Anti-CD44 Antibody Treatment Lowers Hyperglycemia and Improves Insulin Resistance, Adipose Inflammation, and Hepatic Steatosis in Diet-Induced Obese Mice

Diabetes 2015;64:867–875 | DOI: 10.2337/db14-0149

Type 2 diabetes (T2D) is a metabolic disorder characterized by chronic hyperglycemia that is primarily mediated by obesity-induced insulin resistance (IR). Growing evidence has indicated that the causative link between obesity and IR is associated with chronic inflammation in visceral adipose tissue (1). Obesity is associated with impaired lipid storage capacity in subcutaneous adipose tissue. Lipid “spillover” that occurs as a result leads to lipid deposition in visceral fat and, subsequently, the liver (2,3). The excess fat triggers inflammatory pathways in visceral adipose tissue, and the propagation of inflammation signals from adipose into other metabolic tissues induces systemic IR, liver steatosis, and further progression of obesity, creating a vicious cycle (2).

We recently applied a computational system biology method to T2D, meta-analyzing >1,000 T2D case-control gene-expression microarray samples from public data sources. We found that CD44 plays a critical role in the development of adipose tissue inflammation and IR in rodents and humans (4). We also found that CD44 deficiency ameliorates blood glucose levels, IR, adipose tissue inflammation, and liver steatosis in diabetic mice fed a high-fat diet (HFD). We also found, in humans, that CD44-positive inflammatory cells are infiltrated into obese adipose tissue, and that serum CD44 concentration was positively correlated with increasing hyperglycemia.
and IR (4,5). Other researchers have since reproduced these results using the same mouse strain (6,7) and other groups of humans (8).

CD44 is a cell-surface glycoprotein receptor preferentially expressed on cells of the immune system, such as macrophages, neutrophils, and T lymphocytes. It is a major receptor for hyaluronan (HA; an unbranched glycosaminoglycan) and osteopontin (OPN; a T-helper type 1 cytokine) and is involved in the migration and activation of immune cells (9–13). Interestingly, HA and OPN appear to be functionally implicated in the development of IR and T2D in HFD-fed mouse models (14–19). We therefore hypothesized that T2D can be treated with a prototype drug targeting CD44, a novel therapeutic mechanism. To assess this hypothesis, we performed daily injections of anti-CD44 monoclonal antibody (mAb) in an HFD mouse model for 4 weeks. We investigated the therapeutic effects of this antibody on obesity-induced diabetes by comparing CD44 mAb with a control antibody and two oral diabetes drugs, metformin and pioglitazone.

**RESEARCH DESIGN AND METHODS**

**Mice and Treatment Protocols**

Eight-week-old male C57BL/6J (B6J) mice were obtained from The Jackson Laboratory and were fed diets containing 60% kcal fat for 12 weeks (HFD; D12492; Research Diets Inc.) without any treatment. Group 5 was used as controls (wt/wt) pioglitazone (catalog #D08020603Y; Research Diets Inc.); and group 4 (n = 8) were fed an NFD containing 0.5% (wt/wt) metformin (catalog #D11031401; Research Diets Inc.) and OPN: R&D Systems; IL-6 and MCP-1: eBioscience; and nonesterified fatty acid (NEFA) concentrations were determined using enzymatic assay kits (Wako Pure Chemical Co., Ltd.), a mouse resistin ELISA kit (Morinaga Institute of Biological Science), and a mouse resistin immunoassay (R&D Systems).

**Systemic Marker Measurements**

Serum levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), and CD44 ligands HA and OPN were assayed by ELISA kits. Serum triglyceride (TG), total cholesterol (T-ch), and nonesterified fatty acid (NEFA) concentrations were determined using enzymatic assay kits (Wako Pure Chemical Co., Ltd.), a mouse leptin ELISA kit (Morinaga Institute of Biological Science), and a mouse resistin immunoassay (R&D Systems).

**Histological Analysis**

Visceral (epididymal) white adipose tissue (VAT) and liver were removed from mice. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin-eosin (H-E). Adipose in embedded sections was performed as described in a previous study (4) using antibodies for MAC-2 (1:100; catalog #CL8942AP; Cedarlane Laboratories), CD3 (1:1; catalog #N1580; Dako), CD19 (1:100; catalog #250585; Abbiotec), and CD44 (IM7; 1:100; BD Pharmingen, catalog #553131; BD Biosciences), which causes shedding of CD44 (9); group 2 (n = 8) received daily intraperitoneal injections of purified rat anti-mouse CD44 mAb (IM7; BD Pharmingen, catalog #559478; BD Biosciences); group 3 (n = 8) were fed an HFD containing 0.5% (wt/wt) metformin (catalog #D11031401; Research Diets Inc.); and group 4 (n = 8) were fed an HFD containing 0.02% (wt/wt) pioglitazone (catalog #D08020603Y; Research Diets Inc.). In addition, we set up the following two groups of nontreated mice: group 5 (n = 8) was fed an HFD without any treatment and group 6 (n = 3) was fed a normal-fat diet (NFD) (12% kcal fat; CE-2; CLEA Japan, Inc.) without any treatment. Group 5 was used as controls in metabolic measurements (Fig. 1). Groups 5 and 6 served as controls in a differential white blood cell (WBC) count (Supplementary Fig. 2). All treatments were administered for 4 weeks. Mice in groups 1 and 2 were treated with 100 μg of antibody at day 1 and 50 μg from day 2 to day 28. Group 1 had one fewer mouse because our supply of antibody (11.0 mg) could only reliably dose 7 mice for 4 weeks. Weight gain was monitored weekly. The average daily food consumption was determined before treatment (juvenile mice age 8–20 weeks) and during the treatment period (adults age 20–24 weeks). Physical activity was measured as horizontal movements by calculating the average number of times a mouse crossed the x- or y-axis plotted on the center of the bottom of each cage. Body temperature was measured using a rectal thermometer for 5 days before and after the start of treatment. Mice had free access to autoclaved water. They were housed in a barrier facility under specific pathogen-free conditions. The Animal Care and Use Committee of Kitasato University (Tokyo, Japan) approved all animal experiments.

**Metabolic Measurements**

Blood samples were obtained via retro-orbital sinus after a 14-h overnight fast after 4 weeks of therapy, as described above. Glucose tolerance tests were performed by administering glucose (2 g/kg body wt) intraperitoneally after fasting. Venous blood for the measurement of blood glucose was drawn 0, 30, 60, 90, and 120 min later. Blood glucose concentration was determined by the glucose oxidase-peroxidase method. Serum insulin levels were measured with an ultrasensitive mouse insulin ELISA kit (Morinaga Institute of Biological Science). We calculated the quantitative insulin sensitivity check index (QUICKI = 1/log [fasting insulin] + log [fasting glucose]) and used the homeostasis model assessment as an index of IR (log HOMA-IR = log [fasting insulin × fasting glucose/405]) (20). Serum triglyceride (TG), total cholesterol (T-ch), and nonesterified fatty acid (NEFA) concentrations were determined using enzymatic assay kits (Wako Pure Chemical Co., Ltd.), a mouse leptin ELISA kit (Morinaga Institute of Biological Science), and a mouse resistin immunoassay (R&D Systems).
We created digitized images with a BIOQUANT Image Analysis System.

**Hepatic TG content**
Tissue lipids were extracted by the method of Folch et al. (21). Weighed liver samples were homogenized in chloroform/methanol. After overnight extraction, the aqueous layer was aspirated and duplicate aliquots of the chloroform/lipid layer were dried. Lipids were reconstituted in isopropyl alcohol, and TG concentration was measured with a Cholestest TG kit (Sekisui Medical). TG concentration was corrected for liver weight (hepatic TG content; milligrams of TG per gram of liver).

**Real-Time PCR**
Total RNA was isolated using the TRIzol RNA isolation method (Invitrogen) and purified with an RNeasy Mini Kit spin columns (Qiagen) according to the manufacturers’ instructions. RNA quantity and quality were determined by spectrophotometric measurements at optical densities of 260 nm and 280 nm. Its integrity was checked by agarose gel electrophoresis. RNA (2 μg) was reverse transcribed to cDNA using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics). PCR reactions were performed with a LightCycler FastStart DNA Master SYBR Green I system (Roche Diagnostics). Each sample was analyzed in triplicate and normalized to values for GAPDH mRNA expression. Supplementary Table 1 shows the mouse primer sequences used for this study.

**Statistics**
Comparisons between two groups were performed using a two-tailed Welch t test. P values <0.05 were considered significant. All experimental data are represented as the mean ± 1 SE.

**RESULTS**
**CD44 mAb Reduces Hyperglycemia and IR in Obese Model Mice**
High-fat feeding in B6J mice leads to obesity, adipose tissue inflammation, hepatic steatosis, IR, and T2D (4–6,16,22). We fed an HFD to 31 male B6J mice for 12 weeks. Figure 1—Effects of treatments on glucose metabolism and obesity. A: Fasting blood glucose. B: QUICKI results. C: The log HOMA-IR. D: Glucose tolerance tests (intraperitoneal glucose [2 g/kg body wt]) after a 14-h overnight fast. E: Body weight change. F: Daily food intake during treatments. G: Difference in daily food intake during treatment compared with before treatment. Physical activity (H) and body temperature (I) for 5 days before treatment started (day 0) and after treatment started (days 1, 2, 3, and 4). Data from age-matched mice fed only an HFD without any treatment (No Tx) were included in the figures. The effects of treatment with CD44 mAb, metformin (Met), and pioglitazone (Pio) were evaluated with a two-tailed Welch t test by comparison with the IgG2b control group or the No Tx group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. IgG2b. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. No Tx.
weeks and randomly assigned them to one of the following four treatment groups: group 1 received daily intraperitoneal injections of CD44 mAb; group 2 received daily intraperitoneal injections of isotype control antibody; group 3 received metformin mixed in chow; and group 4 received pioglitazone mixed in chow. All treatments were given for 4 weeks, and the HFD continued during treatment. We added group 5 (HFD without any treatment) as controls for measurements of metabolic state (Fig. 1).

We performed metabolic measurements on all mice at the end of the treatment period. Fasting blood glucose levels were lower in CD44 mAb–, metformin–, and pioglitazone–treated mice compared with isotype control–treated mice and mice receiving no treatment (Fig. 1A). The QUICKI was higher in the CD44 mAb–treated group than in the control groups, while the IR index (log HOMA-IR) was lower in the CD44 mAb–treated group (20). These indices were also improved in mice receiving treatment with metformin and pioglitazone (Fig. 1B and C). Glucose tolerance tests indicated that the administration of CD44 mAb and metformin to HFD-fed mice improved their ability to clear intraperitoneally injected glucose compared with controls. Glucose intolerance in HFD-fed mice was also ameliorated by pioglitazone but was not statistically significant (Fig. 1D).

### CD44 mAb Prevents Diet-Induced Obesity

We weighed all mice once weekly throughout the course of the study. Weight gain during treatment was suppressed in the CD44 mAb and metformin groups compared with controls, while weight gain in pioglitazone–treated mice was not significantly different from that of controls (Fig. 1E). Average daily food intake during treatment was not statistically different between the groups (Fig. 1F). However, mice treated with CD44 mAb and metformin did not increase their food intake between, before, and during treatment as much as did mice in the other groups (Fig. 1G). We did not observe significant changes in physical activity and body temperature between the groups (Fig. 1H and I). We also weighed VAT, liver, pancreas, and kidney after treatment. Compared with controls, VAT weight was modestly (but significantly) lower in mice treated with CD44 mAb and metformin. It was also marginally lower in the pioglitazone group, but the difference was not statistically significant. No differences were observed in the weights of the other organs (Supplementary Table 2).

### CD44 mAb Improves Adipose Tissue Inflammation

We performed histological analysis of VAT from all mice. In control mice, we frequently observed accumulations of inflammatory cells forming CLSs surrounding adipocytes in obese visceral adipose tissue (Fig. 2A). However, immune cell infiltration into the stroma of adipose tissue in CD44 mAb–treated mice was strikingly reduced compared with that in controls (Fig. 2A). VAT samples from metformin- and pioglitazone–treated mice also contained fewer CLSs compared with controls, although the degree of inflammation was greater than in CD44 mAb–treated mice (Fig. 2B).

To assess the mechanisms underlying the beneficial effects of anti-CD44 treatment on adipose tissue inflammation, we performed quantitative real-time RT-PCR to measure mRNA expression in adipose tissue for immune cell markers (CD68, F4/80, CD3e, and CD19), proinflammatory cytokines/chemokines (TNF-α, IL-1β, IL-6, IFN-γ, MCP-1, and macrophage inflammatory protein-1α [MIP-1α]), and adipokines (adiponectin, leptin, and resistin). The mRNA expression levels of CD68 and F4/80 were decreased after anti-CD44 treatment compared with the control antibody group (Fig. 2C). CD3e gene expression was low in all the groups (Fig. 2C), and CD19 gene expression was not detected in most samples in all the groups (data not shown). We also found that the expression levels of MCP-1, MIP-1α, TNF-α, IL-1β, and IL-6 were reduced in the group treated with CD44 mAb (Fig. 2D). IFN-γ expression was not detected in most samples (data not shown). There was also a reduction of CD68, MCP-1, IL-1β, and IL-6 gene expression in the pioglitazone group compared with controls. Gene transcript levels of three adipokines were not significantly altered in the CD44 mAb–treated group, but the mRNA of adiponectin was highly expressed in the pioglitazone group (Fig. 2E).

We also determined the systemic levels of three adipokines using sera. Serum adiponectin, leptin, and resistin concentrations were not different in the groups treated with CD44 mAb and metformin compared with those in controls. In pioglitazone–treated mice, serum levels of adiponectin were highly significantly elevated compared with those of controls (Table 1).

We next used RT-PCR to determine whether anti-CD44 treatment affected the expression of CD44 and its ligand, OPN, in adipose tissue. We found that mRNA expression of both was diminished in CD44 mAb–treated group (Fig. 2F).

We further investigated the composition of cell types and the binding affinity of CD44 mAb in the CLS. We conducted an immunohistochemical analysis for MAC-2 (a macrophage marker), CD3, CD19, and CD44 in adipose tissue in obese mice. We found that most infiltrating cells in obese fat tissues were stained with anti-MAC-2 and CD44 antibodies, suggesting that many inflammatory cells in the CLS are macrophages and are positive for CD44 (Fig. 2G). Note that we used the same anti-CD44 antibody (IM7) that was used for therapy. Thus, we confirmed that our therapeutic antibodies can bind to infiltrating cells that are mostly macrophages in CLSs in obese mice (Fig. 2G).

Additionally, to assess whether the CD44 mAb cross-reacted with other proteins, we immunostained adipose tissue from CD44−/− mice using our CD44 mAb (IM7). We confirmed that there was no detectable cross-reactivity.
Figure 2—Effects of treatments on visceral adipose tissue inflammation. A and B: Histological analysis. A: VAT was removed from mice treated with CD44 mAb, metformin (Met), pioglitazone (Pio), or IgG2b control antibody at the end of the therapy protocol. Specimens were stained with H-E. CLSs formed by infiltrated inflammatory cells surrounding adipocytes were frequently observed in samples from mice treated with control antibody. Scale bars = 50 μm. B: Sections were analyzed for the average number of CLSs per low power field (LPF) (magnification ×100). Quantitative real-time RT-PCR analysis for cell markers (C), proinflammatory cytokines/chemokines (D), adipokines (E), and CD44 and OPN (F). n = 5–8. *P < 0.05, **P < 0.01, ***P < 0.001 vs. IgG2b. G: Immunohistochemical analysis for CD44 and other cell markers (MAC-2, CD3, and CD19) in obese fat tissue. Scale bars = 50 μm.
of this antibody to other proteins in the tissue (Supplementary Fig. 1).

**CD44 mAb Reduces Liver Steatosis**

We next examined whether CD44 mAb therapy affected the development of HFD-induced hepatic steatosis. Examination of histological sections clearly showed less lipid accumulation in the livers of CD44 mAb–treated mice compared with that in controls (Fig. 3A). There was also less lipid accumulation in the livers of metformin-treated mice; levels in these mice were similar to those in CD44 mAb–treated mice. We also observed moderately less hepatic lipid accumulation in the pioglitazone-treated group compared with that in controls (Fig. 3A). Lipid analysis indicated that hepatic TG levels in the groups treated with CD44 mAb and metformin were decreased compared with those in controls, while little suppression was observed in the group treated with pioglitazone (Fig. 3B). These findings were consistent with our observations in histological sections.

To assess the effects of anti-CD44 therapy on hepatic steatosis, we performed RT-PCR analysis on liver samples. The mRNA expression of cell markers was not significantly different between groups (Fig. 3C). Proinflammatory cytokine expression decreased in the CD44 mAb–treated group, but the decrease was not statistically significant. We identified a more obvious reduction of levels of proinflammatory cytokines (MCP-1, TNF-α) in the metformin-treated group (Fig. 3D). CD44 mRNA was somewhat decreased in the CD44 mAb–treated group, but this finding was not statistically significant. OPN mRNA expression in liver was not altered by CD44 mAb therapy (Fig. 3E).

In addition, we determined fasting serum TG and T-ch concentrations after therapy. Serum TG concentrations were reduced in the mice treated with CD44 mAb and metformin compared with those in controls. T-ch levels were not changed in the groups treated with CD44 mAb and metformin (Table 1).

**CD44 mAb Improves Systemic Inflammatory State**

IR is associated with low-grade systemic inflammation. To determine the effect of CD44 mAb treatment on systemic inflammation, we analyzed TNF-α, IL-1β, IL-6, IFN-γ, and MCP-1 serum levels and WBC counts in treated mice. TNF-α, IL-1β, and IFN-γ serum levels were below the detectable limit of their respective assays in all the groups. However, we found reductions of serum IL-6 and MCP-1 levels and WBC counts in the CD44 mAb and metformin treatment groups, suggesting that these therapies can improve the systemic inflammatory state in HFD-fed mice (Table 1 and Supplementary Fig. 2).

**DISCUSSION**

We found that CD44 mAb suppressed visceral adipose tissue inflammation and reduced hyperglycemia, IR, body weight gain, and liver steatosis to levels comparable to those induced by metformin and pioglitazone in diet-
**Figure 3**—Effects of treatments on liver steatosis. A: Histological analysis. Hepatic lipid accumulation in liver was evaluated by H-E staining (top panels) and Oil Red O staining (bottom panels). Scale bars = 50 μm. B: Quantitative measurement of hepatic TG content. Hepatic TG content was measured in lipid extracts from livers and defined as mg of TG per gram of liver. RT-PCR analysis for cell markers (C), proinflammatory cytokines/chemokines (D), and CD44 and OPN (E). n = 4–8. *P < 0.05 vs. IgG2b. Met, metformin; Pio, pioglitazone.
Anti-CD44 Antibody Treats Diabetes in Obese Mice

We also conducted several investigations to assess the mechanisms involved in the beneficial effects of anti-CD44.

Adipose tissue macrophages are necessary and sufficient for the development of obesity-induced IR (23,24). In immunohistochemical analysis, we found that most infiltrating cells surrounding adipocytes were CD44-positive macrophages. We used the same anti-CD44 antibody (IM7) in these tests that was used for our therapy protocol, confirming that our therapeutic antibodies had bound to macrophages in obese adipose tissue.

RT-PCR showed that expression of macrophage markers and proinflammatory cytokines (CD68, F4/80, MCP-1, MIP-1α, TNF-α, IL-1β, and IL-6) was downregulated in adipose tissue of CD44 mAb–treated mice. We did not observe statistically significant downregulation of these genes in the liver. Furthermore, systemic levels of the proinflammatory cytokines and NEFA were also decreased in the CD44 mAb–treated group. These data suggested that anti-CD44 mAb can improve glucose metabolism and insulin sensitivity, most likely by reducing adipose tissue macrophage content. It is also likely that improvement of liver steatosis in the CD44 mAb–treated group could have been secondarily induced by the reduction of circulating cytokine levels and diminished NEFA released from inflammatory adipose tissue.

Weight gain in CD44 mAb–treated mice was reduced compared with that in controls. Additionally, members of the CD44 treatment group did not eat as much as members of other treatment groups during the study. In the CD44 mAb–treated group, expression levels of adiponectin were slightly higher, while those of leptin were lower; however, these differences were not statistically significant. Serum levels of two adipokines did not change after treatment with CD44 mAb. The levels of macrophage-related molecules were significantly lower in the CD44 mAb–treated mice. These data suggest that increased insulin sensitivity induced by anti-CD44 treatment was unlikely to result from the difference in adiposity, since this difference did not significantly alter levels of two adipokines in CD44 mAb–treated mice. We therefore speculate that the reduced weight gain and suppressed food intake during CD44 mAb treatment could have been induced by diminished systemic inflammation and improved leptin sensitivity originating from therapy-induced suppression of adipose inflammation. However, we cannot exclude the possibility that side effects of CD44 mAb treatment reduced food intake and weight gain in obese mice, and that this lack of weight gain contributed to the insulin-sensitive effect of CD44 mAb therapy.

It is also possible that systemic blockade of CD44 may impair leukocyte activity. We found that WBC counts were decreased in all the treatment groups compared with control antibody and untreated HFD groups. Interestingly, the decreased numbers of leukocytes post-treatment were comparable to those in mice fed an NFD, suggesting that an anti-inflammatory state can be induced by these treatments. However, we found a somewhat larger reduction of WBC count in CD44 mAb–treated mice, although the count was not statistically different from nontreated mice fed an NFD (Supplementary Fig. 2). This finding may not be explained by only the indirect effect of CD44 mAb through the reduction of systemic inflammation. We speculate that the direct removal of leukocytes from the circulation may also have a minor role in the larger effect of CD44 mAb on the number of leukocytes, as others have indicated (25). Future studies may need to determine the minimum effective dose of CD44 mAb that can improve insulin sensitivity while avoiding adverse effects as much as possible.

This study has demonstrated the potential of CD44 mAb as a treatment for diabetes and obesity. However, our ability to reveal the mechanisms involved in the therapeutic effects of the antibody was limited in this study. Anti-CD44 treatment can induce proteolytic removal of CD44 receptors from leukocyte surfaces and neutralize the HA-binding function of CD44-positive cells (9). CD44 mAb administration can also reduce the infiltration and migration of leukocytes in inflammatory sites (9). Based on this evidence, we believe that CD44 mAb removes CD44 from macrophage surfaces, thereby reducing macrophage activity in CLSs. However, we do recognize that this study did not address the detailed molecular and cellular mechanisms by which CD44 mAb suppressed adipose inflammation and improved diabetes and obesity. Future studies are needed to determine these precise mechanisms.

We found that 4 weeks of therapy with CD44 mAb suppressed visceral adipose tissue inflammation (as assessed by macrophage content) and improved fasting blood glucose levels, obesity, liver steatosis, and IR. Although open questions remain, our findings clearly suggest that CD44 mAb may be useful as a prototype anti-inflammatory drug to break links between obesity and IR, and that the CD44 immune receptor is a possible target for T2D therapy.

Acknowledgments. The authors thank Dr. Damon Tojar of the Department of Clinical Sciences, Lund University, Scania University Hospital, for his suggestions in preparing the manuscript. The authors also thank Dr. Junichiro Irie and Professor Hiroshi Itoh, in the Division of Endocrinology, Metabolism, and Nephrology, Keio University School of Medicine, for their support of the animal experiments. The authors also thank Valerie Natale for copyediting and manuscript preparation.

Funding. This work was supported by grants from the Howard Hughes Medical Institute, the National Library of Medicine (R01-LM009719), the Stanford University Office of Technology Licensing, and the Lucile Packard Foundation for Children’s Health.

Duality of Interest. Stanford University has filed a patent on anti-CD44 as a treatment for T2D. K.K. and A.J.B. are inventors. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. K.K. helped to design and perform the experiments, analyze the data, draft and review the manuscript, and approve the final version of the manuscript. K.T. helped to perform the experiments and analyze the data, drafted part of the manuscript, reviewed the draft, and approved the final version of the manuscript. S.M. and S.Y. performed some of the experiments, reviewed the draft of the manuscript, and approved the final version of the manuscript.
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