital sodium, and the portal vein was cannulated. The liver was perfused with a ethylene glycol bis (beta-aminoethyl ether)-N, N, N', N' -tetraacetic acid (EGTA) solution, and digested with a 0.5 g/L collagenase solution. Differential centrifugation on Nycodenz (Pharma, Oslo, Norway) density gradients was performed as described before[20]. We evaluated the purity of the isolated Kupffer cell population by counting CD68 positive cells, ranging from 88.5% to 93.0%. Kupffer cells from each rat were maintained and treated separately. The Kupffer cells were maintained at 37°C under 50 mL/L CO_2 in Dulbecco's modified Eagle medium containing 100 mL/L fetal calf serum with or without adiponectin (10 mg/L) for 24 h. After a 24 h pretreatment with and without adiponectin, LPS was added to the culture medium at a concentration of 10 mg/L. The doses of adiponectin and LPS were determined in previous studies and our preliminary study[6,17]. TNF-α, IL-10, IFN-γ concentrations in the culture medium were measured 0 and 4 h after the commencement of LPS stimulation. Total RNA of Kupffer cells was isolated at 0, 0.5, 1, and 4 h after the commencement of LPS stimulation.

Measurement of plasma concentrations of ALT and cytokines

Plasma ALT concentrations were measured by using a transaminase CII-test kit according to the protocol provided by the manufacturer (Wako Pure Medical, Osaka, Japan). Circulating levels of TNF-α, IL-10, and IFN-γ were assessed using commercial enzyme-linked immunosorbent assay (ELISA) kits for mice (Biosource Int., Camarillo, CA). Their concentrations in the culture media of Kupffer cells were quantified using ELISA kits for the rat (Biosource Int.).

Quantification of gene expression levels

Total RNA from whole liver, spleen and Kupffer cells were extracted using a Qiagen (Hilden, Germany) QIAshredder and an RNA-easy Mini kit according to the instructions provided by the manufacturer. The reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously[21]. The Quantitect PCR probe kit and Quantitect gene assay kit for mice TNF-α, IL-10, and IFN-γ were purchased from Qiagen and used in a real-time PCR analysis of the mouse samples. Primers for rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH), TNF-α, IL-10, IFN-γ, and mice GAPDH were designed using the computer program Primer Express (Applied Biosystems, Foster city, CA). Dynamo SYBR Green qPCR kit (Finzymes, Espoo, Finland) was used for the real-time PCR analysis of rat TNF-α, IL-10, IFN-γ, GAPDH and mice GAPDH. Real-time PCR was performed using a DNA Engine Opticon 2 real-time PCR Detection System (MJ Research, Waltham, MA). Relative gene expression was quantified using GAPDH as an internal control. The expressions of adiponectin receptors, AdipoR1 and AdipoR2 were assessed with RT-PCR as previously published[22].

TUNEL assay

Quantification of apoptotic hepatocytes in liver sections was performed by counting the number of TUNEL-positive cells. The data were expressed as the average number of TUNEL-positive cells per five high power fields (HPF) (×100). The TUNEL assay was performed based on the instructions provided by the manufacturer (In Situ Apoptosis Detection Kit; TaKaRa, Shiga, Japan, and Liquid DAB Substrate kit; Zymed Lab. Inc., South San Francisco, CA).

Statistical analysis

The results are presented as the mean ± SE. Analysis of variance (ANOVA) for the groups was performed by the

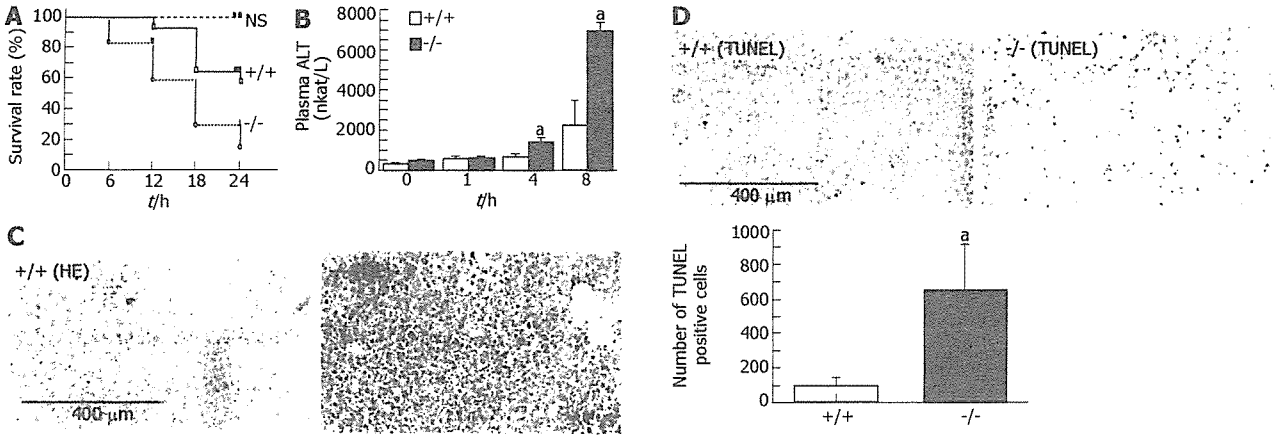

Figure 1 GalN/LPS induced changes in mice. **A:** Survival rate (n = 10); **B:** Plasma ALT (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT; **C:** Histology of the liver. Massive liver injury in adiponectin-/- mice. (Hematoxylin-eosin × 100); **D:** Large numbers of apoptotic hepatocytes in adiponectin-/- mice (TUNEL × 100). Scale bar = 400 μm. $^aP < 0.05$, *vs* WT mice.

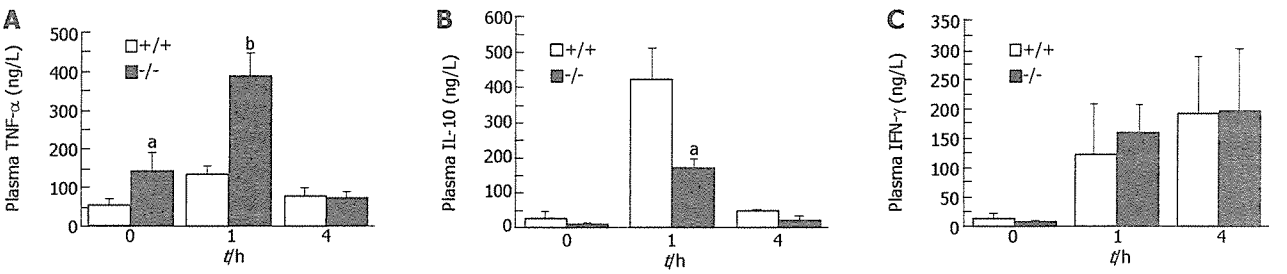

Figure 2 Plasma concentrations of TNF-α, IL-10 and IFN-γ in mice after GalN/LPS administration. (mean ± SE, n = 6). $^aP < 0.05$, $^bP < 0.01$, *vs* WT.

Mann Whitney test, followed by Scheffé's test for multiple comparisons to allow pairwise test for significant differences between groups. The statistical significance of the lethality rates was determined by a Log-rank test. Statistical significance was defined as $P < 0.05$.

RESULTS

GalN/LPS induces severe liver injury in KO mice

The survival rate of adiponectin-/- mice after GalN/LPS administration was significantly lower than that of WT mice 24 h after the administration (P = 0.041; log-lank test; Figure 1A). GalN/LPS administration resulted in an elevation of plasma ALT concentrations in both WT and adiponectin-/- mice, although increases at 4 and 8 h after administration were significantly higher in adiponectin-/- mice than in WT mouse (at 4 h; WT, 693.5 ± 472.8 *vs* adiponectin-/-, 1458.6 ± 541.8 nkat/L; $P < 0.01$, at 8 h; WT, 2255.5±3242.3 *vs* adiponectin-/-, 6988.1 ± 978.5; $P < 0.05$; Figure 1B). A histological examination revealed that GalN/LPS administration induced massive liver injury in adiponectin-/- mice mice (Figure 1C). The number of TUNEL-positive hepatocytes in adiponectin-/- mice livers was significantly higher than that in WT mouse livers 8 h after GalN/LPS administration (WT, 95 ± 118 *vs* adiponectin-/- mice, 683 ± 729 apoptotic cells/HPF; $P < 0.05$; Figure 1D).

Effects of GalN/LPS on plasma TNF-α, IL-10 and IFN-γ

Prior to GalN/LPS administration, plasma TNF-α concentrations were significantly higher in adiponectin-/- mice than in WT mice ($P < 0.05$; Figure 2A). Plasma TNF-α concentrations increased reaching peak levels 1 h after the administration, and were significantly higher in adiponectin-/- mice mice than in WT mice at their peak ($P < 0.01$; Figure 2A). Plasma IL-10 concentrations also increased, reaching peak levels 1 h after the administration of GalN/LPS. In adiponectin-/- mice, IL-10 plasma concentrations were significantly lower than those of WT mice at their peak ($P < 0.05$; Figure 2B). Plasma IFN-γ concentrations increased continuously up to 4 h after GalN/LPS administration, and no significant difference between adiponectin-/- mice and WT mice was found (Figure 2C).

Effects of GalN/LPS on liver TNF-α, IL-10, IFN-γ and splenic IFN-γ mRNA expression

The TNF-α mRNA expression level in the liver increased, reaching a peak level 1 h after GalN/LPS administration, and this level was significantly higher in adiponectin-/- mice than in WT mice 1 h after the administration ($P < 0.01$; Figure 3A). The IL-10 mRNA expression level in the liver also increased, reaching a peak level 4 h after GalN/LPS administration, and the levels at 1 and 4 h were significantly higher in WT mice than in adiponectin-/- mice (at 1 h;

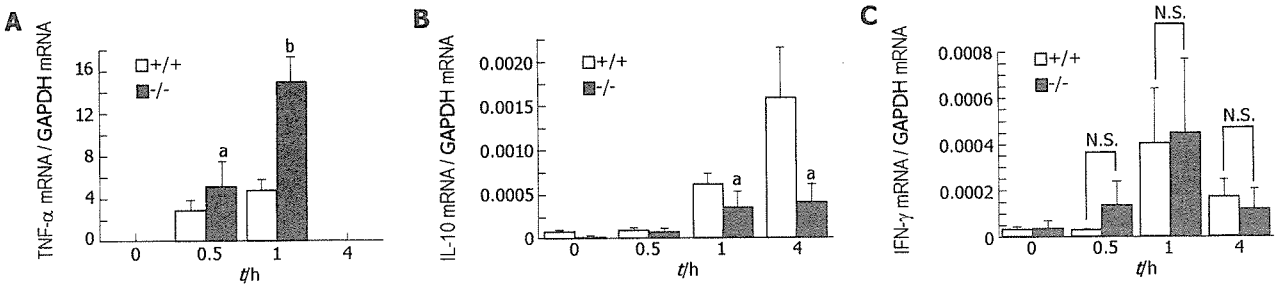

Figure 3 TNF-α, IL-10 and IFN-γ in the liver (**A-C**) of mice following GalN/LPS administration. (mean ± SE, $n = 6$). $^aP < 0.05$, $^bP < 0.01$, *vs* WT (Scheffé's test).

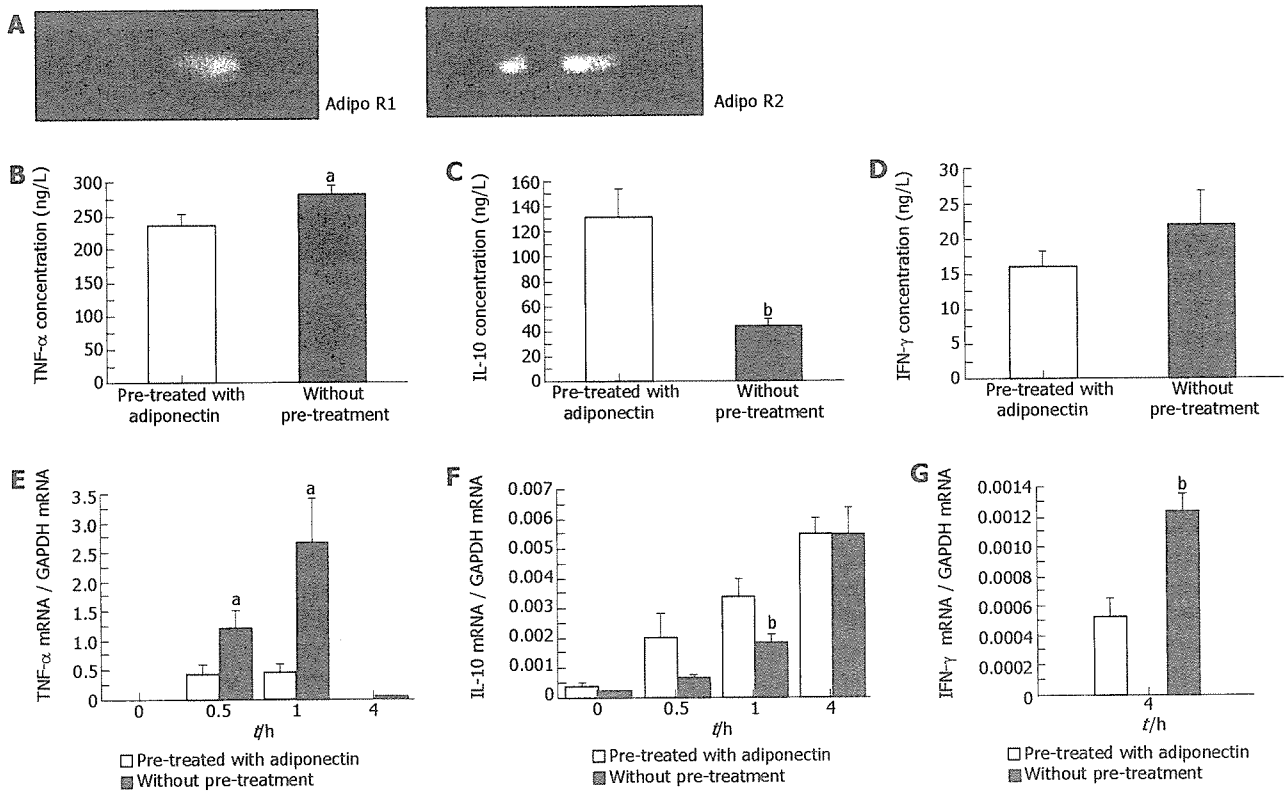

Figure 4 The expressions of adipoR1 and AdipoR2 in Kupffer cells (**A**). Effect of adiponectin on TNF-α, IL-10 and IFN-γ production by LPS-stimulated Kupffer cells *in vitro* (**B-D**). Pre-treated with or without adiponectin (10 mg/L) for 24 h followed by the stimulation with LPS (10 mg/L). TNF-α, IL-10 and IFN-γ mRNA expression in Kupffer cells after LPS stimulation (**E-G**). (mean ± SE, $n = 6$). $^aP < 0.05$, $^bP < 0.01$ (Scheffé's test).

$P < 0.05$; Figure 3B). The IFN-γ mRNA expression level in the liver increased, reaching peak levels 1 h after GalN/LPS administration, but no significant difference between adiponectin-/- mice and WT mice was found (Figure 3C). In the spleen, the IFN-γ mRNA expression levels also reached peak levels at 1 h after the administration, but there was no significant difference between adiponectin-/- mice and WT mice (data not shown).

Adiponectin receptors expression in Kupffer cell

We comfirmed the expressions of AdipoR1 and AdipoR2 in rat Kupffer cells using RT-PCR (Figure 4A).

Adiponectin inhibits LPS-induced TNF-α production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin led to a reduction in the levels of TNF-α released in response to LPS stimulation. TNF-α levels in the culture media of adiponectin-pretreated Kupffer cells were significantly lower than those for untreated cells 4 h after LPS stimulation ($P < 0.05$; Figure 4B). The LPS-induced TNF-α mRNA expression level in Kupffer cells was markedly suppressed by adiponectin pre-treatment. The expression level of TNF-α mRNA in Kupffer cells increased, reaching peak levels 1 h after LPS stimulation, but the levels at 0.5 and 1 h after the commencement of LPS stimulation in culture media of adiponectin-pretreated Kupffer cells was significantly lower than in those of untreated cells (at 0.5 h; $P < 0.05$, at 1 h; $P < 0.05$; Figure 4E).

Adiponectin increases LPS-induced IL-10 production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin increased the release of IL-10 in response to LPS stimulation.

IL-10 concentrations in the culture media of adiponectin-pretreated Kupffer cells were significantly higher at 4 h after LPS stimulation than those without adiponectin pre-treatment ($P < 0.01$; Figure 4C). The expression level of LPS-induced IL-10 mRNA in Kupffer cells was markedly increased as a result of adiponectin pre-treatment. The expression level of IL-10 mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 1 h after LPS stimulation than in untreated cells ($P < 0.05$; Figure 4F).

Lack of effect of adiponectin on LPS-induced IFN-γ production in Kupffer cells

Pretreatment of Kupffer cells with adiponectin had no significant effect on IFN-γ concentrations in the culture media (Figure 4D). But the expression level of LPS-induced IFN-γ mRNA in Kupffer cells was significantly higher in adiponectin-pretreated Kupffer cells 4 h after LPS stimulation than in untreated cells. IFN-γ gene expression in Kupffer cells at 0 and 1h after LPS stimulation could not be determined quantitatively by RT-PCR ($P < 0.01$; Figure 4G).

DISCUSSION

The major findings of the present study were that a lack of adiponectin accelerates LPS-induced liver injury, and that adiponectin has an anti-inflammatory effect on Kupffer cells. Our results demonstrate that adiponectin-pretreated Kupffer cells produced less TNF-α and more IL-10 as a result of LPS treatment. These results suggest that adiponectin has an anti-inflammatory effect on LPS-treated Kupffer cells. An intraperitoneal injection of GalN/LPS in mice resulted in a more serious liver injury that was associated with a significantly higher mortality in adiponectin-/- mice than in WT mice. The plasma levels of TNF-α were significantly increased and those of IL-10 were significantly decreased in adiponectin-/- mice compared to WT mice, and TNF-α gene expression in the liver was significantly higher and those for IL-10 were lower in adiponectin-/- mice than in WT mice. These results indicate that a lack of adiponectin enhances LPS-induced liver injury and that the altered production of cytokines in Kupffer cells caused by a lack of adiponectin affect the severity of LPS-induced liver injury.

TNF-α causes liver cell apoptosis in GalN-sensitized mice[7,23]. Mice that are deficient in TNF-α receptors are protected against GalN/LPS-induced liver damage[24]. In our study, plasma TNF-α levels increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice, suggesting that a lack of adiponectin causes an over response of TNF-α production. Indeed, TNF-α gene expression in the liver increased significantly in adiponectin-/- mice after GalN/LPS administration compared with WT mice. Sennello *et al.* recently reported that adiponectin protects hepatocytes from TNF-α induced cell death[25]. Our results are in agreement with this conclusion. We previously reported that, in peripheral macrophages, adiponectin inhibits LPS-induced TNF-α production possibly through the suppression of TNF-α-induced IκB-α-NF-κB activation via the cAMP-dependent pathway[17,26]. In the present study, adiponectin pre-treated Kupffer cells showed a lower response to LPS than the controls. The TNF-α concentration in the culture media of adiponectin pre-treated Kupffer cells showed a significantly lower elevation than those of untreated cells, and TNF-α gene expression levels after the LPS stimulation of adiponectin pre-treated Kupffer cells were significantly lower than those of untreated cells. The TLR4 receptor system plays an important role in innate immunity[27]. Kupffer cells of mice express TLR4 mRNA and respond to LPS. The LPS-binding protein (LBP) complex associates with the CD14, and via TLR4, activates Kupffer cells to secrete TNF-α[7]. Adiponectin may inhibit this receptor system directly by suppressing IκB-α-NF-κB activation, although further studies will be needed to confirm this conclusion.

IL-10 exhibits a hepatoprotective role in GalN/LPS-induced liver injury by inhibiting the release of TNF-α[10,11,28]. In our study, plasma IL-10 levels were significantly diminished in adiponectin-/- mice after the administration of GalN/LPS compared with WT mice. GalN/LPS treatment of adiponectin-/- mice significantly reduced IL-10 gene expression in the liver compared with WT mice. Kupffer cells are known to release substantial amounts of IL-10 following LPS stimulation[9]. In our study, IL-10 concentrations in the culture medium of adiponectin pre-treated Kupffer cells showed a significantly higher response to LPS stimulation than the control cells. We recently reported that adiponectin increases IL-10 gene expression in human macrophages[18]. IL-10 gene expression in adiponectin pre-treated Kupffer cells was also significantly higher than in untreated cells. These data show that physiological concentrations of adiponectin are sufficient to induce IL-10 production in Kupffer cells in response to LPS stimulation, and that a lack of adiponectin resulted in a lower IL-10 response to LPS stimulation in Kupffer cells. These results suggest that adiponectin inhibits TNF-α production directly as well as indirectly through the induction of IL-10. Further studies will be required to elucidate the precise mechanism of this cross-talk.

IFN-γ is also involved in the toxic effects of LPS on the liver[29], and IFN-γ monoclonal antibodies reduce LPS-induced mortality in mice[30]. The overexpression of IFN-γ activates STAT-1 in the liver and promotes hepatocyte apoptosis following GalN/LPS administration[12]. IL-10 is reported to inhibit IFN-γ[31]. However, Sennelo *et al.* recently reported that, in a model of Concavaline A induced hepatitis, there was no significant difference in serum IFN-γ levels between lipodystrophic aP2-nSREBP-1c transgenic mice, with higher serum adiponectin, and *ob/ob* mice, with lower serum adiponectin levels[25]. Our study found no significant differences in plasma levels of IFN-γ between adiponectin-/- mice and WT mice, or IFN-γ gene expression levels in the liver and splenic lymphocytes between adiponectin-/- mice and WT mice. We found no significant difference in IFN-γ concentration in the culture media of adiponectin pre-treated Kupffer cells and untreated Kupffer cells. However, 4 h after the LPS stimulation, IFN-γ gene expression levels of adiponectin pre-treated Kupffer cells were significantly lower than

those of untreated cells. Further studies will be required to elucidate the effect of adiponectin on IFN-γ production in Kupffer cells.

Obesity is an independent risk factor for the development of chronic liver diseases such as NASH and alcoholic liver disease[1,2]. Serum adiponectin levels are decreased in obesity, and alcohol decreases the expression of adiponectin[13,14]. Adiponectin has been reported to confer protective effects against alcoholic liver injury, and hypoadiponectinemia is involved in the progression of non-alcoholic fatty liver disease (NAFLD) to steatohepatitis[32,33]. Our results suggest that the hypoadiponectinemia in obesity induces an altered response of Kupffer cells to LPS, and is possibly involved in the development of alcoholic liver disease and NASH. Adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) have been cloned by expression cloning[15]. AdipoR1 is expressed abundantly in skeletal muscle, and is also expressed in the liver. AdipoR2 is predominantly expressed in the liver. Thakur V *et al* showed the expressions of adipoR1 and R2 in Kupffer cells and we also found these expressions in Kupffer cells[34]. Recently Wolf AM *et al* reported the up-regulation of adiponectin in liver in ConA mediated acute liver failure[35]. In GalN/LPS induced acute liver injury, the expressions of adiponectin in the liver could not be determined quantitatively by RT-PCR (data not shown). To elucidate the effect of adiponectin produced in the liver in this model, further studies will be needed. Masaki *et al* reported that adiponectin prevents LPS-induced hepatic injury in a KK-Ay obese mice model[36]. KK-Ay obese mice show not only hypoadiponectinemia but also hyperglycemia and insulin resistance. Hyperglycemia and hyperinsulinemia should have an effect on the response of inflammatory cytokines to endotoxemia[37]. We used adiponectin-/- mice to rule out the effects of such other factors. In this model, we were able to clarify the direct effect of adiponectin deficiency on LPS-induced liver injury *in vivo*.

In conclusion, a deficiency of adiponectin could enhance LPS-induced liver injury possibly through modulation of cytokine production by Kupffer cells. The development of adiponectin receptor agonists or molecules that can induce adiponectin secretion might be helpful in controlling LPS-related liver diseases such as NASH and alcoholic liver injury.

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Effect of Adiponectin on Murine Colitis Induced by Dextran Sulfate Sodium

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Background & Aims: Adiponectin, an adipose tissue–derived hormone, exhibits anti-inflammatory properties and has various biological functions, such as increasing insulin sensitivity, reducing hypertension, and suppressing atherosclerosis, liver fibrosis, and tumor growth. The aim of the present study was to determine the effect of adiponectin on intestinal inflammation. **Methods:** We investigated the effect of adiponectin on dextran sulfate sodium (DSS)-induced colitis by using adiponectin-knockout (APN-KO) mice and an adenovirus-mediated adiponectin expression system. We also examined the contribution of adiponectin deficiency to trinitrobenzene sulfonic acid (TNBS)-induced colitis. In vitro, we examined the effect of adiponectin on intestinal epithelial cells. **Results:** After administration of 0.5% DSS for 15 days, APN-KO mice developed much more severe colitis compared with wild-type mice. The messenger RNA expression levels of chemokines were significantly higher in the colonic tissues of DSS-treated APN-KO mice compared with wild-type mice, accompanied by increased cellular infiltration, including macrophages. Adenovirus-mediated supplementation of adiponectin significantly attenuated the severity of colitis, but there were no differences in the severity of TNBS-induced colitis between the 2 groups. Adiponectin receptors were expressed in intestinal epithelial cells, and adiponectin inhibited lipopolysaccharide-induced interleukin-8 production in intestinal epithelial cells. **Conclusions:** Adiponectin is protective against DSS-induced murine colitis, probably due to the inhibition of chemokine production in intestinal epithelial cells and the following inflammatory responses, including infiltration of macrophages and release of proinflammatory cytokines.

Inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, are disorders of unknown etiology characterized by chronic relapsing inflammation of the gastrointestinal tract.[1] The current literature suggests that various immune, genetic, and environmental factors influence both the initiation and progression of colitis.[2–4] During the past half century, the incidence of inflammatory bowel disease has markedly increased in developed countries,[2–4] where a marked increase in the proportion of people with obesity and/or obesity-associated metabolic syndrome has become a social problem.[5,6] This suggests that changes in lifestyle may contribute to the development of inflammatory bowel disease and obesity.

There is ample evidence that adipose tissue produces and secretes a variety of biologically active molecules[5,6] (conceptualized as adipocytokines[7] and include adiponectin,[8] leptin,[6] plasminogen activator inhibitor 1,[7] and tumor necrosis factor α[9]). A series of studies have shown that dysregulated production of these adipocytokines participates in the pathogenesis of obesity-associated metabolic diseases. Adiponectin is an adipocyte-specific secretory factor that we identified in human complementary DNA.[8] The mouse homologue of adiponectin was independently cloned as ACRP30 or AdipoQ.[10,11] We and others have shown that adiponectin has a wide array of biological functions, such as increasing insulin sensitivity in the liver and skeletal muscles,[12,13] preventing atherosclerosis,[14] and inhibiting fatty liver[15] and liver fibrosis.[16] Recent data have pointed to the anti-inflammatory effects of adiponectin, especially in endothelial cells[17] and macrophages.[18–20] In addition, we recently reported that the hypertrophied mesenteric adipose tissue of patients with Crohn's disease produces and secretes high levels of adiponectin, and the expression level of adiponectin in mesenteric adipose tissue inversely correlates with disease severity.[21] This suggests that adiponectin might have a potential role in the suppression of colitis.

Intestinal epithelial cells produce a variety of immunomodulatory substances, including the chemokine interleukin (IL)-8, and play crucial roles in regulating the inflammatory response in the pathogenesis of inflammatory bowel disease.[22,23] Production of the chemokine macrophage inflammatory protein (MIP)-2, which acts in the mouse in a manner similar to IL-8 in humans, in intestinal epithelial cells has been implicated as an important mechanism of dextran sulfate sodium (DSS)-induced murine colitis.[24] It has been reported that adiponectin inhibits IL-8 secretion from the endothelial cells,[25] but whether it affects IL-8 production in intestinal epithelial cells has not yet been defined.

In the present study, we investigated the effects of adiponectin on experimental colitis induced by DSS or trinitrobenzene sulfonic acid (TNBS) using adiponectin-knockout (APN-KO) mice and an adenovirus-mediated adiponectin expression system. To determine the direct effect of adiponectin on intestinal epithelium, we examined the effect of adiponectin on IL-8 production in HT-29 cells in vitro.

Materials and Methods

Animals

APN-KO mice were generated as described previously and backcrossed to the C57BL/6J strain for more than 5 gen-

Abbreviations used in this paper: Ad-APN, adenovirus-adiponectin; Ad-β-gal, adenovirus-β-galactosidase; APN-KO, adiponectin-knockout; DAI, disease activity index; DSS, dextran sulfate sodium; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RT-PCR, reverse-transcription polymerase chain reaction; TNBS, trinitrobenzene sulfonic acid; WT, wild-type.

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erations.[13] The control C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and maintained for 1–2 weeks in the same circumstances before starting DSS treatment. Mice were maintained on a 12-hour light/dark cycle under pathogen-free conditions and had ad libitum access to a standard diet and water until reaching the desired age (8–10 weeks).

Induction of Colitis by DSS

Experiment 1. Male mice were treated with 0.5% DSS (mol wt, 36–50 kilodaltons; MP Biomedicals, Aurora, OH) dissolved in sterile filter-purified distilled water (Millipore Corp, Bedford, MA). There was no restriction regarding the dose taken, and the DSS solution was provided ad libitum for 15 days. Fresh DSS solutions were prepared on day 5 and day 10. Control mice (not treated with DSS) received sterile filter-purified distilled water alone.

To determine the optimum dose of DSS and duration of treatment, we performed several preliminary experiments. When treated with 2.5% DSS for 7 days, both APN-KO and wild-type (WT) mice developed colitis that was too severe to allow us to see any differences between the 2 groups. Then, we tried another protocol with a lower dose of DSS for a longer duration. When treated with 1% DSS for 10 days, the disease activity index (DAI) of APN-KO mice tended to be higher than that of WT mice, although the difference was not statistically significant. Next, when treated with 0.5% DSS for 15 days, WT mice developed only minimal colitis and APN-KO mice developed significantly severe colitis. We stopped treatment and analyzed all mice on day 15, when one APN-KO mouse died of colitis. Based on these data in our preliminary studies, we judged that a protocol of 0.5% DSS for 15 days was optimal for examining the susceptibility of APN-KO mice to DSS-induced colitis.

Experiment 2. To investigate the disease severity during recovery and the survival rate, male mice were treated with 2.5% DSS for 5 days and water for the following 7 days.

Calculation of DSS Load

DSS load for all DSS-treated mice was calculated using the following formula: *Load = ([Total Drinking Volume {mL} × DSS {mg} per 100 mL]/Initial Body Wt [g])*. The drinking volume was recorded every 5 days using a calibrated water bottle.

Assessment of Inflammation in DSS-Induced Colitis

Daily clinical evaluations included measurement of body weight, observation of stool consistency, and determination of the presence of blood in the stool by a guaiac paper test (ColoScreen; Helena Laboratories Inc, Beaumont, TX). A previously validated clinical DAI ranging from 0 to 4 was scored using the parameters of weight loss, stool consistency, and the presence or absence of fecal blood.[26]

Experiment 1. On day 15, mice were killed, colons removed, and colon length and weight measured before processing for histopathologic analysis and inflammatory-related gene messenger RNA (mRNA) expression.

Experiment 2. We evaluated the DAI and the survival rate.

Histopathologic Analysis of DSS-Induced Colitis

Five samples were collected from each animal from each region of the colon (proximal, middle, and distal colon) for histopathologic examination. The samples were fixed in 10% buffered formalin and embedded in paraffin and then assessed in a blinded fashion using a scoring system described previously.[27] Briefly, 3 parameters were measured: severity of inflammation (0, none; 1, slight; 2, moderate; 3, severe), extent of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural), and crypt damage (0, none; 1, basal one third damaged; 2, basal two thirds damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (×1, 0–25%; ×2, 26%–50%; ×3, 51%–75%; ×4, 76%–100%), and all numbers were summed. The combined histopathologic score ranged from 0 to 40.

Immunohistochemical Analysis

The monoclonal antibody F4/80 (Serotec, Oxford, England) was used to track macrophage infiltration. After a limited trypsin (Sigma-Aldrich, St. Louis, MO) digestion for 10 minutes, endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 minutes. Sections were incubated with 1.5% (vol/vol) normal sheep serum to reduce nonspecific reactions and then incubated overnight with biotinylated rat anti-mouse F4/80 (1:200) at 4°C. Further steps were performed according to the instructions provided on the labeling of the Vectastain Elite ABC (avidin/biotin complex) System (Vector Laboratories, Burlingame, CA). Sections were then washed in water, counterstained with methyl green, dehydrated, and mounted.

Preparation and Administration of Adenovirus

Adenovirus producing the full-length mouse adiponectin was prepared by using the Adenovirus Expression Vector Kit (Takara, Kyoto, Japan). To test the effect of adiponectin, 5×10^7 plaque-forming units of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-β-gal) were injected into the jugular vein of mice 2 days before the administration of DSS. On day 17 after the virus injection (on day 15 after administration of DSS), mice were killed for analysis.

Immunoblotting

Immunoblotting analysis of adiponectin was performed as described previously.[13] The protein bands were quantified using an imaging densitometer (FluorChem; Alpha Innotech Corp, San Leandro, CA).

Cell Cultures and Preparation of Recombinant Adiponectin

In the present study, we used several human intestinal epithelial cell lines (CaCo-2, T-84, SW-480, and HT-29 cells) and a human hepatoma cell line (HepG2 cell). All cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C using 5% CO_2 in the following media supplemented with 10% fetal bovine serum (Sigma Chemical Co, St Louis, MO) and 100 U/mL each of penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan): Dulbecco's

modified Eagle medium (Sigma Chemical Co) for CaCo-2, T-84, HT-29, and HepG2 cells and RPMI 1640 medium (Sigma Chemical Co) for SW-480 cells. To measure mRNA expression of adiponectin receptors,[28] CaCo-2, T-84, SW-480, and HepG2 cells were grown to confluence for RNA isolation in standard 6-well plates. To investigate the effect of adiponectin on intestinal epithelium, HT-29 cells were cultured in standard 12-well plates until 80% confluence and then cultured in the media containing 1% fetal bovine serum in the presence of 10 μg/mL recombinant adiponectin or vehicle for 18 hours. Then, cells were stimulated with 1μg/mL lipopolysaccharide (LPS) (Sigma Chemical Co) for 4 hours for total RNA isolation. Recombinant human adiponectin with His-Tag was purified from the supernatant of baculovirus-infected Sf9 cells using His-Tag column and finally eluted to phosphate-buffered saline.

RNA Isolation and Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated with an RNA STAT-60 kit (Tel-Test, Friendswood, TX). First-strand complementary DNA was synthesized using the ThermoScript RT-PCR System (Invitrogen, San Diego, CA). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed on LightCycler using the FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) according to the instructions provided by the manufacturer. The primers used are listed in Table 1.

Measurement of IL-8 in the Media

HT-29 cells were cultured in standard 12-well plates until 80% confluence and then cultured in the media containing 1% fetal bovine serum in the presence of 10 μg/mL recombinant adiponectin or vehicle for 18 hours. Then, cells were stimulated with 1 μg/mL LPS (Sigma Chemical Co) in the fresh media, and the media were collected after LPS stimulation for 24 hours. IL-8 enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions (BioSource, Camarillo, CA).

Induction of Colitis by TNBS

Colitis was induced by TNBS using the method described previously.[29] Briefly, mice were anesthetized following a 24-hour fast; then an infant tube (4F) was inserted into the anus, and the tip was advanced to 4 cm proximal in the colon. TNBS (Sigma Chemical Co) dissolved in 50% ethanol was instilled into the colon through the cannula (2 mg of TNBS in a volume of 100 μL). After the instillation, the mice were held upside down by their tails for 60 seconds and then returned to their cages. All mice were killed 1 week after administration of TNBS. Control mice received an equal amount of 50% ethanol alone.

Assessment of Inflammation in TNBS-Induced Colitis

For assessment of the severity of colitis, we evaluated body weight changes, the survival rate, and histologic findings of colonic tissues in the distal colon.

Statistical Analysis and Ethical Consideration

Results are expressed as mean ± SEM. Differences between groups were examined for statistical significance using the Student *t* test or analysis of variance with Fisher protected least significant difference test. A *P* value less than .05 was considered statistically significant. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Table 1. Primers Used in RT-PCR

Primers		Sequence
Mouse		
IL-1β	Forward	5′-TCGTGCTGTCGGACCCATAT-3′
	Reverse	5′-GTGTGCCGTCTTTCATTACA-3′
IL-6	Forward	5′-ACAACCACGGCCTTCCCTACTT-3′
	Reverse	5′-CACGATTTCCCAGAGAACATGTG-3′
Tumor necrosis factor	Forward	5′-GCCACCACGCTCTTCTG-3′
	Reverse	5′-GGTGTGGGTGAGGAGCA-3′
E-selectin	Forward	5′-TGCATGGCTCAGCTCAACTTGA-3′
	Reverse	5′-CACTGTGCCGAAAACTGCTGTTC-3′
Intercellular adhesion molecule	Forward	5′-TGAATGCCAGCTCGGAGGATCAC-3′
	Reverse	5′-CGTGCAGTTCCAGGGTCTGGTT-3′
Vascular cell adhesion molecule	Forward	5′-ATCTGGGTCAGCCCCTCTCCTATAC-3′
	Reverse	5′-TGTCTGCTCCACAGGATTTTGG-3′
MIP-2	Forward	5′-GGCAAGGCTAACTGACCTGGAAAGG-3′
	Reverse	5′-ACAGCGAGGCACATCAGGTACGA-3′
MCP-1	Forward	5′-TTCCTCCACCACCATGCAG-3′
	Reverse	5′-CCAGCCGGCAACTGTGA-3′
CD68	Forward	5′-AGGGACACTTCGGGCCATGTTT-3′
	Reverse	5′-TTGTCGTCTGCGGGTGATGCA-3′
AdipoR1	Forward	5′-ACGTTGGAGAGTCATCCCGTAT-3′
	Reverse	5′-CTCTGTGTGGATGCGGAAGAT-3′
AdipoR2	Forward	5′-TCCCAGGAAGATGAAGGGTTTAT-3′
	Reverse	5′-TTCCATTCGTTCGATAGCATGA-3
Human		
AdipoR1	Forward	5′-TTCTTCCTCATGGCTGTGATGT-3′
	Reverse	5′-AAGAAGCGCTCAGGAATTCG-3′
AdipoR2	Forward	5′-ATAGGGCAGATAGGCTGGTTGA-3′
	Reverse	5′-GGATCCGGGCAGCATACA-3
IL-8	Forward	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′
	Reverse	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′
Cyclophilin	Forward	5′-CCCACCGTGTTCTTCGACAT-3′
	Reverse	5′-CCAGTGCTCAGAGCACGAAA-3′
Human, mouse		
18S	Forward	5′-CGGCTACCACATCCAAGGAA-3′
	Reverse	5′-GCTGGAATTACCGCGGCT-3′

Results

Starting Body Weight and DSS Load

Starting body weight and DSS load are listed in Table 2. There were no significant differences in starting body weight and DSS load between the groups.

Macroscopic Evaluation of DSS-Induced Colitis

In experiment 1, untreated WT and APN-KO mice showed no clinical signs of spontaneous intestinal inflammation, as evidenced by recordings of body weight, DAI, and colon length and weight-to-length ratio (Figure 1*A–D*). On the other

Table 2. Starting Body Weight and DSS Load

Group	Body wt (*g*)	DSS load (*mg/g body wt*)
Experiment 1		
WT + water (n = 6)	23.4 ± 0.4	0
WT + DSS (n = 11)	23.6 ± 0.5	16.4 ± 0.5
APN-KO + water (n = 6)	23.9 ± 0.2	0
APN-KO + DSS (n = 11)	22.9 ± 0.6	16.5 ± 0.6
Experiment 2		
WT + DSS (n = 5)	25.0 ± 0.2	24.3 ± 0.2
APN-KO + DSS (n = 5)	24.7 ± 0.3	24.6 ± 0.6

NOTE. Data are expressed as mean ± SEM.

hand, DSS-treated APN-KO mice showed a significant weight loss of 10.1%, while no weight loss was observed in DSS-treated WT mice (Figure 1*A*). The DAI is a well-characterized tool for quantifying disease severity in this experimental colitis model, and it correlates well with histopathologic findings.[30] From day 5 of DSS treatment, DSS-treated APN-KO mice exhibited a significantly higher DAI compared with DSS-treated WT mice; the DAI of DSS-treated APN-KO mice on day 15 reached 2.21 ± 0.25, while that of DSS-treated WT mice was 0.36 ± 0.16 (Figure 1*B*). To assess the severity of colitis, the colon length and colon weight-to-length ratio were measured as indices of macroscopic inflammation. There was no significant difference in both indices between control and DSS-treated WT mice on day 15. In contrast, DSS-treated APN-KO mice showed a marked reduction in colon length and an increase in colon weight-to-length ratio compared with control APN-KO mice (Figure 1*C* and *D*).

In experiment 2, treatment with 2.5% DSS induced severe colitis similarly in both groups of mice, as assessed by the DAI (Figure 1*E*). However, 80% of WT mice survived until day 12, whereas 80% of APN-KO mice died on day 9 and all were dead by day 10 (Figure 1*F*).

Histopathologic Evaluation of DSS-Induced Colitis

Histopathologically, DSS-induced colitis is characterized by edema, infiltration of inflammatory cells into the mucosa and submucosa, destruction of epithelial cells, and mucosal thickening. The colon sections of untreated WT and APN-KO mice did not show any signs of inflammation (data not shown). On the other hand, the colon sections of DSS-treated WT mice showed shortening of crypts in some local lesions and mild inflammatory cell infiltration but no destruction of the epithelial cells (Figure 2*A* [a]). In contrast, the colon sections of DSS-treated APN-KO mice showed complete crypt loss, destruction of the epithelial cells, and severe inflammatory cell infiltration (Figure 2*A* [b]). Semiquantitative analysis of these changes showed that in DSS-treated APN-KO mice, the severity of colitis, as assessed by total score, inflammation, extent, and crypt damage, was significantly higher than that in DSS-treated WT mice (Figure 2*B* and *C*). Figure 2*C* depicts parameter values of the distal colon, where colitis was the most severe (parameter values of the proximal and middle colon are not shown).

Evaluation of Macrophage Infiltration

The number of infiltrated macrophages in the inflamed mucosa was examined using anti-F4/80 antibody, which recognizes mature macrophages. Only a few macrophages were detected in the colonic tissues of both WT and APN-KO mice without DSS treatment (Figure 2*D* [a and c]). In the colonic tissues of DSS-treated WT and APN-KO mice, the number of F4/80-positive macrophages was increased. The number of macrophages was significantly greater in DSS-treated APN-KO mice than in DSS-treated WT mice (Figure 2*D* [b and d]). A significant increase of CD68 mRNA expression in the colonic tissues of DSS-treated APN-KO mice confirmed these findings (Figure 3, bottom right).

Increased Expression of Inflammatory-Related Molecules in DSS-Treated APN-KO Mice

The mRNA expression levels of several genes were measured in whole colonic tissues of mice on day 15 by real-time PCR. The mRNA expression levels of proinflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor α),

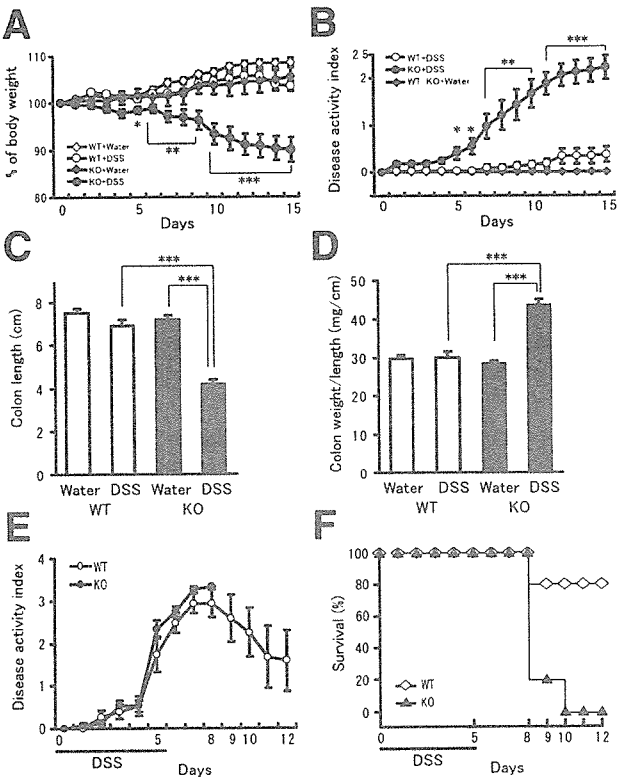

Figure 1. Severe DSS-induced colitis in APN-KO mice. Mice were administered 0.5% DSS dissolved in water for 15 days. Control mice received water alone. (*A*) Changes in percentage of body weight. (*B*) Changes in the DAI. (*C*) Changes in colon length. (*D*) Changes in colon weight-to-length ratio. Data are expressed as mean ± SEM. $^{*}P < .05$ vs WT + DSS, $^{**}P < .01$ vs WT + DSS, $^{***}P < .001$ vs WT + DSS. WT + water, n = 5; APN-KO + water, n = 5; WT + DSS, n = 11; APN-KO + DSS, n = 11. To investigate the disease severity during recovery and the survival rate, we treated mice with 2.5% DSS for 5 days and water for the following 7 days. (*E*) Changes in the DAI of mice treated with 2.5% DSS. (*F*) The survival rate. WT + DSS, n = 5; APN-KO + DSS, n = 5.