periportal or perisinusoidal fibrosis; stage 2, periportal plus perisinusoidal fibrosis; and stage 3, bridging fibrosis.[24]

Quantitative RT-PCR

Total RNA was extracted from the liver and epididymal white adipose tissue using Sepasol reagent (Nacalai Tesque, Inc., Kyoto, Japan). Quantitative RT-PCR was performed using the ABI Prism 7000 Sequence Detection System with PCR Master Mix Reagent (Applied Biosystems, Inc., Foster City, CA) as described previously.[23] Primers used are given in Table 1. mRNA levels were normalized to those of 36B4 mRNA.

Measurement of Serum Hydroperoxides

The total amount of organic hydroperoxides in the serum was measured at spectrophotometry using the derivatives of reactive oxygen metabolites test (FREE Carpe Diem; Diacron International SAS, Grosseto, Italy). Hydroperoxides are intermediate oxidative products of lipids, peptides, and amino acids, and their concentrations represent an index of oxidative injury. This method is described in detail elsewhere.[25] In brief, 20-μL serum samples were added to 1 mL assay mixture, gently mixed, and incubated for 3 minutes at 37°C. The absorbance increase at 505 nm was monitored for 2 minutes. The concentrations were expressed in conventional units [Carratelli units (U.CARR)], where 1 U.CARR corresponds to 0.8 mg/L H_2O_2.

TG Secretion Rate

The TG secretion rate was measured as previously described.[26] In brief, 500 mg/kg body weight tyloxapol (Triton WR-1339; Sigma-Aldrich Corp., St. Louis, MO) was injected via the tail vein into mice that had been fasted for 5 hours. Serum TG concentrations were measured at 60 minutes after injection. The TG secretion rate was calculated from the increment in TG concentration per minute multiplied by the serum volume of mice (estimated as 3.5% of body weight in grams) and expressed in milligrams per minute per 100 g body weight.

Statistical Analysis

Data are given as mean ± SE. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using analysis of variance followed by Scheffé's test.

Table 1. Primers Used in the Present Study

Genes	Primers
ACC1	
Forward	5′-TGAGATTGGCATGGTAGCCTG-3′
Reverse	5′-CTCGGCCATCTGGATATTCAG-3′
Catalase	
Forward	5′-GGAGGCAGAAACTTTCCCATT-3′
Reverse	5′-GGCCAAACCTTGGTCAGATC-3′
COL1A1	
Forward	5′-CCTCAGGGTATTGCTGGACAAC-3′
Reverse	5′-ACCACTTGATCCAGAAGGACCTT-3′
CPT1A	
Forward	5′-CCTGCATTCCTTCCCATTTG-3′
Reverse	5′-TGCCCATGTCCTTGTAATGTG-3′
F4/80	
Forward	5′-CTTTGGCTATGGGCTTCCAGT-3′
Reverse	5′-GCAAGGAGGACAGAGTTTATCGTG-3′
FAS	
Forward	5′-CCTGGATAGCATTCCGAACCT-3′
Reverse	5′-AGCACATCTCGAAGGCTACACA-3′
gp91^{phox}	
Forward	5′-CCAGTGCGTGTTGCTCGA-3′
Reverse	5′-AGTGAGGTTCCTGTCCAGTTGTCT-3′
MMP-2	
Forward	5′-CCCCATGAAGCCTTGTTTACC-3′
Reverse	5′-TTGTAGGAGGTGCCCTGGAA-3′
MTP	
Forward	5′-ACAGGTCCTCGAGCGTGTCT-3′
Reverse	5′-CAGTGCTCCGCCAGAGAAG-3′
p22^{phox}	
Forward	5′-CATGGAGCGATGTGGACAGA-3′
Reverse	5′-CCCGAAAAGCTTCACCACAG-3′
p40^{phox}	
Forward	5′-CAGCCAACATCGCTGACATC-3′
Reverse	5′-CAAAGTGGCTGGTGAAGCCC-3′
p47^{phox}	
Forward	5′-ACTCTCACTGAATACTTCAACG-3′
Reverse	5′-TCATCAGGCCGCACTTT-3′
p67^{phox}	
Forward	5′-AAGCAAAAAGAGCCCAAGGAA-3′
Reverse	5′-CATGTAAGGCATAGGCACGCT-3′
PPARα	
Forward	5′-AGGAAGCCGTTCTGTGACAT-3′
Reverse	5′-AATCCCCTCCTGCAACTTCT-3′
SOD1	
Forward	5′-GCAGGACCTCATTTTAATCCTCACT-3′
Reverse	5′-AGGTCTCCAACATGCCTCTCTTC-3′
SREBP1c	
Forward	5′-GGCACTAAGTGCCCTCAACCT-3′
Reverse	5′-GCCACATAGATCTCTGCCAGTGT-3′
TGFβ1	
Forward	5′-CCTGAGTGGCTGTCTTTTGACG-3′
Reverse	5′-AGTGAGCGCTGAATCGAAAGC-3′
TIMP1	
Forward	5′-CATCACGGGCCGCCTA-3′
Reverse	5′-AAGCTGCAGGCACTGATGTG-3′
TNFα	
Forward	5′-ACCCTCACACTCAGATCATCTTC-3′
Reverse	5′-TGGTGGTTTGCTACGACGT-3′
36B4	
Forward	5′-GGCCCTGCACTCTCGCTTTC-3′
Reverse	5′-TGCCAGGACGCGCTTGT-3′

Results

Metabolic Phenotypes of MC4R-KO Mice

Throughout the experimental period, MC4R-KO mice demonstrated accelerated body weight gain relative to WT mice fed either the standard diet or the HFD (Figure 1A). Both the WT mice fed the HFD and the MC4R-KO mice fed the standard diet exhibited increased adiposity relative to the WT mice fed the standard diet (Figure 1B). Although increased adiposity was observed in MC4R-KO mice at 8 weeks of HFD feeding, there was no further increase or decrease in adipose tissue weight thereafter (Figure 1B). In contrast, MC4R-KO mice exhibited a time-dependent increase in liver

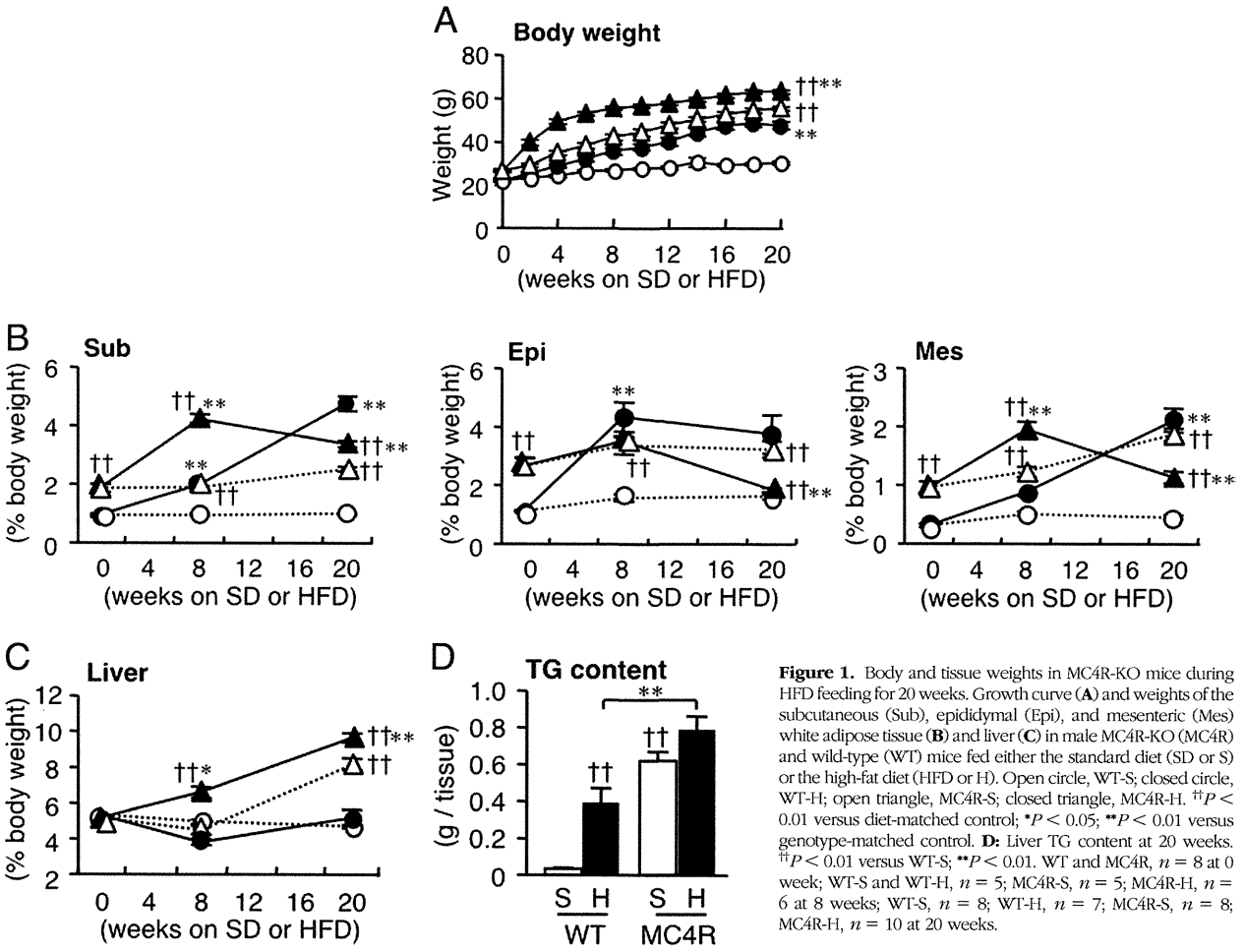

Figure 1. Body and tissue weights in MC4R-KO mice during HFD feeding for 20 weeks. Growth curve (**A**) and weights of the subcutaneous (Sub), epididymal (Epi), and mesenteric (Mes) white adipose tissue (**B**) and liver (**C**) in male MC4R-KO (MC4R) and wild-type (WT) mice fed either the standard diet (SD or S) or the high-fat diet (HFD or H). Open circle, WT-S; closed circle, WT-H; open triangle, MC4R-S; closed triangle, MC4R-H. $^{\dagger\dagger}P < 0.01$ versus diet-matched control; $^{*}P < 0.05$; $^{**}P < 0.01$ versus genotype-matched control. **D:** Liver TG content at 20 weeks. $^{\dagger\dagger}P < 0.01$ versus WT-S; $^{**}P < 0.01$. WT and MC4R, $n = 8$ at 0 week; WT-S and WT-H, $n = 5$; MC4R-S, $n = 5$; MC4R-H, $n = 6$ at 8 weeks; WT-S, $n = 8$; WT-H, $n = 7$; MC4R-S, $n = 8$; MC4R-H, $n = 10$ at 20 weeks.

weight and hepatic TG content relative to WT mice fed either diet (Figure 1, C and D; and data not shown). Both genotypes fed the HFD exhibited insulin resistance, and MC4R-KO mice fed the HFD exhibited a significant increase in serum FFA concentrations relative to WT mice (Table 2). Dysregulation of adipocytokines was also marked in MC4R-KO mice fed the HFD relative to WT mice (Table 2, and Supplemental Table S2 at *http://ajp.amjpathol.org*). Serum alanine aminotransferase concentrations were significantly increased in MC4R-KO mice fed the HFD relative to any other groups (Table 2). In essence, these observations are consistent with previous reports that described the metabolic phenotypes in the liver in MC4R-KO mice, which were generated using different strategies.[18,19] Collectively, our data indicate that MC4R-KO mice fed

Table 2. Serologic Parameters of MC4R-KO and WT Mice Fed the HFD for 20 Weeks

Variable	WT mice		MC4R-KO mice	
	SD	HFD	SD	HFD
Blood glucose (*ad lib*, mg/dL)	117.3 ± 5.8	133.0 ± 8.3	167.4 ± 10.3*	170.1 ± 12.4
HOMA-IR	0.8 ± 0.3	15.5 ± 3.7*	17.0 ± 4.0†	21.8 ± 3.7
TG (mg/dL)	84.6 ± 9.8	46.3 ± 1.2*	135.8 ± 18.4*	92.7 ± 8.1
FFA (mEq/L)	0.27 ± 0.02	0.26 ± 0.01	0.34 ± 0.02	0.51 ± 0.06‡§
TC (mg/dL)	51.6 ± 2.8	189.7 ± 6.4†	143.0 ± 9.5*	294.5 ± 9.2‡¶
Adiponectin (μg/mL)	15.4 ± 1.7	19.8 ± 1.7	13.5 ± 1.0	8.2 ± 0.9‡§
Leptin (ng/mL)	1.7 ± 0.3	97.2 ± 8.3†	57.7 ± 6.0†	112.6 ± 9.2¶
IL-6 (pg/mL)	0.84 ± 0.64	2.21 ± 1.68	3.25 ± 0.75	6.80 ± 0.71‡¶
ALT (IU/L)	36.9 ± 1.1	129.9 ± 19.6	191.9 ± 29.2*	623.9 ± 50.8‡¶

$n = 7$–10. Data are expressed as mean ± SE.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$ versus WT-SD; $^{\ddagger}P < 0.01$ versus WT-HFD; $^{\S}P < 0.05$; $^{\P}P < 0.01$ versus MC4R-SD.
ALT, alanine aminotransferase; FFA, free fatty acid; HFD, high-fat diet; HOMA-IR, homeostasis model assessment–insulin resistance; IL-6, interleukin-6; SD, standard diet; TC, total cholesterol; TG, triglyceride; WT, wild-type.

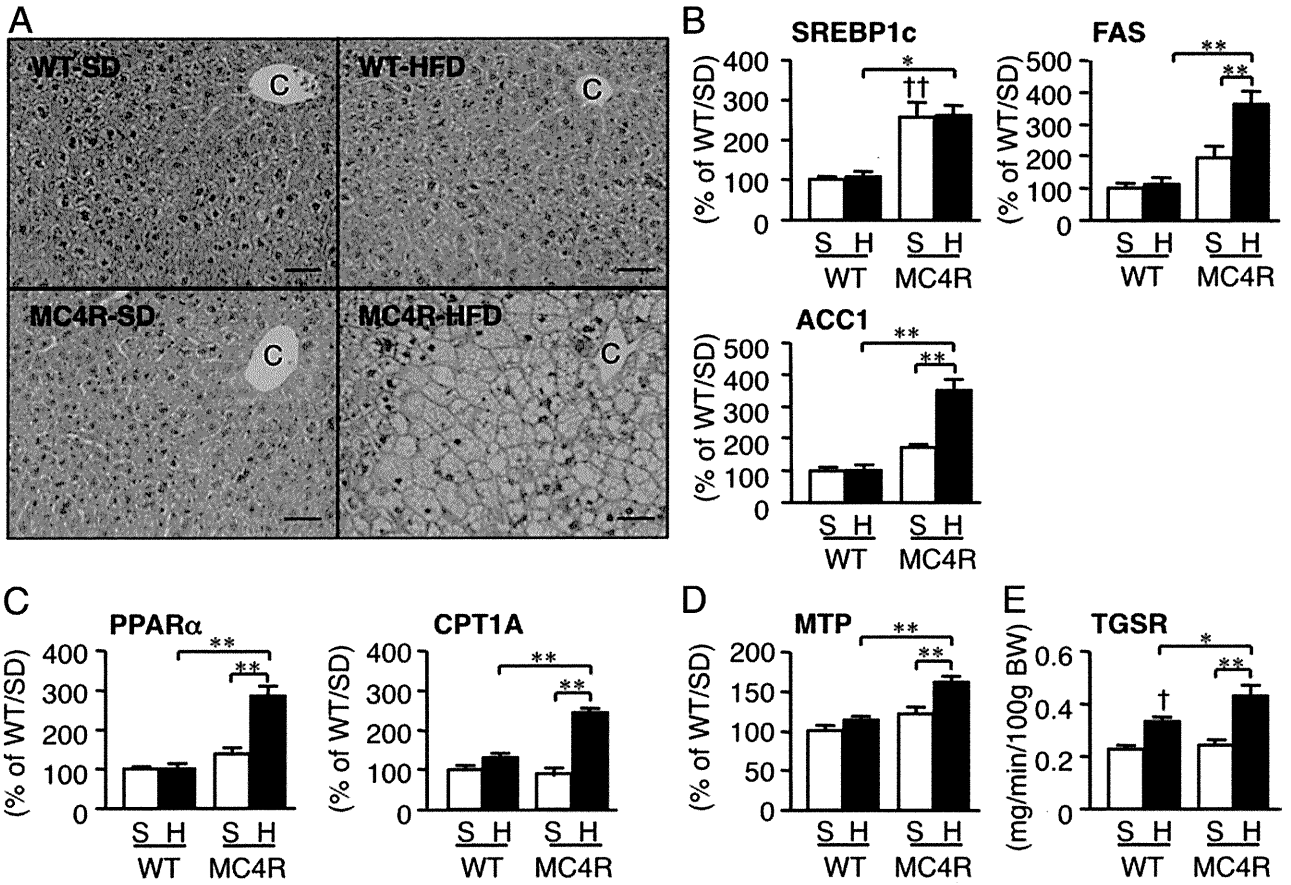

Figure 2. Hepatic histologic features and mRNA expression of genes related to lipid metabolism in MC4R-KO and WT mice fed the HFD for 8 weeks. **A:** H&E staining of the liver. C, central vein. Original magnification, × 200. Scale bar = 50 μm. Hepatic mRNA expression of genes for *de novo* lipogenesis (sterol regulatory element binding protein 1c, fatty acid synthase, and acetyl-CoA carboxylase 1) (**B**), oxidative metabolism (peroxisome proliferator-activated receptor-α and carnitine palmitoyltransferase 1A) (**C**), and TG secretion (microsomal TG transport protein) (**D**). **E:** Triglyceride secretion rate from the liver at 2 weeks of HFD feeding. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ versus WT-S. WT-S and WT-H, $n = 5$; MC4R-S, $n = 5$; MC4R-H, $n = 6$.

the HFD exhibit metabolic characteristics similar to those in obese humans.

Lipid Metabolism and Oxidative Stress in Liver from MC4R-KO Mice

Next examined was lipid metabolism and oxidative stress in liver from MC4R-KO mice fed the HFD. Although histologic examinations revealed minimal lipid accumulation in liver from WT mice fed the HFD for 8 weeks, liver from MC4R-KO mice exhibited massive microvesicular steatosis in the centrilobular and portal areas (Figure 2A). Expression of mRNAs for *de novo* lipogenesis (fatty acid synthase and acetyl-CoA carboxylase 1) was markedly increased in liver from MC4R-KO mice relative to WT mice at 8 weeks (Figure 2B), as previously reported.[18,19] In addition, expression of mRNAs for fatty acid oxidation (peroxisome proliferator–activated receptor-α and carnitine palmitoyltransferase 1A), and TG secretion (microsomal triglyceride transport protein) and TG secretion rate were increased in liver from MC4R-KO mice (Figure 2, C–E). These observations are consistent with lipid metabolism in human NASH.[27] In contrast to the changes in hepatic expression of lipogenic genes in MC4R-KO mice (Figure 2B), WT mice exhibited up-regulation of lipogenic genes only after 20 weeks of HFD feeding (data not shown), which suggests that hepatic steatosis develops much faster in MC4R-KO mice than in WT mice. There was a marked increase in mRNA expression of the NADPH oxidase components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$, and $p22^{phox}$), and a slight increase in mRNA expression of antioxidant enzymes (superoxide dismutase 1 and catalase) in the HFD-fed MC4R-KO mice relative to any other groups (see Supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*). Serum concentrations of derivatives of reactive oxidative metabolite were significantly increased in MC4R-KO mice fed the HFD ($P < 0.01$; see also Supplemental Figure S1C at *http://ajp.amjpathol.org*).

Lipid Accumulation and Fibrosis in Liver from MC4R-KO Mice

After HFD feeding for 20 weeks, microvesicular steatosis was observed uniformly, and moderate inflammatory cell infiltration in liver from WT mice (Figure 3A), whereas liver fibrosis was rarely observed at this time point (Figure 3, B–D). In contrast, liver from MC4R-KO mice fed the HFD exhibited microvesicular and macrovesicular steatosis, ballooning degeneration, and

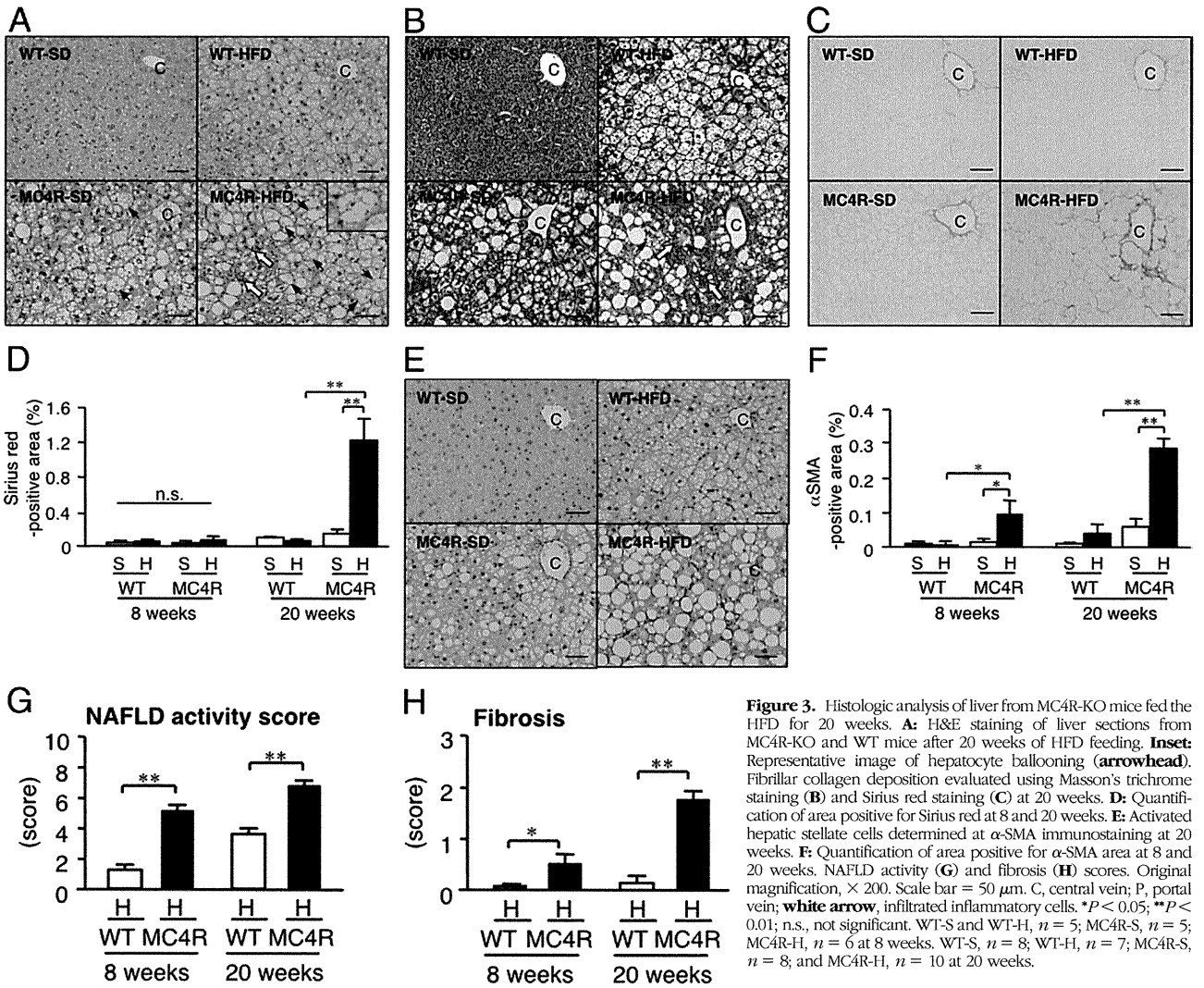

Figure 3. Histologic analysis of liver from MC4R-KO mice fed the HFD for 20 weeks. **A:** H&E staining of liver sections from MC4R-KO and WT mice after 20 weeks of HFD feeding. **Inset:** Representative image of hepatocyte ballooning (**arrowhead**). Fibrillar collagen deposition evaluated using Masson's trichrome staining (**B**) and Sirius red staining (**C**) at 20 weeks. **D:** Quantification of area positive for Sirius red at 8 and 20 weeks. **E:** Activated hepatic stellate cells determined at α-SMA immunostaining at 20 weeks. **F:** Quantification of area positive for α-SMA area at 8 and 20 weeks. NAFLD activity (**G**) and fibrosis (**H**) scores. Original magnification, × 200. Scale bar = 50 μm. C, central vein; P, portal vein; **white arrow**, infiltrated inflammatory cells. $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant. WT-S and WT-H, $n = 5$; MC4R-S, $n = 5$; MC4R-H, $n = 6$ at 8 weeks. WT-S, $n = 8$; WT-H, $n = 7$; MC4R-S, $n = 8$; and MC4R-H, $n = 10$ at 20 weeks.

massive infiltration of inflammatory cells (Figure 3A). Masson's trichrome and Sirius red staining revealed marked pericellular fibrosis in liver from MC4R-KO mice fed the HFD (Figure 3, B–D). In addition, the area positive for α-SMA was markedly increased in MC4R-KO mice relative to WT mice fed the HFD for 8 and 20 weeks ($P < 0.05$ and $P < 0.01$, respectively; Figure 3, E and F). Histologic analysis demonstrated a significant increase in the NAFLD activity and fibrosis scores in MC4R-KO mice at 8 and 20 weeks of HFD feeding (Figure 3, G and H). Expression of mRNAs for fibrogenic genes (transforming growth factor-β1; collagen, type 1, α1; metalloproteinase-2; and tissue inhibitor of metalloproteinase 1), and inflammatory genes (macrophage marker F4/80 and tumor necrosis factor-α) was increased in liver from MC4R-KO mice relative to WT mice after 20 weeks of HFD feeding (Figure 4). Together, these observations suggest that MC4R-KO mice fed the HFD develop liver fibrosis accompanied by histologic pathognomonic features of human NASH: inflammatory cell infiltration, hepatocyte ballooning, and pericellular fibrosis.[1,24]

Development of HCC in MC4R-KO Mice

After feeding the HFD for 1 year, mild fibrosis was observed in WT mice, which was much more accelerated in MC4R-KO mice (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Moreover, multiple liver tumors were observed in all of the MC4R-KO mice examined ($n = 5$) (Figure 5A). Microscopic analysis revealed that the tumors form discrete nodules surrounded by non-tumor liver tissue (Figure 5B). Normal liver architecture was lost, and irregular and thick trabeculae were observed in the tumors (Figure 5C). The tumor cells exhibited severe dysplasia, with an increased nuclear-cytoplasmic ratio, enlarged and hyperchromatic nuclei, and fat deposition in the cytoplasm (Figure 5C). Immunohistochemical analysis revealed that the tumors express α-fetoprotein, a widely recognized marker of HCC (Figure 5D). In contrast, at 20 weeks and even at 1 year, there was no appreciable collagen deposition and tumor development in liver from MC4R-KO mice fed the standard diet (Figures 3; see also Supplemental Figure S2 at *http://ajp.amjpathol.org*). To elucidate the involvement of the inher-

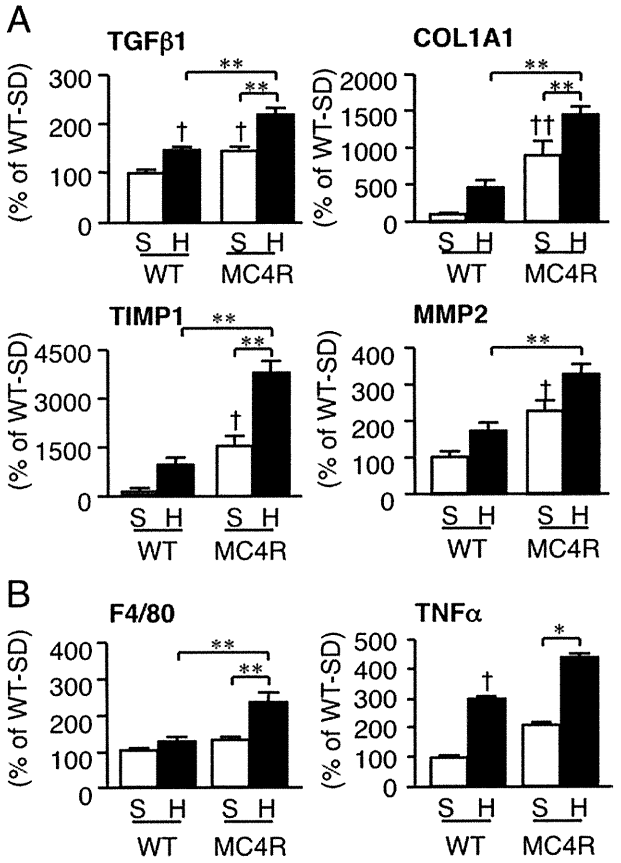

Figure 4. Hepatic mRNA expression in MC4R-KO mice fed the HFD for 20 weeks. Hepatic mRNA expression levels were measured using quantitative PCR after 20 weeks of HFD feeding. mRNA expression of fibrogenic factors (transforming growth factor-β1; collagen, type 1, α1; tissue inhibitor of metalloproteinase 1;, and metalloproteinase 2) (**A**) and inflammatory markers (F4/80 and tumor necrosis factor-α) (**B**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ versus WT-S. WT-S, $n = 8$; WT-H, $n = 7$; MC4R-S, $n = 8$; and MC4R-H, $n = 10$.

ent dysregulation of inflammation and fibrogenesis in MC4R-KO mice, we examined the liver and adipose tissue phenotypes in 8 week-old MC4R-KO and WT mice. There was no significant difference in mRNA expression of pro-fibrotic and inflammatory genes between the genotypes at this time point (see Supplemental Figure S3 at *http://ajp.amjpathol.org*). These observations suggest that in addition to liver fibrosis, MC4R-KO mice fed the HFD develop multiple liver tumors with histologic characteristics of well-differentiated HCC.

Inflammatory and Fibrotic Changes in Adipose Tissue from MC4R-KO Mice

Recent evidence has suggested that obese adipose tissue exhibits chronic inflammatory changes characterized by macrophage infiltration and fibrosis, which may contribute to ectopic lipid accumulation and systemic insulin resistance.[28,29] In the present study, macrophage infiltration and pro-inflammatory cytokine expression were markedly increased in the epididymal white adipose tissue from MC4R-KO mice relative to WT mice after 8 weeks of HFD feeding, although the epididymal white adipose tissue weight was almost comparable between the genotypes (Figure 1B and Figure 6, A and B). Moreover, a marked increase was observed in collagen deposition and in transforming growth factor β_1 and in collagen, type 1, α1 mRNA expression in the epididymal white adipose tissue from MC4R-KO mice relative to WT mice (Figure 6, C and D). These observations suggest that adipose tissue inflammation is markedly enhanced in MC4R-KO mice relative to WT mice during HFD feeding, along with dysregulation of adipocytokine production in MC4R-KO mice (see Supplemental Table S2 at *http://ajp.amjpathol.org*).

Discussion

According to the two-hit hypothesis, the pathogenesis of NASH may involve at least two processes: excessive accumulation of lipids in the liver and enhanced liver fibrosis. To understand the molecular mechanism underlying the development of NASH, many animal models of hepatic steatosis and liver fibrosis have been described. Herein we demonstrate that MC4R-KO mice fed an HFD for a relatively short time (20 weeks) exhibit a liver condition similar to human NASH, in addition to obesity and insulin resistance. As a model of hyperphagic obesity, *ob/ob* mice exhibit severe hepatic steatosis, although

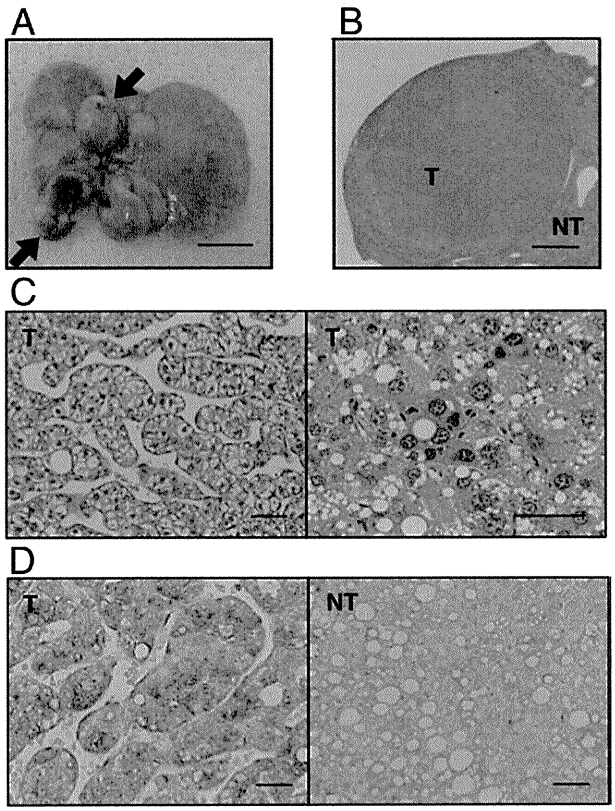

Figure 5. Development of hepatocellular carcinoma in liver from MC4R-KO mice after 1 year of HFD feeding. All MC4R-KO mice ($n = 5$) developed multiple liver tumors (**arrows**). **A:** Representative macroscopic image of liver from MC4R-KO mice. H&E staining (**B** and **C**) and α-fetoprotein immunostaining (**D**) of the tumor. NT, non-tumor liver; T, tumors. Scale bars: 1 cm (**A**); 1 mm (**B**); 50 μm (**C** and **D**).

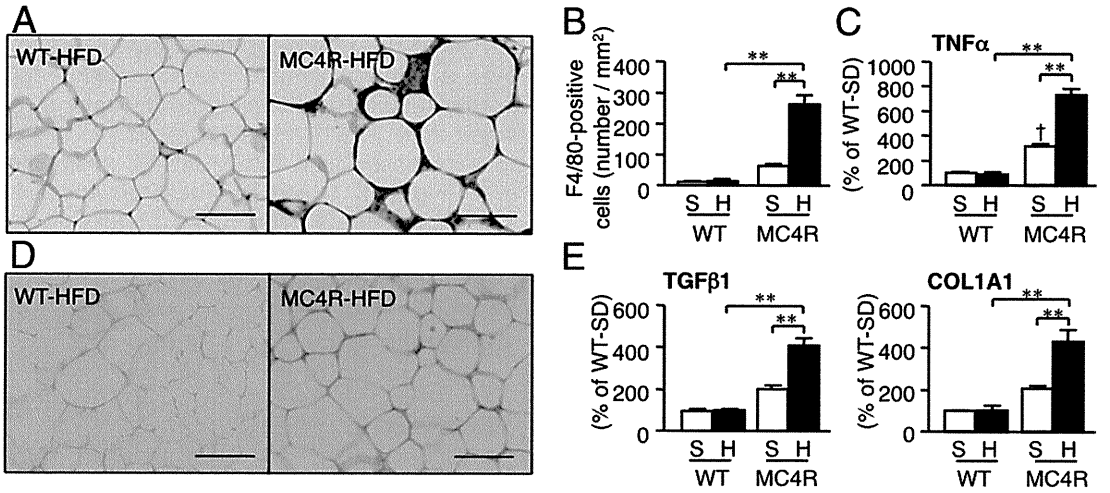

Figure 6. Inflammatory changes in epididymal white adipose tissue from MC4R-KO mice fed the HFD. Representative F4/80 immunostaining (**A**) and quantification of F4/80-positive cells (**B**) of the epididymal white adipose tissue from MC4R-KO and WT mice fed the HFD for 8 weeks. Original magnification, ×200. Scale bar = 100 μm. **C:** Tumor necrosis factor-α mRNA expression in the epididymal white adipose tissue. Sirius red staining of epididymal white adipose tissue (**D**) and mRNA expression of fibrogenic genes (transforming growth factor-β1; and collagen, type 1, α1) (**E**). $^{**}P < 0.01$; $^{\dagger}P < 0.05$ versus WT-S. WT-S and WT-H, $n = 5$; and MC4R-S, $n = 5$, MC4R-H, $n = 6$.

they are resistant to liver fibrosis.[3] Moreover, animals with diet-induced obesity exhibit hepatic steatosis and develop mild liver fibrosis only after long-term (usually longer than 1 year) HFD feeding,[30,31] as was observed in the present study. In contrast, chemically induced liver fibrosis is not accompanied by obesity, insulin resistance, and hepatic steatosis.[3] Dietary deficiency of methionine and choline also contribute to development of steatosis and mild fibrosis, without obesity and insulin resistance.[3] In this regard, Larter et al[32] recently reported that *foz/foz* mice, carrying truncating mutation in *Alms1*, demonstrated both obesity and NASH-like liver histologic features during long-term feeding of an HFD. This study has established MC4R-KO mice fed the HFD for a relatively short time as a novel rodent model of NASH with obesity, insulin resistance, and excessive accumulation of lipids and enhanced fibrosis in the liver.

NASH is a severe form of NAFLD, and can progress to cirrhosis and HCC. However, there have been no appropriate animal models in which NASH progressed to HCC during the course of obesity. Indeed, there are a few genetic models of liver fibrosis and HCC: mice with liver-specific disruption of phosphatase and tensin homolog deleted from chromosome 10 and those lacking methionine adenosyltransferase 1A, although they do not exhibit obesity and insulin resistance.[33,34] In the present study, we have demonstrated that MC4R-KO mice, when fed the HFD over the long term, develop multiple liver tumors with histologic characteristics of well-differentiated HCC. In MC4R-KO mice fed the HFD, enhanced pro-inflammatory cytokine production and fibrotic changes preceded development of multiple liver tumors, which is consistent with a recent report by Park et al[35] that increased production of tumor necrosis factor-α and IL-6 in obesity is involved in promotion of carcinogen-induced hepatic tumorigenesis. Collectively, MC4R-KO mice would provide a novel mouse model of NASH with which to investigate the sequence of events that make up diet-induced hepatic steatosis, liver fibrosis, and HCC or how overnutrition leads to hepatic steatosis and liver fibrosis and eventually to HCC.

During the course of obesity, there might be complex interactions between the adipose tissue and the liver (the adipohepatic axis) in the pathogenesis of NASH.[6,7] In the present study, enhanced adipose tissue inflammation, ie, increased macrophage infiltration and fibrotic changes, was observed in MC4R-KO mice relative to WT mice during the HFD feeding. We previously have demonstrated that cross-talk between adipocytes and macrophages in obese adipose tissue results in marked up-regulation of pro-inflammatory adipocytokines such as tumor necrosis factor-α and significant down-regulation of anti-inflammatory adiponectin.[36] Of note, saturated fatty acids, which are released in large quantities from hypertrophied adipocytes via macrophage-induced lipolysis, may act as an endogenous ligand for the Toll-like receptor 4 complex.[37] Moreover, FFAs, when overproduced in the visceral fat depots through the cross-talk between adipocytes and macrophages, may enter the liver via the portal vein. Indeed, MC4R-KO mice demonstrated increased serum concentrations of FFA relative to WT mice during the HFD feeding. It is, therefore, likely that enhanced adipose tissue inflammation in MC4R-KO mice causes dysregulation of adipocytokine production and FFA release, thereby contributing to the NASH-like hepatic phenotype. Also observed was a marked increase in collagen deposition in white adipose tissue from MC4R-KO mice. Khan et al[38] recently reported that in collagen VI–deficient mice extracellular matrix components, when increased in obese adipose tissue, inhibited adipose tissue expansion. This is consistent with a recent clinical study that demonstrated that adipose tissue fibrosis is negatively correlated with adipocyte diameter in obese humans.[39] Moreover, McQuaid et al[40] reported that the lipid storage function in adipose tissue is negatively associated with ectopic lipid accumulation in obese

humans. It is, therefore, conceivable that both adipose tissue macrophage infiltration and fibrotic changes increase the release of FFAs, thereby contributing to excessive fat accumulation in the liver in MC4R-KO mice. Together, these observations suggest that enhanced adipose tissue inflammation has a role in development of NASH as both the first and second hits in MC4R-KO mice. These data suggest that MC4R-KO mice are useful for investigation of the role of the adipohepatic axis in the development of NASH.

Because MC4R mRNA expression is restricted to the hypothalamus and other brain regions[41] and is undetectable in liver from WT mice fed the HFD (Itoh et al, unpublished data, 2010), it is likely that the hepatic phenotype in MC4R-KO mice results from loss of function of MC4R in the brain. In the present study, MC4R-KO mice demonstrated increased expression of mRNAs for *de novo* lipogenesis, fatty acid oxidation, and TG secretion relative to WT mice fed the HFD. This is consistent with a recent report by Nogueiras et al[42] that short-term pharmacologic blockade of the central melanocortin system in WT mice results in increased expression of *de novo* lipogenic genes and microsomal TG transport protein in the liver. Evidence has accumulated suggesting that the brain and liver interact via neuronal pathways, central insulin signaling regulates glucose metabolism in the liver, and peroxisome proliferator-activated receptor-γ activation in the liver modulates energy expenditure and systemic insulin sensitivity via the sympathetic nervous system.[43,44] In this regard, we previously reported that the central melanocortin system is involved in renal macrophage infiltration in a mouse model of renal fibrosis.[21] This discussion also supports the concept that MC4R signaling in the brain is involved in excessive accumulation of lipids and fibrosis in the liver in MC4R-KO mice.

Because MC4R-KO mice exhibited hyperleptinemia, leptin signaling may have a role in the pathogenesis of the hepatic liver phenotype in MC4R-KO mice. Indeed, deficiency of leptin signaling protects against hepatic fibrosis in several rodent models of chronic liver injury.[9–11] Exogenous leptin administration also accelerates carbon tetrachloride–induced liver fibrosis in WT mice.[45] These findings led us to speculate that leptin acts as a pro-inflammatory and pro-fibrotic cytokine in liver fibrosis. However, obesity is associated with the reduced ability of circulating leptin to regulate energy homeostasis or central leptin resistance.[46] Inasmuch as leptin receptors are widely expressed in peripheral tissues including the liver,[47] leptin may act directly on the liver to induce inflammation and fibrosis. If so, it would be important to know whether leptin is effective in the peripheral tissues in obesity. Further studies will be required to elucidate how hyperleptinemia is involved in development of the hepatic phenotype in MC4R-KO mice. It would be interesting to generate tissue-specific leptin receptor–deficient mice and/or MC4R-KO mice crossed with *ob/ob* mice for the next phase of study.

In conclusion, the present study is the first to demonstrate that MC4R-KO mice develop a liver condition similar to human NASH when fed an HFD, which is associated with obesity, insulin resistance, and dyslipidemia. Of note, they develop well-differentiated HCC after long-term HFD feeding. Our data support the concept that NASH develops from a combination of excessive lipid accumulation in the liver and systemic and/or local chronic inflammation. Thus, MC4R-KO mice would provide a novel mouse model of NASH with which to investigate the sequence of events that make up diet-induced hepatic steatosis, liver fibrosis, and HCC, and thus aid in understanding its pathophysiologic features, pursuing specific biomarkers, and evaluating potential therapeutic strategies.

Acknowledgments

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Review Article

Adipose Tissue Remodeling as Homeostatic Inflammation

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Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation. Obese adipose tissue is characterized by dynamic changes in cellular composition and function, which may be referred to as "adipose tissue remodeling". Among stromal cells in the adipose tissue, infiltrated macrophages play an important role in adipose tissue inflammation and systemic insulin resistance. We have demonstrated that a paracrine loop involving saturated fatty acids and tumor necrosis factor-α derived from adipocytes and macrophages, respectively, aggravates obesity-induced adipose tissue inflammation. Notably, saturated fatty acids, which are released from hypertrophied adipocytes via the macrophage-induced lipolysis, serve as a naturally occurring ligand for Toll-like receptor 4 complex, thereby activating macrophages. Such a sustained interaction between endogenous ligands derived from parenchymal cells and pathogen sensors expressed in stromal immune cells should lead to chronic inflammatory responses ranging from the basal homeostatic state to diseased tissue remodeling, which may be referred to as "homeostatic inflammation". We, therefore, postulate that adipose tissue remodeling may represent a prototypic example of homeostatic inflammation. Understanding the molecular mechanism underlying homeostatic inflammation may lead to the identification of novel therapeutic strategies to prevent or treat obesity-related complications.

1. Introduction

The metabolic syndrome is a constellation of visceral fat obesity, insulin resistance, atherogenic dyslipidemia, and hypertension, which all independently increase the risk of atherosclerotic diseases [1–5]. The adipose tissue secretes a number of bioactive substances or adipocytokines, and unbalanced production of pro- and anti-inflammatory adipocytokines in obese adipose tissue may critically contribute to many aspects of the metabolic syndrome [1–5]. Obesity is now viewed as a state of systemic, chronic low-grade inflammation [1–4]. In contrast to acute inflammation which resolves by an active termination program, chronic inflammation may involve persistent stress and/or impaired resolution process, thereby resulting in functional maladaptation and tissue remodeling [6]. On the other hand, during the course of obesity, adipose tissue is characterized by adipocyte hypertrophy, followed by increased angiogenesis, immune cell infiltration, and extracellular matrix overproduction [1, 2, 7, 8], which may be referred to as adipose tissue remodeling.

Pathogen sensors or pattern-recognition receptors (PRRs), which are important for the recognition of pathogen-associated molecular patterns (PAMPs) in innate immunity, are also capable of recognizing endogenous ligands, damage-associated molecular patterns (DAMPs) or danger signals (Figure 1) [6, 9, 10]. Interaction between endogenous ligands and pathogen sensors may play a role in the basal homeostatic state as well as diseased tissue remodeling, which has been referred to as homeostatic inflammation [6, 11]. This paper summarizes the molecular mechanism and pathophysiologic implication of adipose tissue remodeling as a prototypic example of homeostatic inflammation.

2. Adipose Tissue Inflammation and Adipose Tissue Remodeling

In addition to lipid-laden mature adipocytes, the adipose tissue is composed of various stromal cells, including

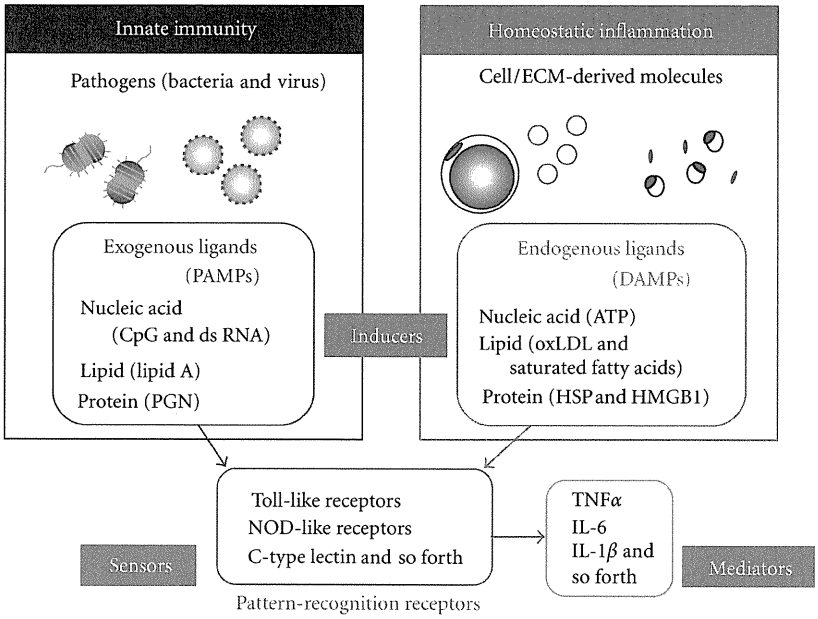

FIGURE 1: Adipose tissue inflammation as homeostatic inflammation. In innate immunity, exogenous ligands (pathogen-associated molecular patterns; PAMPs) are sensed by pattern-recognition receptors (PRRs), thereby inducing inflammatory changes. On the other hand, damage-associated molecular patterns (DAMPs) released from damaged or stressed cells and tissues can activate PRRs, thereby inducing homeostatic inflammation ranging from the basal homeostatic state to diseased tissue remodeling. For instance, free fatty acids (FFAs) released from hypertrophied adipocytes can report, as a danger signal, their diseased state to macrophages via Toll-like receptor 4 (TLR4) complex during the course of obesity. dsRNA, double-strand RNA; PGN, peptidoglycan; ATP, adenosine tri-phosphate; oxLDL, oxidized low-density lipoprotein; HSP, heat shock protein; HMGB1, high-mobility group box-1.

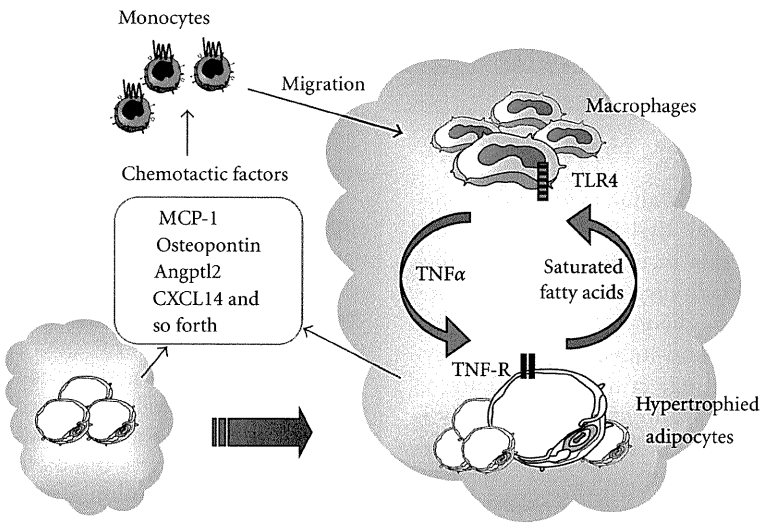

FIGURE 2: Molecular mechanism underlying adipose tissue inflammation. During the course of obesity, adipose tissue secretes several chemotactic factors to induce macrophage infiltration into adipose tissue. Circulating monocytes migrate and infiltrate into adipose tissue through adhesion process to endothelial cells. Macrophages enhance the inflammatory changes through the crosstalk with parenchymal adipocytes. For example, the macrophage-derived tumor necrosis factor-α (TNFα) induces the release of saturated fatty acids from adipocytes via lipolysis, which, in turn, induces inflammatory changes in macrophages via TLR4. Such a paracrine loop between adipocytes and macrophages constitutes a vicious cycle, thereby further accelerating adipose tissue inflammation. TNF-R, TNFα receptor.

preadipocytes, endothelial cells, fibroblasts, and immune cells [12]. Obese adipose tissue exhibits functional and morphological changes, thereby leading to unbalanced production of pro- and anti-inflammatory adipocytokines [1, 2, 7, 8]. The morphological changes found in obese adipose tissue are reminiscent of the chronic inflammatory responses in atherosclerotic vascular walls termed vascular remodeling, which arise from the complex interactions among vascular endothelial cells, vascular smooth muscle cells, lymphocytes, and monocyte-derived macrophages [4]. Vascular remodeling is considered to be an adaptive process in response to long-term changes in hemodynamic conditions and lipid metabolism, thereby contributing to the pathophysiology of vascular diseases [13]. Thus, the dynamic changes seen in obese adipose tissue can be referred to as adipose tissue remodeling. Notably, macrophage infiltration and inflammation-related gene expression in the adipose tissue precedes the development of insulin resistance in animal models [14, 15], suggesting that macrophages should play a central role in adipose tissue remodeling. It is, therefore, important to know the pathophysiologic role of macrophages infiltrated into the adipose tissue during the course of adipose tissue remodeling.

3. Macrophage Infiltration into Obese Adipose Tissue

Evidence has accumulated that adipocytes *per se* secrete pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), during the course of adipocyte hypertrophy [1–3]. Increased production of chemokines in obese adipose tissue has been implicated in the regulation of monocyte recruitment to adipose tissue [14]. The involvement of MCP-1/chemokine receptor 2 (CCR2) pathway has been extensively studied as the mechanism underlying macrophage infiltration into obese adipose tissue (Figure 2) [16–19]. Moreover, several reports have suggested the role of other chemotactic factors in obesity-induced macrophage infiltration: osteopontin, angiopoietin-like protein 2 (Angptl2), and CXC motif chemokine ligand-14 (CXCL14) (Figure 2) [20–22]. Inhibition of macrophage infiltration into obese adipose tissue through genetic and/or pharmacologic strategies has improved the dysregulation of adipocytokine production, thereby leading to the amelioration of obesity-induced adipose tissue inflammation and insulin resistance. Indeed, macrophage infiltration and inflammation-related gene expression in the adipose tissue precedes the development of insulin resistance in animal models [14, 15]. Understanding the molecular mechanisms underlying increased macrophage infiltration into obese adipose tissue may lead to the identification of novel therapeutic strategies to prevent or treat obesity-induced adipose tissue inflammation.

4. Interaction between Adipocytes and Macrophages

The adipose tissue macrophages also represent a major source of pro-inflammatory cytokines, which play important roles in chronic inflammatory responses in obese adipose tissue. Using an *in vitro* coculture system composed of adipocytes and macrophages, we have demonstrated that a paracrine loop involving saturated fatty acids and TNFα derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes (Figure 2) [23]. Among numerous cytokines derived from infiltrated macrophages in obese adipose tissue, TNFα acts on TNF receptor in hypertrophied adipocytes, thereby inducing pro-inflammatory cytokine production and adipocyte lipolysis via nuclear factor-κB- (NF-κB-) dependent and independent (possibly mitogen-activated protein kinase- (MAPK-) dependent) mechanisms, respectively [24]. On the other hand, saturated fatty acids released from adipocytes serve as a naturally occurring ligand for Toll-like receptor 4 (TLR4) complex, which is essential for the recognition of lipopolysaccharide (LPS), to induce NF-κB activation in macrophages [24].

The interaction between adipocytes and macrophages results in marked upregulation of pro-inflammatory adipocytokines and significant downregulation of anti-inflammatory adipocytokines, which lead to development of obesity-related complications in multiple organs, such as atherosclerosis and hepatic steatosis [1–4]. For instance, adiponectin is a well-established anti-inflammatory adipocytokine, which is markedly downregulated in obese adipose tissue, and supplementation of adiponectin in obese mice effectively reverses insulin resistance in the skeletal muscle and liver [25, 26]. On the other hand, overproduction of MCP-1 induces macrophage infiltration into the adipose tissue and directly induces insulin resistance in the skeletal muscle and liver [17, 18, 27]. Thus, dysregulation of adipocytokine production as a result of inflammatory changes in the adipose tissue may be involved in the pathogenesis of metabolic derangements in obesity.

5. Heterogeneity of Adipose Tissue Macrophages

Recent studies have pointed to the phenotypic change of macrophages in lean and obese adipose tissue; M1 or classically activated (pro-inflammatory) macrophages and M2 or alternatively activated (anti-inflammatory) macrophages (Figure 3) [28]. Adipocytes in lean adipose tissue produce humoral factors that induce M2 activation of macrophages, such as interleukin-4 (IL-4) and interleukin-13 (IL-13), and M2 activated macrophages release anti-inflammatory mediators, such as interleukin (IL-10) [29]. On the other hand, hypertrophied adipocytes secrete pro-inflammatory saturated fatty acids, cytokines, and chemokines to induce M1 polarization of macrophages [29]. Activated M1 macrophages in turn produce pro-inflammatory cytokines and

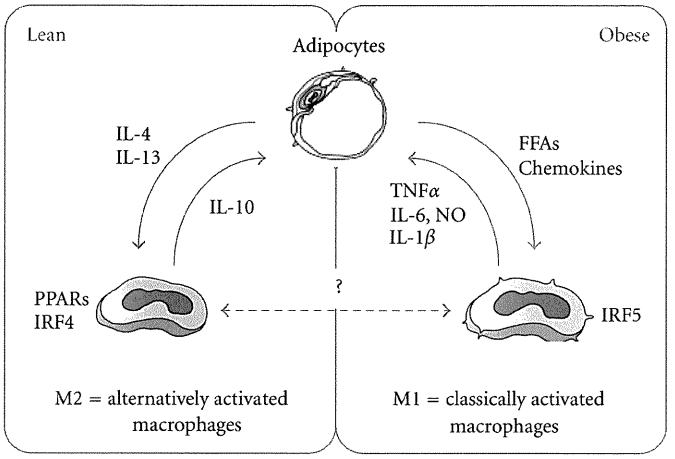

FIGURE 3: Regulation of macrophage polarity in adipose tissue. Recent evidence has also pointed to the heterogeneity of adipose tissue macrophages, that is, M1 or classically activated (pro-inflammatory) macrophages and M2 or alternatively activated (anti-inflammatory) macrophages. Under lean condition, adipocytes secrete factors that promote M2 activation of macrophages, such as interleukin-4 (IL) and interleukin-13 (IL-13). M2 macrophages secrete anti-inflammatory mediators. On the other hand, adipocytes secrete pro-inflammatory FFAs, chemokines, and cytokines under obese condition. Activated M1 macrophages produce large amounts of pro-inflammatory cytokines, thereby accelerating inflammatory responses in adipose tissue through paracrine interaction between adipocytes and macrophages.

chemokines, thereby accelerating adipose tissue inflammation.

We have recently identified activating transcription factor 3 (ATF3), a member of ATF/cAMP response element-binding protein family of basic leucine zipper-type transcription factors, as a target gene of saturated fatty acids/TLR4 signaling in adipose tissue macrophages and found that ATF3 attenuates obesity-induced macrophage activation in obese adipose tissue [30]. On the other hand, peroxisome proliferator-activated receptor γ (PPARγ) and peroxisome proliferator-activated receptor β/δ (PPARβ/δ) can stimulate M2 polarization of adipose tissue macrophages and thus systemic insulin sensitivity [31–34]. Indeed, the activation of PPARγ by pioglitazone, a thiazolidinedione class of insulin sensitizer, improves the unbalanced M1/M2 phenotype of adipose tissue macrophages in diet-induced obese mice [35]. Interestingly, circulating blood monocytes, precursors of infiltrated macrophages to the site of chronic inflammation, also express both M1 and M2 markers [36, 37]. Moreover, monocytes in obese mice and/or obese type 2 diabetic patients show significantly higher expression of M1 markers and lower expression of M2 markers relative to normal-weight controls [36]. Thus, pioglitazone treatment improves the unbalanced M1/M2 phenotype of monocytes, which may contribute to its antidiabetic and antiatherogenic effect [36, 37].

Recent studies have found other molecules that regulate macrophage polarization; that is, Jumonji domain containing-3 (Jmjd3) is essential for M2 activation through demethylation of interferon-regulatory factor 4 (IRF4) under infectious condition [38], and interferon-regulatory factor 5 (IRF5) is crucial for conversion from M2 to M1 activation in response to LPS [39]. It is interesting to know their importance in the regulation of macrophage polarization and plasticity during the course of obesity. Modulating macrophage activation state in obese adipose tissue would be a novel therapeutic target to treat or prevent the progression of obesity-induced complications such as diabetes and atherosclerosis.

6. Adipose Tissue Remodeling and Ectopic Lipid Accumulation

The adipose tissue is primarily an energy reservoir that stores fatty acids in the form of triglyceride, which is facilitated by insulin. However, obesity induces insulin-resistant state and inflammation in the adipose tissue, both of which lead to increased fatty acid release from the adipose tissue [1, 23, 29]. Moreover, recent studies have suggested that increased expression of genes related to ECM components and fibrotic changes in the adipose tissue from obese subjects and animals [40–43]. It is reported that adipose tissue fibrosis is negatively correlated with adipocyte diameters in human adipose tissue [44], suggesting that increased ECM components may limit adipose tissue expandability. Indeed, Khan et al. reported that mice lacking collagen VI, which is expressed predominantly in the adipose tissue, exhibit the uninhibited adipose tissue expansion and substantial improvements in whole-body energy homeostasis during a high-fat diet feeding [43]. It is conceivable that the rigid extracellular environment limits adipocyte expansion, and triggers adipocyte cell death and inflammatory responses through MAPK activation by increased shear stress and membrane stretching [43, 44]. Recent evidence suggests that impaired lipid storage in the adipose tissue may contribute to ectopic lipid accumulation in the skeletal muscle, liver, and pancreatic β-cells, where lipotoxicity impairs their metabolic functions [45–47]. This discussion supports the emerging view that metabolic problems associated with obesity become overt when adipose tissue cannot fully meet demands for

additional lipid storage in addition to the dysregulation of adipocytokine production.

7. Adipose Tissue Remodeling as Homeostatic Inflammation

TLR4 is a pattern-recognition receptor essential for the recognition of LPS, which is reported to play an important role in obesity-induced adipose tissue inflammation and systemic glucose and lipid metabolism *in vivo* [24, 48–50]. In obese adipose tissue, TLR4 expressed in macrophages is capable of sensing saturated fatty acids (FAs) released from adipocytes to induce chronic inflammatory responses [24, 51, 52], suggesting that saturated fatty acids could be a danger signal. On the other hand, free fatty acids (FFAs) are an important energy source mobilized from triglycerides stored in the adipose tissue, particularly under starvation conditions. Kosteli et al. have recently suggested that FFAs released from adipocytes during fasting recruit macrophages into the adipose tissue, which may be involved in the regulation of local lipid concentrations [53]. In this regard, FFAs, when released physiologically during fasting or starvation via adipocyte lipolysis, may be involved in the regulation of metabolic homeostasis within the adipose tissue rather than a danger signal. Under overnutrition conditions, increased concentrations of FFAs also activate inflammatory pathways to maintain adipose tissue homeostasis such as tissue repair and regulation of metabolism. When cellular and/or tissue stresses are excessive and/or sustained and adaptive responses are no longer possible, inflammatory responses are prolonged (i.e., chronic inflammation), thereby leading to diseased tissue remodeling [6].

Recently, we have reported that macrophage-inducible C-type lectin (Mincle; also called Clec4e and Clecsf9), a pathogen sensor for pathogenic fungi and Mycobacterium tuberculosis, is induced in adipose tissue macrophages in obesity at least partly through the saturated fatty acid/TLR4/NF-κB pathway, thereby suggesting its pathophysiologic role in obesity-induced adipose tissue inflammation [54]. Yamasaki et al. reported that Mincle serves as a receptor for SAP130, a component of small nuclear ribonucleoprotein released from damaged cells, to sense cell death and induce pro-inflammatory cytokine production [55]. Since dead adipocytes are surrounded by macrophages in the adipose tissue of obese humans and mice (crown-like structure) [7, 8, 56], it is conceivable that Mincle plays a role in sensing adipocyte-derived endogenous ligand(s) during adipocyte death.

The above discussion supports the concept that interaction between endogenous ligands and pathogen sensors in the adipose tissue involves multiple stages of adipose tissue remodeling, ranging from normal metabolic homeostasis to diseased tissue remodeling, which may be referred to as homeostatic inflammation. It is interesting to identify other endogenous danger signals and pathogen sensors that contribute to the pathophysiology of adipose tissue inflammation.

8. Homeostatic Inflammation and Other Metabolic Disorders

Recent evidence has provided new insight into the interaction between endogenous ligands and pathogen sensors in a variety of chronic inflammatory diseases such as atherosclerosis, diabetes mellitus, malignant cancers, autoimmune diseases, and even neurodegenerative diseases. Similar to the interaction between saturated fatty acids and TLR4, oxidized low-density lipoprotein (LDL), known as a ligand for the scavenger receptor CD36, is reported to trigger inflammatory signaling through a newly identified heterodimer of TLR4 and TLR6 in macrophages [57] and also to trigger CD36-TLR2-dependent apoptosis in macrophages under endoplasmic reticulum stress [58]. On the other hand, Schulthess et al. reported that CXC motif chemokine ligand-10 (CXCL10), when upregulated in diabetic pancreatic islet, is capable of binding to TLR4 in β cells in pancreatic islets to induce apoptosis [59].

In Nod-like receptor family, the NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome is well characterized. The NLRP3 inflammasome is a cytosolic protein complex consisting of the regulatory subunit NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and the effector subunit caspase-1. It is activated by pathogen-derived DNA and endogenous DAMPs such as components of necrotic cells and damaged tissues [60, 61]. Several lines of evidence have suggested that the NLRP3 inflammasome plays an important role in the pathogenesis of obesity-related diseases. NLRP3 deficient mice show improved glucose tolerance and insulin sensitivity [62]. It is also reported that ceramide and islet amyloid polypeptide (IAPP) activate as danger signals for NLRP3 inflammasome in adipose tissue macrophages and pancreatic islets, respectively, which results in insulin resistance [63–65]. In atherogenesis, it is reported that crystalline cholesterol acts as an endogenous danger signal and its deposition in arteries or elsewhere is an early cause rather than a late consequence of inflammation [66]. A better understanding of the molecular basis underlying homeostatic inflammation would allow more efficient multidisciplinary approach to and a better assessment of the metabolic syndrome.

9. Concluding Remarks

Obesity may be viewed as a chronic low-grade inflammation as well as a metabolic disease. Although considerable progress has been made in understanding the cellular and molecular events that are involved in acute inflammation caused by infection, there is no clear understanding of their physiological counterpart of the systemic chronic inflammatory state, which could be referred as homeostatic inflammation. The interaction between parenchymal and stromal cells through a number of endogenous ligands and pathogen sensors may contribute to inflammatory responses in obese adipose tissue as well as other metabolic organs. Understanding

the molecular mechanism underlying adipose tissue remodeling as homeostatic inflammation may lead to novel therapeutic strategies to prevent or treat obesity-related complications.

Abbreviations

Angptl2: Angiopoietin-like protein 2
ASC: Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATF3: Activating transcription factor 3
CCR2: Chemokine receptor 2
CXCL10: CXC motif chemokine ligand-10
CXCL14: CXC motif chemokine ligand-14
DAMP: Damage-associated molecular pattern
ECM: Extracellular matrix
FFA: Free fatty acid
IAPP: Islet amyloid polypeptide
IL-4: Interleukin-4
IL-6: Interleukin-6
IL-10: Interleukin-10
IL-13: Interleukin-13
IRF4: Interferon-regulatory factor 4
IRF5: Interferon-regulatory factor 5
Jmjd3: Jumonji domain containing-3
LDL: Low-density lipoprotein
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemoattractant protein-1
NF-κB: Nuclear factor-κB
NLRP3: NACHT, LRR and PYD domain-containing protein 3
PAMP: Pathogen-associated molecular pattern
PPARγ: Peroxisome proliferator-activated receptor γ
PPARβ/δ: Peroxisome proliferator-activated receptor β/δ
PRR: Pattern-recognition receptor
SVF: Stromal vascular fraction
TLR4: Toll-like receptor 4
TNFα: Tumor necrosis factor-α.

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Increased Expression of Macrophage-Inducible C-type Lectin in Adipose Tissue of Obese Mice and Humans

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OBJECTIVE—We have provided evidence that saturated fatty acids, which are released from adipocytes via macrophage-induced adipocyte lipolysis, serve as a naturally occurring ligand for the Toll-like receptor (TLR) 4 complex in macrophages, thereby aggravating obesity-induced adipose tissue inflammation. The aim of this study was to identify the molecule(s) activated in adipose tissue macrophages in obesity.

RESEARCH DESIGN AND METHODS—We performed a cDNA microarray analysis of coculture of 3T3-L1 adipocytes and RAW264 macrophages. Cultured adipocytes and macrophages and the adipose tissue of obese mice and humans were used to examine mRNA and protein expression.

RESULTS—We found that macrophage-inducible C-type lectin (Mincle; also called Clec4e and Clecsf9), a type II transmembrane C-type lectin, is induced selectively in macrophages during the interaction between adipocytes and macrophages. Treatment with palmitate, a major saturated fatty acid released from 3T3-L1 adipocytes, induced Mincle mRNA expression in macrophages at least partly through the TLR4/nuclear factor (NF)-κB pathway. Mincle mRNA expression was increased in parallel with macrophage markers in the adipose tissue of obese mice and humans. The obesity-induced increase in Mincle mRNA expression was markedly attenuated in C3H/HeJ mice with defective TLR4 signaling relative to control C3H/HeN mice. Notably, Mincle mRNA was expressed in bone-marrow cell (BMC)-derived proinflammatory M1 macrophages rather than in BMC-derived anti-inflammatory M2 macrophages in vitro.

CONCLUSIONS—Our data suggest that Mincle is induced in adipose tissue macrophages in obesity at least partly through the saturated fatty acid/TLR4/NF-κB pathway, thereby suggesting its pathophysiologic role in obesity-induced adipose tissue inflammation. ***Diabetes* 60:819–826, 2011**

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Adipose tissue of obese animals and humans is characterized by adipocyte hypertrophy, followed by increases in angiogenesis, macrophage infiltration, and extracellular matrix and unbalanced production of pro- and anti-inflammatory adipocytokines (1–3). The dynamic change seen in adipose tissue during the course of obesity has been referred to as adipose tissue remodeling (4). Given their multifunctional roles in a variety of biological contexts, macrophages should play a central role in adipose tissue remodeling, thereby regulating adipocytokine production (2,4). Recent studies have pointed to at least two different polarization states of adipose tissue macrophages: M1 or "classically activated" (or proinflammatory) macrophages (5), which are induced by proinflammatory mediators such as lipopolysaccharide (LPS) and Th1 cytokine interferon (IFN)-γ, and M2 or "alternatively activated" (or anti-inflammatory) macrophages, which are generated in vitro by exposure to Th2 cytokines such as interleukin (IL)-4 and IL-13. It is noteworthy that macrophages, which are infiltrated into the adipose tissue during the course of obesity, exhibit the phenotypic switch from M2 to M1 polarization (6).

To explore the molecular mechanism underlying the crosstalk between adipocytes and macrophages during the course of adipose tissue remodeling, we have developed an in vitro coculture system composed of 3T3-L1 adipocytes and RAW264 macrophages and provided evidence that a paracrine loop involving saturated fatty acids and tumor necrosis factor (TNF)-α derived from adipocytes and macrophages, respectively, establishes a vicious cycle, thereby accelerating the inflammatory change in the adipose tissue in obesity (7). Interestingly, saturated fatty acids, which are released via macrophage-induced adipocyte lipolysis, may act as naturally occurring ligands for the Toll-like receptor (TLR) 4 complex, which is essential for the recognition of LPS, to induce nuclear factor (NF)-κB activation in macrophages (8). With the aid of the coculture system, we recently have identified activating transcription factor 3, a member of basic leucine zipper-type transcription factors, which is induced in adipose tissue macrophages through the saturated fatty acid/TLR4 pathway, thereby regulating transcriptionally the obesity-induced macrophage activation (9). We, therefore, think the coculture system would provide a unique in vitro experimental system with which to investigate the molecular basis underlying obesity-induced adipose tissue inflammation.

Through a combination of cDNA microarray analyses of the coculture of 3T3-L1 adipocytes and RAW264 macrophages (7), we found that macrophage-inducible C-type

lectin (*Mincle*; also called *Clec4e* and *Clecsf9*), a type II transmembrane C-type lectin, is induced selectively in macrophages during the interaction between adipocytes and macrophages. Mincle originally was identified as a transcriptional target of CCAAT/enhancer binding protein β in macrophages in response to proinflammatory stimuli such as LPS, TNF-α, IL-6, and IFN-γ (10). Our data also suggest that Mincle is induced in M1 macrophages in the adipose tissue in obesity through the saturated fatty acid/TLR4/NF-κB pathway. This study is the first detailed analysis of a C-type lectin in adipose tissue macrophages, thereby providing a novel insight into the molecular mechanism underlying adipose tissue inflammation.

RESEARCH DESIGN AND METHODS

Reagents. LPS (from *Escherichia coli* O111: B4) and BAY11-7085, an NF-κB inhibitor, were purchased from Sigma (San Diego, CA) and Merck (Whitehouse Station, NJ), respectively. Palmitate and oleate were purchased from Sigma, solubilized in ethanol, and conjugated with fatty acid- and immunoglobulin-free BSA (Sigma) at a molar ratio of 10 to 1 (fatty acids to BSA) in a low-serum medium as described (7). The concentrations of palmitate and oleate used in this study (<200 μmol/L) were within the physiologic range. All other reagents were purchased from Sigma or Nacalai Tesque (Kyoto, Japan), unless otherwise described.

Animals. Male C3H/HeJ (HeJ) mice, which have defective LPS signaling attributed to a missense mutation in the *TLR4* gene (11), and control C3H/HeN (HeN) mice were purchased from CLEA Japan (Tokyo, Japan). Male C57BL/6 J leptin-deficient *ob/ob* mice and their wild-type littermates were purchased from Charles River Japan (Tsukuba, Japan). The animals were housed in individual cages in a temperature-, humidity-, and light-controlled room (12-h light and 12-h dark cycle) and were allowed free access to water and standard diet (Oriental MF; 362 kcal/100 g, 5.4% energy as fat; Oriental Yeast, Tokyo, Japan), unless otherwise noted. In some experiments, mice were given free access to water and either the standard diet (SD) or the high-fat diet (HFD) (D12492; 556 kcal/100 g, 60% energy as fat; Research Diets, New Brunswick, NJ) for 16 weeks. All animal experiments were conducted according to the guidelines of the Tokyo Medical and Dental University Committee on Animal Research (no. 100098).

Cell culture. The RAW264 macrophage cell line (Riken BioResource Center, Tsukuba, Japan) and 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque) containing 10% FBS (7,8). Generation of RAW264 macrophages overexpressing a superrepressor form of the inhibitor of κB (IκB)-α (SR-IκBα; a degradation-resistant mutant of IκBα) were reported previously (9,12,13). Peritoneal and bone-marrow–derived macrophages were prepared as described (8,14,15). For macrophage polarization experiments, bone-marrow cell (BMC)-derived macrophages were cultured for 24 h in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) containing 5% FBS supplemented with 10 ng/mL LPS and 20 ng/mL IFN-γ (for M1 polarization) or 10 ng/mL IL-4 (for M2 polarization) (5). Control macrophages were prepared in Iscove's modified Dulbecco's medium containing 5% FBS alone.

Coculture of adipocytes and macrophages. Coculture of 3T3-L1 adipocytes and macrophages (RAW264 or peritoneal macrophages) in the contact system was performed as described (7,8). In brief, serum-starved differentiated 3T3-L1 adipocytes (~0.5 × 10^6 cells) were cultured in a 35-mm dish, and macrophages (1.0 × 10^5 cells) were plated onto 3T3-L1 adipocytes (contact coculture) (Fig. 1*A*). The cells were cultured with direct cell-to-cell contact and harvested after a 24-h incubation, unless otherwise described. As a control (control culture), adipocytes and macrophages, the numbers of which were equal to those in the coculture, were cultured separately and mixed after harvest. Our previous data suggest that there is no apparent difference in macrophage cell number between the coculture and control culture (7).

In some experiments, we cocultured RAW264 macrophages with adipose tissue explants in the transwell system, in which adipose tissue explants of 8-week-old C57BL/6 J mice were plated in a transwell insert with a 0.4-μm porous membrane (Corning, Corning, NY) to separate them from RAW264 macrophages (Supplementary Fig. 1*A*). After incubation for 8 h, RAW264 macrophages in the lower well were harvested.

cDNA microarray analysis. cDNA microarray analysis was performed using mouse genome 430A 2.0 (Affymetrix, Santa Clara, CA) as described (8,9). Total RNA was prepared from the contact coculture of 3T3-L1 adipocytes and RAW264 macrophages, the control culture, and 3T3-L1 adipocytes alone (Fig. 1*A*). In this experiment, we cocultured the cells for 8 h. We also performed a hierarchical clustering analysis using GeneSpring (Agilent Technologies, Palo Alto, CA).

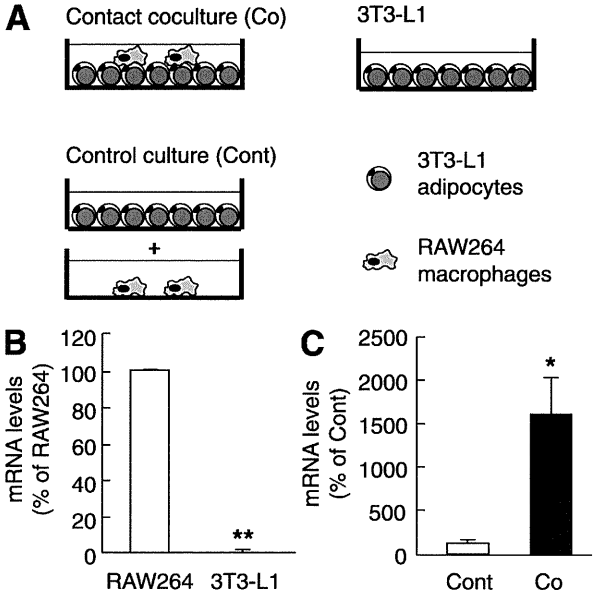

FIG. 1. Identification of *Mincle* as a target gene upregulated in the coculture of 3T3-L1 adipocytes and RAW264 macrophages. *A*: Illustration of the contact coculture system composed of 3T3-L1 adipocytes and RAW264 macrophages. *B*: Mincle mRNA expression in RAW264 and 3T3-L1. *C*: Effect of coculture on Mincle mRNA expression. $n = 4$. *$P < 0.05$; **$P < 0.01$.

Quantitative real-time PCR. Total RNA was extracted from various tissues and cultured cells using a TRIzol reagent (Invitrogen), and quantitative real-time PCR was performed with an ABI Prism 7000 Sequence Detection System using PCR Master Mix reagent (Applied Biosystems, Foster City, CA) as described (7–9). Primers used to detect mouse and human mRNAs are described in Supplementary Tables 1 and 2, respectively. Levels of mRNA were normalized to those of 36B4 mRNA (for mouse) or β-actin (for humans).

Preparation of rabbit polyclonal anti-mouse Mincle antibody and Western blot analysis. Details are shown in the Supplementary Research Design and Methods.

Human studies on Mincle expression in the adipose tissue and circulating monocytes. Details are shown in Supplementary Research Design and Methods and Supplementary Table 3. The study protocol was approved by the ethical committee on human research of The University of Tokushima Graduate School, Kyoto Medical Center, and the Tokyo Medical and Dental University.

Statistical analysis. Data were expressed as the means ± SE. Statistical analysis was performed using ANOVA. $P < 0.05$ was considered to be statistically significant. In the human study, linear regression analysis was used to evaluate the relationship between Mincle mRNA levels and BMI.

RESULTS

Coculture-induced Mincle mRNA expression in macrophages. To screen the gene(s) that are upregulated selectively in macrophages during the interaction between adipocytes and macrophages, we performed cDNA microarray analysis of the coculture of 3T3-L1 adipocytes and RAW264 macrophages in the contact system (Fig. 1*A*). There were 316 genes upregulated (>1.5-fold) in the coculture of 3T3-L1 adipocytes and RAW264 macrophages relative to the control culture, including chemokines, proinflammatory cytokines, and acute-phase reactants (Supplementary Table 4). We also compared the control culture with 3T3-L1 adipocytes alone to examine macrophage selective expression (Supplementary Table 4). In this study, we have focused on Mincle, a type II transmembrane C-type

lectin in macrophages, for the following reasons. First, Mincle is induced by LPS (10). Second, Mincle is selectively expressed in macrophages (Supplementary Table 4). We confirmed by real-time PCR that Mincle exhibits highly selective expression in RAW264 macrophages relative to 3T3-L1 adipocytes and is markedly upregulated by the coculture ($P < 0.01$ and $P < 0.05$) (Fig. 1*B* and *C*, respectively). Last, Mincle acts as a pathogen sensor to induce proinflammatory cytokine and chemokine expression such as TNF-α and macrophage inflammatory protein 2 (16–19). Indeed, cluster analysis revealed that Mincle shows a similar expression pattern with eight genes (Supplementary Fig. 2), which are all related to inflammatory responses. These observations, taken together, suggest that Mincle is selectively induced in macrophages during the interaction between adipocytes and macrophages.

Role of the saturated fatty acid/TLR4/NF-κB pathway in Mincle expression. Because saturated fatty acids are a major adipocyte-derived paracrine mediator of inflammation in macrophages (8,20), we examined the effect of palmitate, a major saturated fatty acid released from 3T3-L1 adipocytes, on Mincle mRNA expression in RAW264 or peritoneal macrophages. Treatment with palmitate for 24 h induced Mincle mRNA and protein expression in RAW264 macrophages in a dose-dependent manner (Fig. 2*A–C*). On the other hand, there was no apparent change in Mincle mRNA and protein expression when it was treated with unsaturated fatty acid oleate (Fig. 2*A–C*). We also found that palmitate, as well as LPS, induces Mincle mRNA expression in peritoneal macrophages of control C3H/HeN mice. Importantly, the induction was markedly inhibited in peritoneal macrophages of TLR4 signal–deficient C3H/HeJ mice ($P < 0.01$) (Fig. 2*D*). Moreover, treatment with BAY11-7085, an NF-κB inhibitor, significantly inhibited the palmitate-induced Mincle mRNA expression in RAW264 macrophages ($P < 0.05$) (Fig. 2*E*). The palmitate-induced Mincle mRNA expression also was significantly reduced in RAW264 macrophages overexpressing SR-IκBα, a dominant-negative form of IκBα, relative to those without SR-IκBα expression ($P < 0.01$) (Fig. 2*F*). These observations suggest that the TLR4/NF-κB pathway is involved in the saturated fatty acid–induced Mincle expression in macrophages.

To elucidate the role of TLR4 in the coculture-induced Mincle mRNA expression, we performed the coculture of 3T3-L1 adipocytes and peritoneal macrophages of C3H/HeJ or C3H/HeN mice. Coculture of 3T3-L1 adipocytes with C3H/HeN peritoneal macrophages resulted in the marked upregulation of Mincle mRNA, which was significantly inhibited in the coculture with C3H/HeJ peritoneal macrophages ($P < 0.05$) (Fig. 2*G*). Moreover, treatment with BAY11-7085 effectively inhibited the coculture-induced Mincle mRNA expression ($P < 0.05$) (Fig. 2*H*). These observations, taken together, suggest the role of the TLR4/NF-κB pathway in the coculture-induced Mincle mRNA expression.

Mincle expression in adipose tissue of obese mice. Because there is no previous report on the tissue distribution of Mincle expression in vivo, we next examined the tissue distribution of Mincle mRNA in genetically obese *ob/ob* mice and wild-type mice (Fig. 3*A*). Real-time PCR analysis revealed that Mincle mRNA is expressed most abundantly in the spleen of lean wild-type mice. Other organs such as the liver, colon, intestine, and adipose tissue also expressed Mincle mRNA, although to a lesser extent than in the spleen. Similar to macrophage marker F4/80 and M1 macrophage marker CD11c, Mincle mRNA expression was markedly increased in the adipose tissue of *ob/ob* mice relative to wild-type mice. We also observed a significant upregulation of Mincle mRNA in the adipose tissue of diet-induced obese mice ($P < 0.01$) (Fig. 3*B*). Collagenase digestion of adipose tissue, which is validated by F4/80 and adiponectin mRNA expression (9), revealed that Mincle mRNA expression is predominantly detected in the stromal-vascular fraction, which was markedly increased in *ob/ob* mice relative to wild-type mice ($P < 0.05$) (Fig. 3*C*). In this study, there was a significant increase in Mincle mRNA expression in the heart and liver ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 3*A*). These observations, taken together, suggest that Mincle is markedly upregulated mostly in adipose tissue macrophages in obesity.

Role of TLR4 in obesity-induced Mincle mRNA expression in vivo. Using C3H/HeJ and C3H/HeN mice fed an HFD or an SD for 16 weeks, we examined the involvement of TLR4 signaling in obesity-induced Mincle mRNA expression in vivo. We previously demonstrated that the weight gain of C3H/HeJ mice as a result HFD feeding is roughly comparable with that of C3H/HeN mice (9,21). We also found that there is no appreciable difference in the number of F4/80-positive macrophages between the genotypes (9,21). In this study, Mincle mRNA expression in adipose tissue on an HFD was significantly attenuated in C3H/HeJ mice relative to C3H/HeN mice ($P < 0.05$), whereas there was no appreciable difference between the genotypes on an SD (Fig. 4*A*). Similarly, mRNA expression of the M1 macrophage marker, CD11c, tended to be decreased in the adipose tissue of C3H/HeJ mice relative to C3H/HeN mice, although CD11c mRNA expression was significantly increased on an HFD relative to an SD in both genotypes (Fig. 4*B*). These observations suggest that TLR4 signaling plays an important role in the obesity-induced Mincle mRNA expression in adipose tissue macrophages in vivo.

Mincle mRNA expression in BMC-derived M1 macrophages in vitro. To further explore Mincle mRNA expression in M1 versus M2 macrophages, we examined Mincle mRNA expression in BMC-derived M1 and M2 macrophages in vitro. We confirmed that TNF-α and inducible nitric oxide (NO) synthase mRNAs are expressed exclusively in BMC-derived M1 macrophages (Fig. 5*A*), whereas BMC-derived M2 macrophages show substantial expression of arginase 1 and mannose receptor mRNAs (Fig. 5*B*). In this study, Mincle mRNA was predominantly expressed in BMC-derived M1 macrophages (Fig. 5*C*). In contrast, no appreciable amount of Mincle mRNA was detected in BMC-derived M2 macrophages (Fig. 5*C*). These observations suggest that Mincle is expressed in M1 macrophages rather than in M2 macrophages in vitro.

Mincle mRNA expression in the adipose tissue and circulating monocytes of obese subjects. We also examined Mincle mRNA expression in human subcutaneous adipose tissue. We did not observe significant differences in blood pressure and serum triglycerides, HDL cholesterol, LDL cholesterol, and HbA_{1c} levels between the groups (Table 1). On the other hand, there was a tendency of increased expression of CD11c and CD68 mRNAs in the adipose tissue of obese subjects relative to nonobese subjects (Fig. 6*A*). In this study, Mincle mRNA expression was markedly increased in the adipose tissue of obese subjects relative to nonobese subjects ($P < 0.01$) (Fig. 6*A*). Linear regression analysis also revealed a significantly positive correlation between Mincle mRNA levels and BMI ($r^2 = 0.3589$, $P < 0.01$) (Fig. 6*B*).

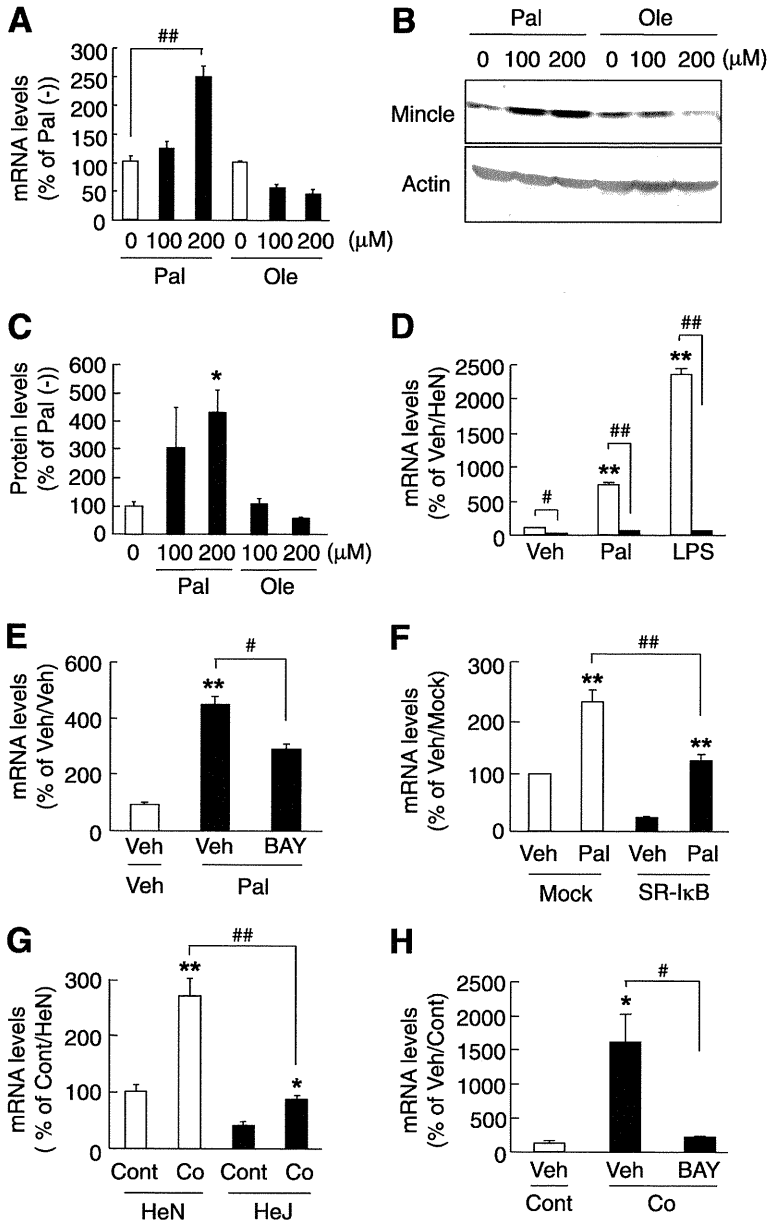

FIG. 2. Effect of palmitate on Mincle expression in cultured macrophages. *A–C*: Effect of palmitate (Pal) and oleate (Ole) on Mincle mRNA (*A*) and protein (*B* and *C*) expression in RAW264 macrophages. Representative Western blots (*B*) and quantitative relative protein expression (*C*) of Mincle. *D*: Mincle mRNA expression stimulated by palmitate (200 μmol/L) and LPS (10 ng/mL) in peritoneal macrophages prepared from TLR4 signal–deficient C3H/HeJ (■, HeJ) and control C3H/HeN (□, HeN) mice. *E*: Effect of BAY11-7085 (BAY; an NF-κB inhibitor) on the palmitate-induced Mincle mRNA expression in RAW264 macrophages. *F*: Effect of palmitate on Mincle mRNA expression in SR-IκBα- (a dominant-negative form of IκBα) and mock-overexpressing RAW264 (SR-IκB and Mock, respectively) macrophages. *G*: Effect of coculture of 3T3-L1 adipocytes and peritoneal macrophages of C3H/HeJ and C3H/HeN mice on Mincle mRNA expression. *H*: Effect of BAY (1 μmol/L) on coculture-induced Mincle mRNA expression. Co, coculture; Cont, control culture; Veh, vehicle. n = 3–4. *$P < 0.05$; **$P < 0.01$ vs. each control; #$P < 0.05$; ##$P < 0.01$.

We further examined Mincle mRNA expression in circulating monocytes of nondiabetic subjects. We did not observe significant differences in diastolic blood pressure and serum lipid levels between the groups, whereas systolic blood pressure was significantly high in obese subjects relative to nonobese subjects ($P < 0.05$) (Supplementary Table 3). In this study, Mincle mRNA expression was significantly increased in the circulating monocytes of obese subjects relative to nonobese subjects ($P < 0.05$) (Fig. 7). In this setting, circulating monocytes of obese subjects exhibited higher TNF-α and IL-6 mRNA expression and lower IL-10 mRNA expression than those of nonobese subjects (Fig. 7). Collectively, these observations suggest that Mincle expression is increased in the adipose tissue and circulating monocytes in obese subjects.

DISCUSSION

During the course of adipose tissue remodeling in obesity, infiltrating macrophages may participate in the inflammatory pathways that are activated in the adipose tissue (1,2,7).