Matrix Metalloproteinase-3 Enhances the Free Fatty Acids-Induced VEGF Expression in Adipocytes Through Toll-Like Receptor 2

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Infiltrated macrophages (Mϕ) are believed to cause pathological changes in the surrounding adipocytes through the secretion of active molecules in visceral fat. Matrix metalloproteinase (MMP)-3 is secreted from Mϕ, and enhances expression of the inflammatory cytokines through the activation of toll-like receptor (TLR) 2. Visceral adipocytes express high levels of vascular endothelial growth factor (VEGF), and the degree of visceral fat accumulation is associated with the plasma VEGF concentration in obese subjects. The aim of the study is to clarify the role of MMP-3 in the enhancement of the free fatty acids (FFAs)-induced VEGF expression through TLR2 in visceral adipocytes. One mM FFAs induced VEGF mRNA and protein expression in 3T3-L1 adipocytes. The FFAs-induced VEGF expression was mostly mediated by TLR2. A high fat intake increased the VEGF mRNA expression in visceral fat and the VEGF concentration in plasma, accompanied with the increase in the plasma FFAs concentration in mice. These increases were largely inhibited in TLR2-deficient mice. The FFAs-induced VEGF expression was increased in the presence of Mϕ-conditioned medium (CM) in adipocytes, and the enhancement was inhibited by a MMP-3 inhibitor or a neutralizing antibody against MMP-3. The active form of MMP-3 induced the VEGF mRNA expression, as well as TLR2, in adipocytes. The increase in the VEGF expression by MMP-3 was inhibited by the treatment with siRNA for TLR2. The enhancement of FFAs-induced TLR2 expression by Mϕ-CM was inhibited by blocking of the MMP-3. The increase in the VEGF mRNA expression by Mϕ-CM or MMP-3 was partially inhibited by a neutralizing antibody against TNF-α. These results indicate that MMP-3 in Mϕ-CM enhances the FFAs-induced VEGF expression in adipocytes through the increase in the TLR2 expression. The MMP-3 secreted from the infiltrated Mϕ may be a regulator of the VEGF expression in visceral adipocytes. Exp Biol Med 233:1213–1221, 2008

Key words: matrix metalloproteinase-3; toll-like receptor 2; macrophage; vascular endothelial growth factor; adipocyte; visceral fat

Introduction

Adipocytes secrete a wide variety of cytokines, and some cause metabolic disturbance through the development of insulin resistance (1–3). The expression of toll-like receptor (TLR) 2 gene is associated with the gene expression of TNF-α (4), one of the cytokines that causes an inhibition of insulin signals in fat, muscle and liver (1–3, 5). High fat feeding leads to an increased population of TLR2/TNF-α co-expressing adipocytes in visceral fat, but not in subcutaneous fat in mice (4). A microarray analysis revealed that the accumulated visceral fat is accompanied with drastic changes in expression of matrix metalloproteinase (MMP) family genes, among which MMP-3 potentiated free fatty acids (FFAs)-induced TNF-α secretion from adipocytes (6). Infiltrated macrophage (Mϕ) has a potential for a pathological link with surrounding adipocytes through the secretion of MMP-3 followed by TNF-α expression in adipocytes in visceral fat tissue (7).

Vascular endothelial growth factor (VEGF) is abundantly secreted from adipocytes, and it plays a key role in the process of fat tissue formation through the regulation of angiogenesis in the tissues (8). The inhibition of angiogenesis leads to a decreased volume of developing fat tissue or fat grafting after transplantation in mice (9, 10). VEGF is highly expressed in visceral fat, and the circulating VEGF concentration is positively correlated with visceral fat

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volume in obese mice (11). These observations suggest that the TLR2 expression in adipocytes is related to the expression of VEGF as well as TNF-α. In this context, the tight association between visceral fat accumulation and the plasma VEGF level is in fact observed in obese human subjects (12). However, the mechanism for the induction of VEGF expression in visceral fat has not been elucidated.

FFAs have been shown to activate the downstream signals of TLR2 (13, 14). The aim of this study was to clarify the role of MMP-3 in the enhancement of the FFAs-induced VEGF expression through TLR2 in visceral adipocytes. The significance of TLR2 for the FFAs-induced VEGF expression was at first analyzed in cultured adipocytes and the visceral fat of TLR2-deficient mice. Next, the possible pathological role of the MMP-3 secreted from Mϕ in the FFAs-induced VEGF expression through TLR2 was assessed in a culture system using conditioned medium (CM) of Mϕ.

Materials and Methods

Adipocyte Culture. 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were cultured in high-glucose DMEM (DMEM-H, GibcoBRL, Tokyo, Japan) supplemented with 10% FBS (Sigma, St. Louis, MO) and antibiotics. After the 3T3-L1 preadipocytes were grown to near confluence, the differentiation to mature adipocytes was performed using insulin (Sigma), dexamethasone (Sigma), and 3-isobutyl-1-methyl-xanthine (Sigma), as described previously (7). A vast majority of cells (~90%) had accumulated lipid droplets between 10–14 days after differentiation and were used for further experiments.

Cell Treatment with FFAs. The treatment of 3T3-L1 cells with FFAs was performed essentially according to the method previously described (15). After overnight incubation in serum-free DMEM-H supplemented with 0.1% FFAs-free BSA, the cells were treated in serum-free DMEM-H supplemented with 0.1% FFAs-free BSA with 1 mM FFAs (a cocktail containing 0.5 mM palmitic acid and 0.5 mM myristic acid, Sigma) for indicated times, and then incubated in serum-free DMEM-H supplemented with 0.1% FFAs-free BSA with 1 mM FFAs in the presence or absence of 1 μg/ml zymosan A (Wako, Tokyo, Japan), 10 μg/ml peptidoglycan (PGN, Invitrogen, Tokyo, Japan), or 100 μg/ml active form of MMP-3 purified from human fibroblasts (Sigma), a neutralizing antibody against TNF-α (15) (R&D Systems, Minneapolis, MN) or non-immune IgG (R&D Systems) for indicated times. The composition ratio of FFAs/BSA in the medium corresponds to 6:1 for the first incubation medium.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Relation (RT-PCR). Total RNA was extracted from fat tissue or 3T3-L1 adipocytes using RNeasy Mini kit (QIAGEN, Valencia, CA), and was reverse transcribed using the GeneAmp Gold RNA PCR Reagent kit (PE Applied Biosystems, Foster City, CA) as described previously (7). Quantitative RT-PCR amplifications were performed using TaqMan Universal PCR Master Mix and Assay-on-Demand Gene expression Assay Mix specific for mouse VEGF A (Mm000437304_m1) or TLR2 (Mm00442346_m1) mRNA. All PCRs were performed in an ABI PRISM 7500 sequence detection system (PE Applied Biosystems).

Enzyme-Linked Immunosorbent Assay (ELISA). The VEGF concentration of plasma or cell culture medium was measured using a Quantikine Mouse VEGF Immunoassay kit (R&D Systems).

siRNA Knockdown Treatment. 3T3-L1 adipocytes or THP-1-derived Mϕ were transfected either with 5 nmol siRNA for TLR2 (QIAGEN) or MMP-3 (Invitrogen), or Allstars Negative Control (QIAGEN) siRNA by electroporation as described previously (7). The cells were reseeded after electroporation, and incubated with DMEM-H containing 10% FBS for two days. After overnight incubation in serum-free DMEM-H supplemented with 0.1% FFA-free BSA, the siRNA-treated 3T3-L1 adipocytes were incubated in serum-free DMEM-H supplemented with 0.1% FFA-free BSA and 1 mM FFAs for 6 h. The CM of the siRNA-treated Mϕ was prepared by the incubation for the following 24 h.

Mice. $TLR2^{-/-}$ mice were kindly provided by Dr. Shizuo Akira (Osaka University) (16). Briefly, mouse TLR2 gene was disrupted by introducing a targeted mutation into E14.1 embryonic stem cells. A targeting vector was designed to replace a part of the exon that encodes the transmembrane and the cytoplasmic domain of TLR2 with the *neo* gene. ES cell lines containing a mutant TLR2 allele were microinjected into C57BL/6 blastocysts. Heterozygous mice were intercrossed to produce $TLR2^{-/-}$ mice and $TLR2^{+/+}$ mice. The $TLR2^{-/-}$ mice grew healthy and did not show any obvious abnormality until 20 weeks. TLR2 mRNA could not be detected in either the mesenteric or subcutaneous fat tissue of the $TLR2^{-/-}$ mice. From five weeks of age, female $TLR2^{+/+}$ and $TLR2^{-/-}$ mice were given either a regular (D12450B, Research Diet, New Brunswick, NJ, see Table 1) or a high fat diet (D12492).

Preparation of Mϕ-CM. The preparation of Mϕ-CM was performed essentially as described (7). The human monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 supplemented with l-glutamine (GibcoBRL) and antibiotics and 10% FBS. To allow the monocytes to differentiate into adherent macrophages, THP-1 cells were washed in PBS (calcium- and magnesium-free, GibcoBRL, medium A) and re-suspended in fresh medium A containing 50 ng/ml phorbol 12-myristate–13-acetate (PMA, Sigma) for 3 days (at day 0). The cells were incubated for 3 more days in DMEM supplemented with 2% BSA, and then at day 4, the cells were treated for the CM preparation for 24 h. Control CM was prepared by incubating the THP-1 cells without the differentiation treatment (THP1-CM).

Treatment of Adipocytes with Mϕ-CM. After treatment in serum-free DMEM-H supplemented with

Table 1. Contents of Regular and High Fat Diets

	Regular diet (D12450B)		High fat diet (D12492)	
	g %	kcal %	g %	kcal %
Protein	19.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20
Fat	4.3	10	34.9	60
Total		100		100
kcal/g	3.85		5.24	
	g	kcal	g	kcal
Casein, 80 mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn starch	315	1260	0	0
Maltodextrin 10	35	140	125	500
Sucrose	350	1400	68.8	275.2
Cellulose, BW200	50	0	50	0
Soybean oil	25	225	25	225
Lard	20	180	245	2205
Mineral mix S10026	10	0	10	0
DiCalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium citrate, 1 H2O	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Total	1055.05	4057	773.85	4075

0.1% FFA-free BSA and 1 mM FFAs for 6 h, 3T3-L1 adipocytes were incubated in serum-free DMEM-H supplemented with 0.1% FFA-free BSA and 1 mM FFAs with Mϕ-CM or THP1-CM at 10% of the final volume, or the active form of MMP-3 for the indicated time periods. Mϕ-CM or THP1-CM was treated with an MMP-3 inhibitor, NNGH (*N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycyl-hydroxamic acid; Calbiochem, San Diego, CA) (17, 18), goat anti-MMP-3 polyclonal antibody (Santa Cruz, Santa Cruz, CA) or non-immune goat IgG (Santa Cruz) for 12 h, prior to the addition to adipocytes.

Statistical Analyses. The results are presented as the mean ± SD. Statistical significance between two groups was evaluated by Student's *t*-test. Statistical significance among several groups was performed using a one-way ANOVA. A value of $P < 0.05$ was considered to be significant.

Results

FFAs-Induced VEGF mRNA Expression Is Mediated by TLR2 in 3T3-L1 Adipocytes. FFAs induce the TNF-α expression through the increased TLR2 expression in adipocytes (4). The effect of FFAs on the expression of VEGF mRNA through TLR2 was analyzed in 3T3-L1 adipocytes. The VEGF mRNA expression levels were increased time-dependently in the presence of 1 mM FFAs (a cocktail containing 0.5 mM palmitic acid and 0.5 mM myristic acid) (Fig. 1A). The VEGF concentration in the medium for 72 hr after incubation of 3T3-L1 adipocytes in the presence of 1 mM FFAs was significantly higher than that in the absence of FFAs (Fig. 1B). Zymosan A, a TLR2 ligand that induces the TNF-α mRNA expression in 3T3-L1 adipocytes (4, 19), showed the effect additive to the treatment with FFAs on the increase in the VEGF mRNA expression in the cells (Fig. 1C). Furthermore, the VEGF mRNA expression was increased to 1.8-fold by 10 g/ml peptidoglycan (PGN), another TLR2 ligand (19), in comparison to that without the addition in the cells incubated with FFAs (data not shown). The FFAs-induced increase in the VEGF mRNA expression level was mostly abolished in the adipocytes transfected with TLR2-specific siRNA in comparison to that in the cells transfected with control siRNA (Fig. 1D). These results indicate that FFAs-induced VEGF mRNA expression is most likely mediated by TLR2 in 3T3-L1 adipocytes.

A High Fat Intake Increases the VEGF mRNA Expression in Visceral Fat, Accompanied with an Increase in Plasma VEGF Concentration in TLR2$^{+/+}$ Mice, but Not in TLR2$^{-/-}$ Mice. The above observations for the FFAs-induced VEGF expression through TLR2 in cultured adipocytes suggest that a high fat intake may induce the VEGF mRNA expression through the FFAs-mediated TLR2 expression in fat tissues. The expression levels of VEGF mRNA in the fat tissues of TLR2$^{+/+}$ mice and TLR2$^{-/-}$ mice were investigated after fat feeding for six weeks. Plasma FFAs levels were significantly increased in the high fat-fed TLR2$^{+/+}$ mice in comparison to those in the regular diet-fed TLR2$^{+/+}$ mice, and were not significantly different from the high fat-fed TLR2$^{-/-}$ mice (Fig. 2A). The expression levels of VEGF mRNA in mesenteric fat were significantly increased in the high fat-fed TLR2$^{+/+}$ mice in comparison to those in regular

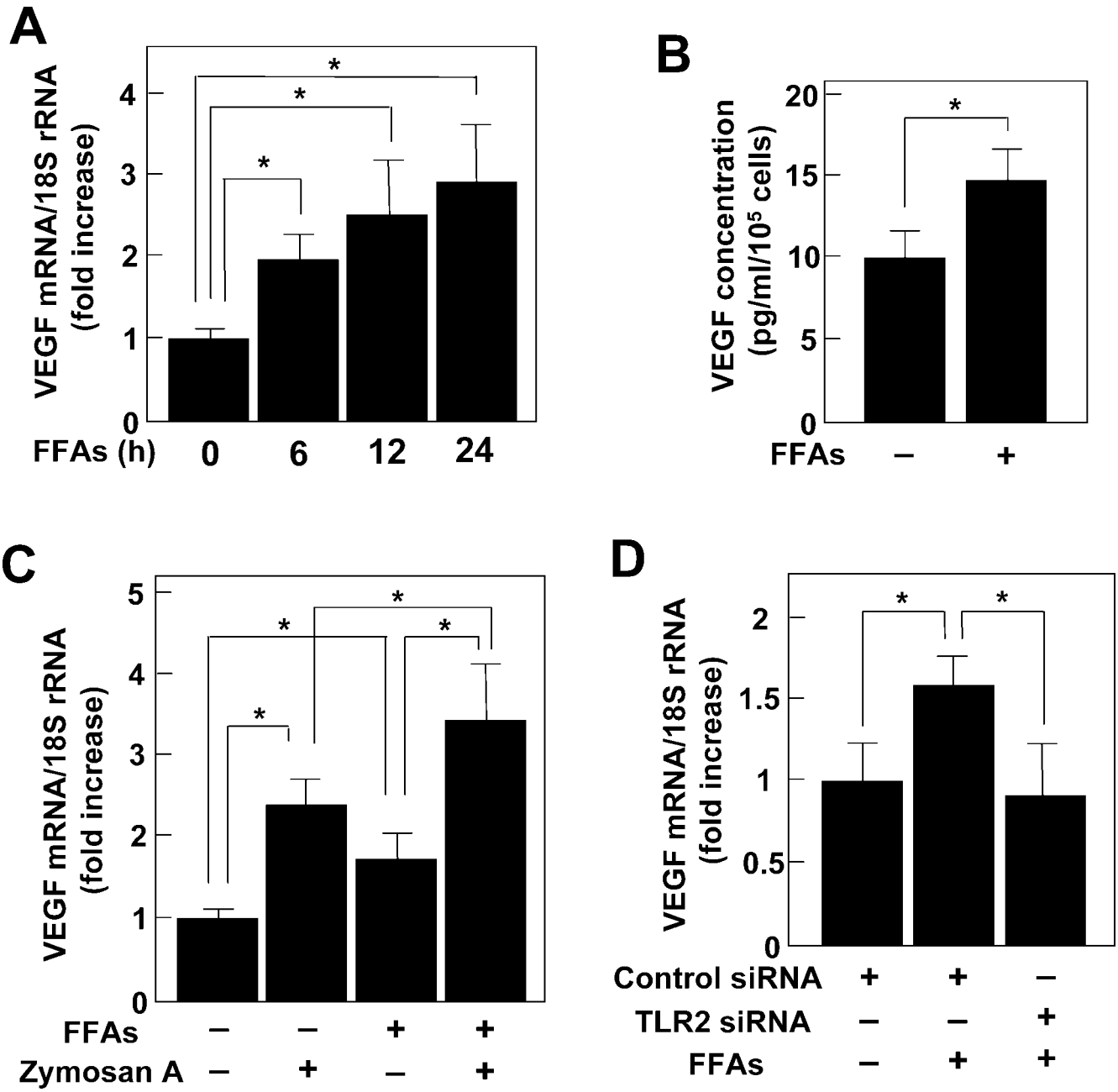

Figure 1. The effect of FFAs with zymosan A or TLR2 knockdown on the VEGF expression in 3T3-L1 adipocytes. (A) Total RNA was extracted from adipocytes after incubation with 1 mM FFAs (a cocktail of 0.5 mM palmitic acid and 0.5 mM myristic acid) for indicated time. The VEGF mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of the control at 0 h. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (B) Conditioned media were collected after incubation of adipocytes with 1 mM FFAs for 72 h. The VEGF concentration was measured using ELISA, and then presented by the correction with the incubated cell number. The cell appearance was not changed after the incubation with FFAs, and no significant difference was observed in the cell counts between the adipocytes incubated with and without FFAs. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (C) Adipocytes were incubated with or without 1 mM FFAs for 6 h, and then with or without 1 mM FFAs in the presence or absence of 1 μg/ml Zymosan A for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. The mRNA levels were calculated as the fold increase of the control in the absence of FFAs or zymosan A. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (D) Adipocytes were transfected either with TLR2-specific siRNA or control siRNA. Total RNA was extracted from the adipocytes after incubation with or without 1 mM FFA for 6 h. The mRNA levels were calculated as the fold increase of those of cells transfected with control siRNA without the FFA treatment. The bars represent the mean ± SD ($n=5$). * $P < 0.05$.

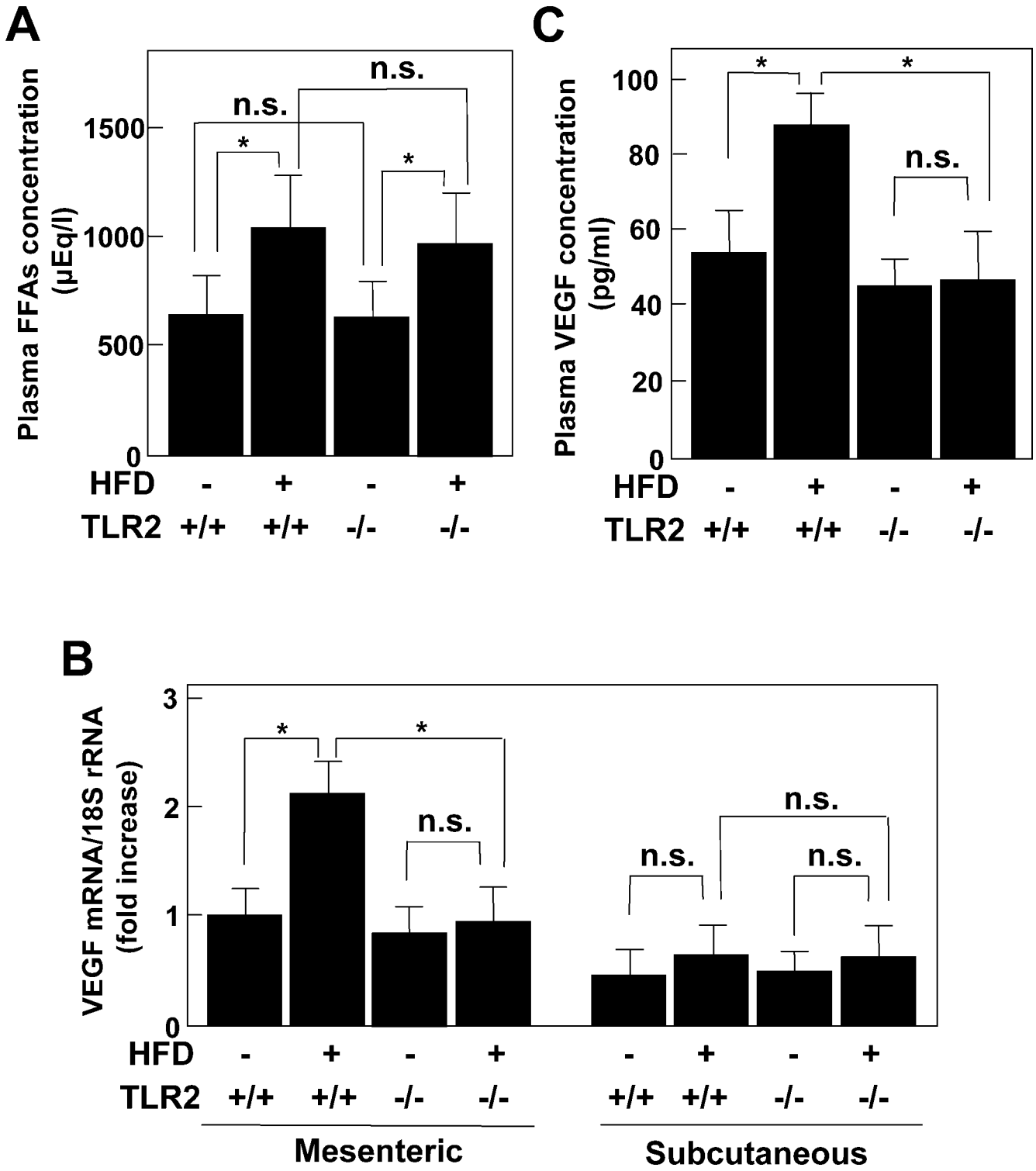

Figure 2. The VEGF concentration in the plasma and the VEGF mRNA expression in fat tissues in the $TLR2^{+/+}$ or $TLR2^{-/-}$ mice fed a high fat diet (HFD) for four weeks. (A) The plasma FFAs concentrations in $TLR2^{+/+}$ and $TLR2^{-/-}$ mice with or without a HFD diet. The bars represent the mean ± SD ($n=5$). * $P < 0.05$. (B) Total RNA was extracted from either mesenteric or subcutaneous fat tissue specimens of mice. The mRNA levels corrected by protein weight of tissues were calculated as the fold increase of the control in mesenteric fat of $TLR2^{-/-}$ mice without a HFD feeding (note: the fold increases of the control for 3T3-L1 preadipocyte and adipocyte are 0.17 ± 0.04 and 0.59 ± 0.18, respectively). The bars represent the mean ± SD ($n=5$). * $P < 0.05$. n.s., not significant. (C) The plasma VEGF concentration was measured using an ELISA. The bars represent the mean ± SD ($n=5$). * $P < 0.05$.

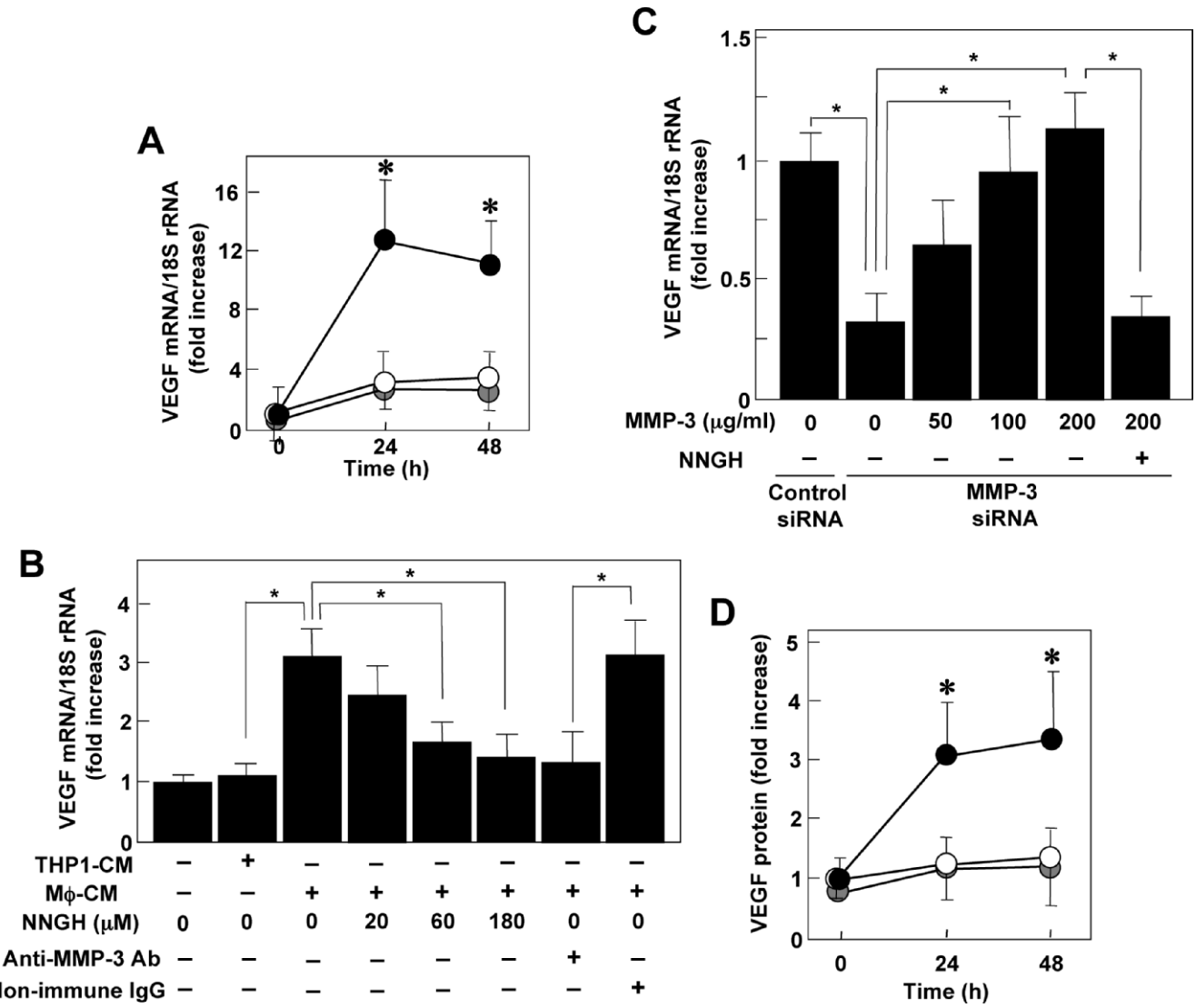

Figure 3. Effect of MMP-3 blocking on the FFAs-induced VEGF expression enhanced by Mϕ-CM in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were incubated with (open and closed circles) or without (gray circle) 1 mM FFAs in the presence of 10% THP1-CM (open circle) or 10% Mϕ-CM (closed and gray circles) for indicated time in the presence (open and closed circles) or absence (gray circle) of preincubation with 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of control at 0 h in the absence of CM after the treatment with FFAs. The bars represent the mean ± SD ($n=3$). * $P < 0.05$ vs. cells in the absence of CM after the treatment with FFAs. (B) After the incubation with 1 mM FFAs for 6 h, 3T3-L1 adipocytes were treated with or without 10% THP1-CM, 10% Mϕ-CM, 20–180 μM NNGH, anti-MMP-3 neutralizing antibody, or non-immune IgG, in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of control in the absence of CM, NNGH or antibody. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (C) 3T3-L1 adipocytes were transfected either with MMP-3-specific or control siRNA. Total RNA was extracted from adipocytes incubated with or without 50–200 μg/ml MMP-3 or 60 μM NNGH in the presence of 1mM FFAs for 6 h after pre-incubation with 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. The mRNA levels were calculated as the fold increase of that in cells transfected with control siRNA in the absence of MMP-3 and NNGH. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (D) 3T3-L1 adipocytes were incubated with (open and closed circles) or without (gray circle) 1 mM FFAs in the presence of 10% THP1-CM (open circle) or 10% Mϕ-CM (closed and gray circles) for indicated time in the presence (open and closed circles) or absence (gray circle) of pre-incubation with 1 mM FFAs for 6 h. The incubated media were collected, and the VEGF concentration was measured using ELISA. The concentration levels were calculated as the fold increase of control at 0 h in the absence of CM after the treatment with FFAs. The bars represent the mean ± SD ($n=3$). * $P < 0.05$ vs. cells in the absence of CM after the treatment with FFAs. * $P < 0.05$.

diet-fed TLR2$^{+/+}$ mice (Fig. 2B). However, the expression levels of VEGF mRNA in the mesenteric fat did not significantly change between the TLR2$^{-/-}$ mice fed a regular diet and a high fat diet. Thus, the increase in the expression levels of VEGF mRNA in the mesenteric fat of the high fat-fed TLR2$^{+/+}$ mice were not observed in high fat-fed TLR2$^{-/-}$ mice. There were no significant differences in the VEGF mRNA expression levels in the subcutaneous fat either between the high fat-fed TLR2$^{+/+}$ mice and the regular diet-fed TLR2$^{+/+}$ mice, or between the high fat-fed

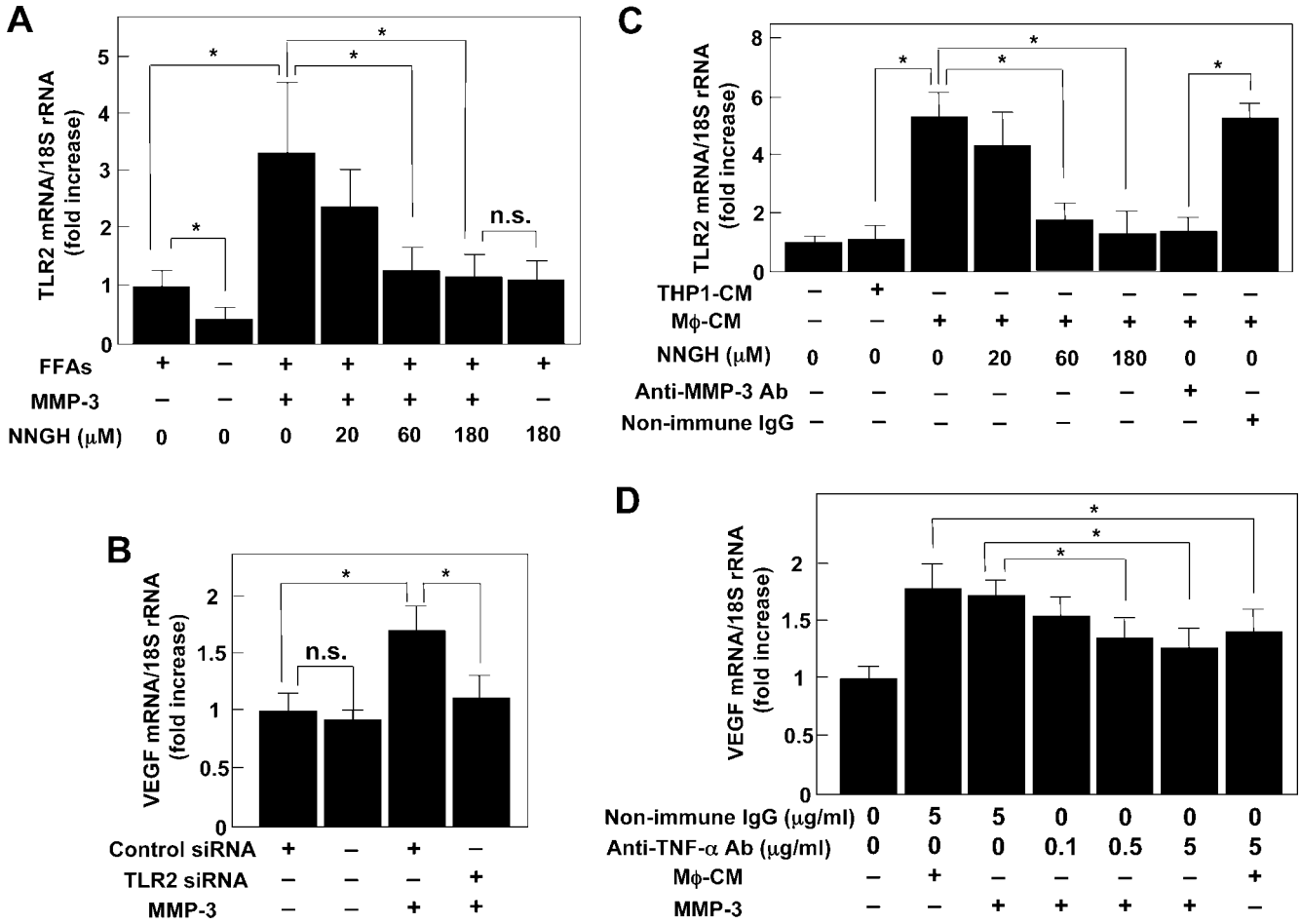

Figure 4. Effect of MMP-3 on the FFAs-induced TLR2 and VEGF expression in 3T3-L1 adipocytes. (A) After the incubation with 1 mM FFAs for 6 h, adipocytes were treated with or without the active form of MMP-3 (100 μg/ml) or 20–180 μM NNGH in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of control without MMP-3 or NNGH. The bars represent the mean ± SD ($n=3$). * $P < 0.05$. (B) Adipocytes were transfected either with TLR2-specific siRNA or control siRNA. After the incubation with 1 mM FFAs for 6 h, cells were treated with or without MMP-3 (100 μg/ml) in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the mRNA levels were calculated as a fold increase of control in cells transfected with control siRNA without MMP-3. The bars represent the mean ± SD ($n=4$). * $P < 0.05$. (C) After the incubation with 1 mM FFAs for 6 h, adipocytes were treated with or without 10% THP1-CM, 10% Mϕ-CM, 20–180 μM NNGH, anti-MMP-3 neutralizing antibody, or non-immune IgG in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the TLR2 mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of the control in the absence of CM or NNGH. The bars represent the mean ± SD ($n=4$). * $P < 0.05$. (D) After the incubation with 1 mM FFAs for 6 h, adipocytes were treated with or without 10% Mϕ-CM, 100 μg/ml MMP-3, 0.5–5 μg/ml anti-TNF-α neutralizing antibody or 5 μg/ml non-immune IgG for 24 h in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from adipocytes, and the VEGF mRNA level was evaluated by quantitative real-time RT-PCR. mRNA levels were calculated as the fold increase of the control in the absence of antibody, CM or MMP-3. The bars represent the mean ± SD ($n=4$). * $P < 0.05$.

$TLR2^{+/+}$ mice and the high fat-fed $TLR2^{-/-}$ mice. The plasma VEGF concentration was significantly increased in the high fat-fed $TLR2^{+/+}$ mice in comparison to those in the regular diet-fed $TLR2^{+/+}$ mice (Fig. 2C). The plasma VEGF level in the high fat-fed $TLR2^{-/-}$ mice did not change in comparison to that in the regular diet-fed $TLR2^{-/-}$ mice, and significantly decreased in comparison to those of the high fat-fed $TLR2^{+/+}$ mice. Thus, the TLR2 ablation abolished an increase in the VEGF mRNA expression in the visceral fat and the plasma VEGF concentration in the mice fed a high fat diet.

Mϕ Enhances the FFAs-Induced VEGF mRNA Expression in Adipocytes Through MMP-3. Infiltrated Mϕ causes the induction of TNF-α expression in the adipocytes of visceral fat tissues (20, 21). A recent study identified that MMP-3 is secreted from Mϕ, and responsible for the induction for the TNF-α expression using co-culture system (7). To assess whether MMP-3 is a regulatory player for the VEGF expression in adipocytes accumulated in mesenteric regions, the blocking effect of MMP-3 on the action of Mϕ-CM for the FFAs-induced VEGF mRNA expression was firstly examined in 3T3-L1 adipocytes. The addition of Mϕ-CM significantly enhanced the VEGF mRNA expression in the adipocytes treated with 1 mM FFAs for 6 h, but not in cells without the treatment with FFAs (Fig. 3A). The stimulatory effect of Mϕ-CM on the FFAs-induced VEGF mRNA expression was dose-dependently inhibited by the treatment of adipocytes with NNGH, a

specific inhibitor of MMP-3 (17), in the range similar to that for the inhibition of MMP-3 in microglia (18) (Fig. 3B). The effect of Mϕ-CM on the FFAs–induced VEGF mRNA expression was also inhibited by the treatment of an anti-MMP-3 neutralizing antibody to an extent similar to that by NNGH. In addition, the stimulatory effect of Mϕ-CM on the FFAs-induced VEGF mRNA expression was not observed using the CM of Mϕ pretreated with siRNA specific for MMP3 (Fig. 3C), and the decreased VEGF mRNA expression increased by the addition of active form of MMP-3 in a dose-dependent manner; the recovered expression level by the addition of 200 μg/ml MMP-3 was again inhibited to that without MMP-3 by the incubation together with NNGH at 60 μM. Finally, the VEGF concentration in the media of 3T3-L1 adipocytes significantly increased in the presence of active form of MMP-3 in addition to the treatment with 1 mM FFAs, in comparison to those in the absence of MMP-3 or without the incubation with FFA (Fig. 3D). These results strongly suggest that Mϕ enhances the FFAs-induced VEGF mRNA expression in adipocytes, in part through the action of secreted MMP-3.

MMP-3, Secreted from Mϕ, Enhances the FFAs-Induced VEGF Expression Through the Expression and Activation of TLR2 in Adipocytes. We have shown that the expression of TLR2 is tightly associated with that of TNF-α in visceral adipocytes, and the population of TLR2/TNF-α co-expressing adipocytes is increased in visceral fat of the high fat-fed mice (4). Therefore, the role of TLR2 in the enhancement of FFAs-induced VEGF mRNA expression by MMP3 was analyzed in 3T3-L1 adipocytes. The TLR2 mRNA expression level after treatment with 1mM FFAs for 6 h was significantly increased by the incubation of adipocytes with active form of MMP-3 (Fig. 4A). The increase in the expression level of TLR2 mRNA in the presence of MMP-3 was dose-dependently inhibited by the treatment together with NNGH. Furthermore, the VEGF mRNA expression induced by MMP-3 was abolished in the 3T3-L1 adipocytes treated with siRNA specific for TLR2 (Fig. 4B). The FFAs-induced TLR2 expression level was significantly increased in the presence of Mϕ-CM in comparison to that in the presence of THP-1-CM (Fig. 4C), and the increase in the FFAs-induced TLR2 expression level by the incubation with Mϕ-CM was decreased by the addition of NNGH or a neutralizing antibody against MMP-3; the dose range of NNGH for the inhibition of Mϕ-CM-mediated TLR2 expression was nearly the same as those for the Mϕ-CM-mediated VEGF mRNA expression and MMP-3-mediated TLR2 expression (Fig. 3B and 4A, respectively). Finally, we evaluated the effect of an addition of a neutralizing antibody against TNF-α in Mϕ-CM on the FFAs-induced VEGF mRNA expression at around 2 μg/ml, which is a concentration that was previously shown to block the TNF-α action in 3T3-L1 adipocytes (15) (Fig. 4D). The enhancement in the FFAs-induced VEGF mRNA expression by Mϕ-CM or MMP-3 was partially, but significantly, inhibited by the blocking of TNF-α. These results indicate that Mϕ, through the action of secreted MMP-3, enhances the FFAs-induced VEGF expression through the TLR2 expression, and in part the following expression of TNF-α in adipocytes.

Discussion

In the series of experiments using a culture system and TLR2-knockout mice, we at first investigated the role of TLR2 in the FFAs-induced VEGF expression in adipocytes. FFAs induced the VEGF mRNA and protein expressions, and the FFAs-induced VEGF expression was mostly mediated by TLR2. Next, a high fat intake caused significant increases in the VEGF mRNA expression in visceral fat and the VEGF concentration in plasma in mice, and the effects of a high fat intake were inhibited in TLR2-deficient mice. The FFAs-induced VEGF expression was increased in the presence of Mϕ-CM in 3T3-L1 adipocytes. The increased expression was almost inhibited by the blocking of MMP-3. Furthermore, active form of MMP-3 enhanced the FFAs-induced VEGF and TLR2 mRNA expression, and the increased VEGF expression by MMP-3 was not observed by the TLR2 knockdown in adipocytes. The enhancement of FFAs-induced TLR2 expression by Mϕ-CM was again almost completely inhibited by the blocking of MMP-3. Finally, the MMP-3-mediated VEGF expression was in part inhibited by the blocking of TNF-α in 3T3-L1 adipocytes. These results indicated that MMP-3, secreted from Mϕ, enhances the FFAs-induced VEGF expression through the induction in the expression of TLR2 and its downstream molecule, TNF-α, in adipocytes.

Adipocytes transplanted to the mesenteric regions express a variety of genes in comparison to those in the subcutaneous regions in mice (6). MMP-3 is one of the highly expressed genes in mesenteric regions, and enhanced the FFA-induced TNF-α secretion from adipocytes (7). The Mϕ infiltration has been observed in the accumulated fat tissues, and active Mϕ causes a change in the surrounding adipocytes in visceral fat, thus leading to the progression of insulin resistance (20, 21). The degree of visceral fat accumulation has shown to be closely associated with the development of insulin resistance (1, 2). A variety of inflammatory bioactive molecules play an important role in the pathological interaction between Mϕ and adipocytes in visceral fat (1–3, 20, 21). In this context, infiltrated Mϕ may thus have a pathological link with the surrounding adipocytes through the secretion of MMP-3 followed by the TLR2 and TNF-α expression in the adipocytes in visceral fat tissues.

The plasma VEGF concentration, as well as the VEGF gene expression in visceral fat, is induced in db/db and KK-Ay mice (11). A high fat intake causes an increase in the number of TLR2/TNF-α co-expressing adipocytes in visceral fat, but not in subcutaneous fat, in mice (4). The current study showed that TLR2 enhances the FFAs-

induced VEGF expression, as well as the TNF-α expression. The ablation of TLR2 expression reduced the FFAs-induced VEGF expression in cultured cells and plasma VEGF levels in high fat-fed mice. The mechanism of VEGF mRNA expression in TLR2-expressing adipocytes has not yet been fully elucidated. A recent study showed that the inflammatory cytokines, IL-6 and oncostatin M, up-regulate VEGF expression in fat tissues via the JAK/STAT pathways, and these effects were reflected by the increased visceral obesity accompanied with the increased plasma VEGF concentration in mice (22, 23). Hypoxia is another potent stimulus for VEGF mRNA expression in human adipocytes (24). There is a possibility that MMP-3 cleaves a protein in the Mϕ-CM that becomes a ligand for the TLR2 receptor or another receptor for the induction of TLR2 expression, in addition to the direct interaction of MMP-3 with the cell surface of adipocytes, in the present culture system. Further analyses to address the regulation of TLR2 expression by MMP-3 are thus called for to elucidate the specific VEGF expression in adipocytes of visceral fat and the relationships between the plasma VEGF concentration and visceral adiposity, which is tightly associated with the development of the insulin resistance associated.

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