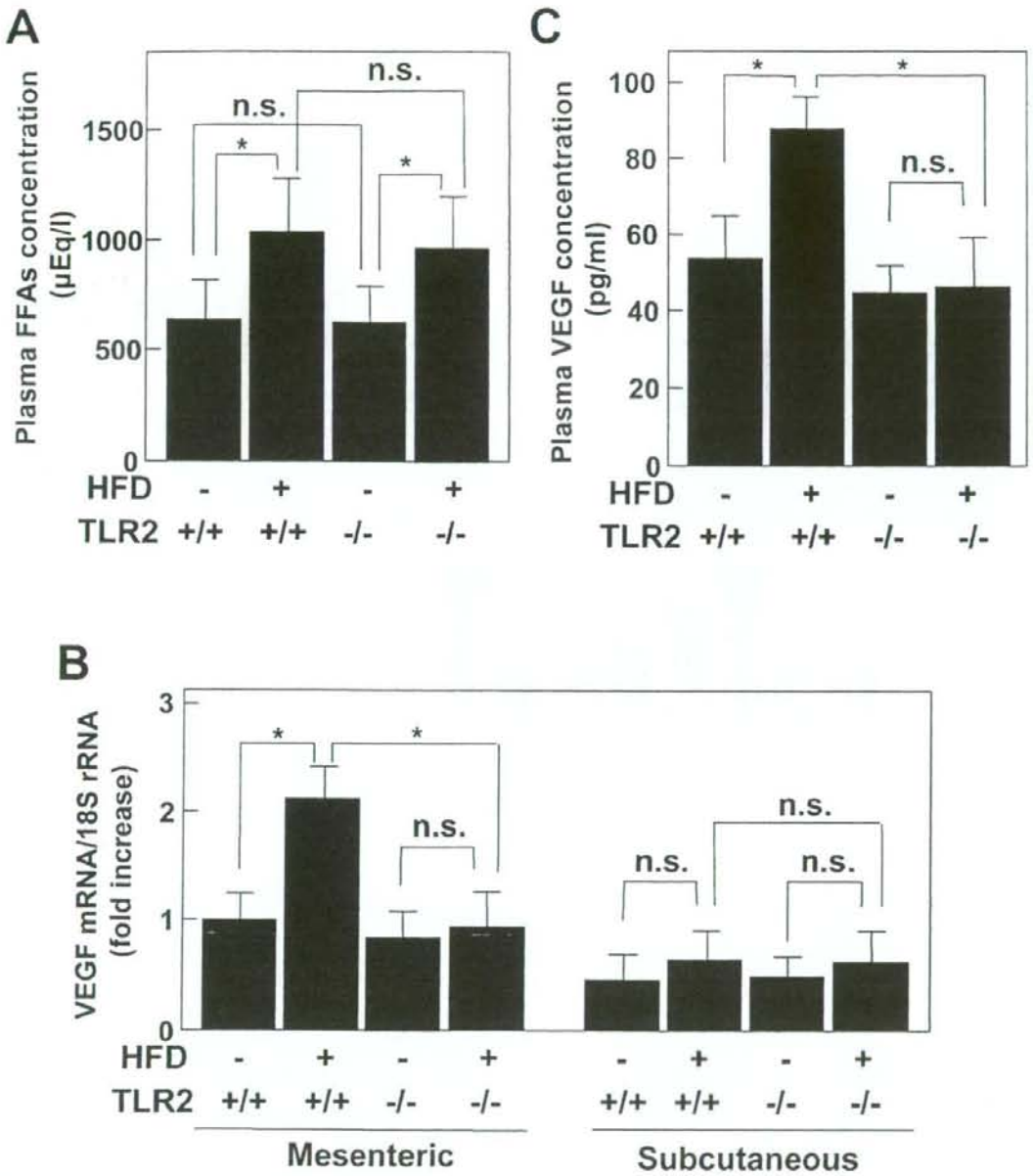

Figure 2. The VEGF concentration in the plasma and the VEGF mRNA expression in fat tissues in the $TLR2^{+/+}$ or $TLR2^{-/-}$ mice fed a high fat diet (HFD) for four weeks. (A) The plasma FFAs concentrations in $TLR2^{+/+}$ and $TLR2^{-/-}$ mice with or without a HFD diet. The bars represent the mean ± 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 ($n = 5$). * $P < 0.05$.

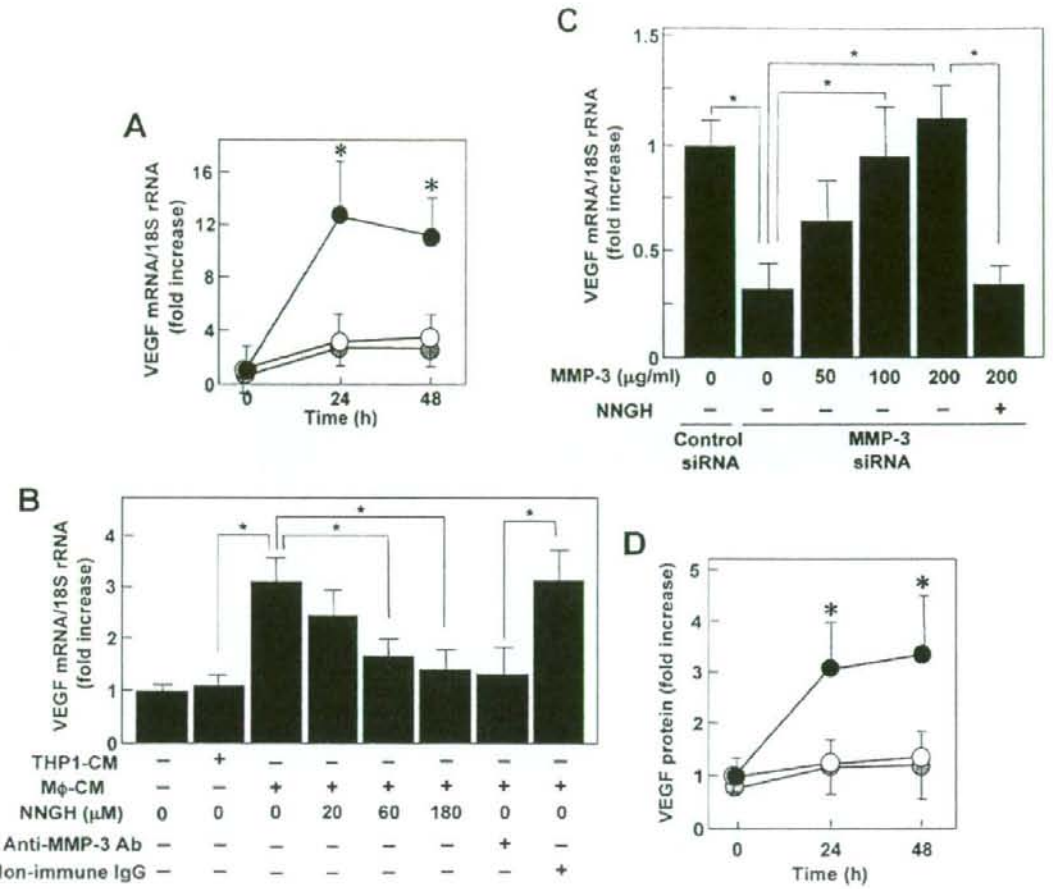

Figure 3. Effect of MMP-3 blocking on the FFAs-induced VEGF expression enhanced by Mϕ-CM in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were incubated with (open and closed circles) or without (gray circle) 1 mM FFAs in the presence of 10% THP1-CM (open circle) or 10% Mϕ-CM (closed and gray circles) for 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

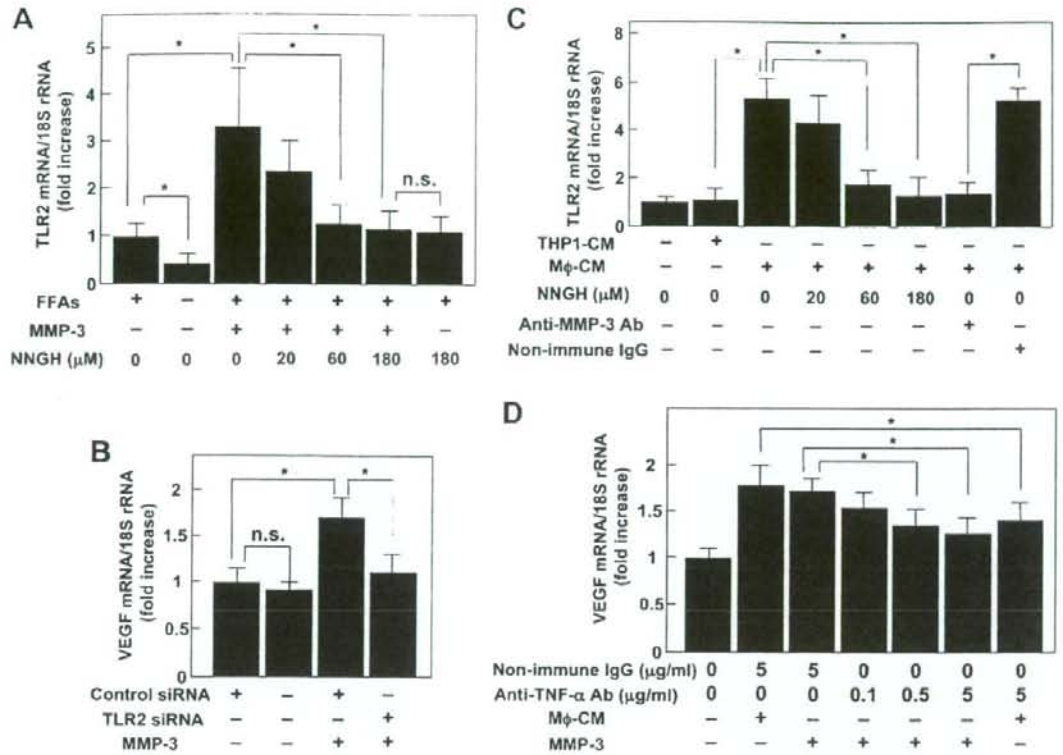

Figure 4. Effect of MMP-3 on the FFAs-induced TLR2 and VEGF expression in 3T3-L1 adipocytes. (A) After the incubation with 1 mM FFAs for 6 h, adipocytes were treated with or without the active form of MMP-3 (100 μg/ml) or 20–180 μM NNGH in the presence of 1 mM FFAs for 6 h. Total RNA was extracted from 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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ORIGINAL ARTICLE

Macrophages regulate tumor necrosis factor-α expression in adipocytes through the secretion of matrix metalloproteinase-3

H Unoki[1,4], H Bujo[2], M Jiang[2], T Kawamura[3], K Murakami[3] and Y Saito[3]

[1]*Division of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba, Japan;* [2]*Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba, Japan and* [3]*Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba, Japan*

Objective: Adipocytes accumulated in the visceral area change their function to induce tumor necrosis factor-α (TNF-α) secretion with concomitant matrix metalloproteinase (MMP)-3 induction in mice. This study was performed to clarify the role of macrophages (Mϕ)-secreted MMP on the functional changes in adipocytes using a culture system.
Design: Cultures of 3T3-L1 adipocytes with THP-1 Mϕ or the Mϕ-conditioned medium were used to investigate the role of Mϕ-MMP on the TNF-α gene in 3T3-L1 adipocytes by the addition of MMP inhibitors. For animal experiments, male C57BL/6J mice were rendered insulin resistant by feeding a high-fat diet, and the expression of an Mϕ marker F4/80, and MMP-3 genes in mesenteric and subcutaneous fat tissue specimens were examined.
Results: Mϕ-conditioned media (Mϕ-CM) increased the levels of TNF-α mRNA expression in 3T3-L1 adipocytes, and these adipocyte responses were abolished by treatment with GM6001, a broad-spectrum MMP inhibitor, or NNGH (*N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid), an MMP-3 inhibitor. The activated form of MMP-3 enhanced glycerol release as well as TNF-α protein secretion from 3T3-L1 adipocytes. The incubation of adipocytes with MMP-3 inhibited insulin-induced glucose uptake in adipocytes. Furthermore, a high-fat intake increased the expression of MMP-3, decreased the insulin-induced glucose uptake of adipocytes and induced expression of F4/80 in mesenteric fat tissue of C57BL/6 mice.
Conclusion: Mϕ may cause a pathological link with surrounding adipocytes through the secretion of MMP-3 followed by TNF-α expression in adipocytes in visceral fat tissue.
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Keywords: macrophage; matrix metalloproteinase-3; tumor necrosis factor-α; adipocyte; insulin resistance

Introduction

Disturbed insulin sensitivity plays an important role in the accumulation of various metabolic disorders, and has been recognized as 'metabolic syndrome'.[1,2] In accordance with the clinical significance of evaluation of visceral fat accumulation in metabolic syndrome, it has become evident that visceral fat has direct interaction with other tissues, such as muscles, liver or vessel walls, through the secretion of several molecules regulating the insulin sensitivity in tissues.[3,4] The transplantation of cultured cells into the intramesenteric space of mature mice has been established as an adequate mode for the analyses of the interaction between visceral fat and insulin sensitivity.[5] The mice with transplanted cultured adipocytes showed that visceral fat, and not subcutaneous fat, secretes the tumor necrosis factor-α (TNF-α), and the secreted molecules actually disturb the insulin sensitivity based on the decreased insulin action in tissues.[5] The accumulated visceral fat caused drastic changes in expression of matrix metalloproteinase (MMP) family genes, among which MMP-3 potentiated free fatty acid-induced TNF-α secretion from adipocytes.[6] Therefore, the MMP-3 activity in visceral fat seems to be directly linked to cytokine expression in adipocytes.

There is an infiltration of macrophages (Mϕ) in the accumulated fat tissues, and active Mϕ cause a pathological inter-relationship with surrounding adipocytes in visceral fat, which leads to the progression of insulin resistance.[7]

Correspondence: Dr H Bujo, Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail: hbujo@faculty.chiba-u.jp
[4]Current address: Laboratory for Diabetic Nephropathy, SNP Research Center, The Institute of Physical and Chemical Research, Kanagawa 230-0045, Japan.
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A variety of inflammatory bioactive molecules plays an important role in pathological interaction between Mϕ and adipocytes in visceral fat.[8–14] An overexpression of monocyte chemoattractant protein (MCP)-1 in adipose tissues causes macrophage recruitment and insulin resistance in mice.[10,11] TNF-α secretion is highly related to the free fatty acid (FFA)-induced inflammatory changes in both adipocytes and Mϕ.[12–15] The peroxisome proliferator-activated receptor activation in Mϕ is able to regulate the FFA-induced TNF-α secretion from adipocytes.[16]

The present study was designed to identify the role of MMP-3 in the interaction between Mϕ and adipocytes for TNF-α gene induction. Conditioned media from Mϕ (Mϕ-CM) increased the TNF-α mRNA expression in adipocytes. The induced levels of TNF-α mRNA were largely abolished by treatment with GM6001, a broad-spectrum MMP inhibitor, or *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH), an MMP-3 inhibitor. The active form of MMP-3 enhanced release of TNF-α and glycerol from 3T3-L1 adipocytes, and inhibited insulin-induced glucose uptake into the cells. The MMP-3 expression in Mϕ, in addition to adipocytes, is potentially important for the development of a pathological link between Mϕ and adipocytes through TNF-α secretion in visceral fat tissue.

Methods

Cell culture and preparation of Mϕ conditioned media

3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured and differentiated into adipocytes as described previously.[16] The human monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 supplemented with l-glutamine (GibcoBRL, Tokyo, Japan) penicillin/streptomycin (100 U per 100 mg ml^{-1}; GibcoBRL) and 10% fetal bovine serum (GibcoBRL, medium A). To allow the monocytes to differentiate into adherent macrophages, THP-1 cells were washed in phosphate-buffered saline (calcium- and magnesium-free; GibcoBRL, buffer A) and resuspended in fresh medium A containing phorbol 12-myristate-13-acetate (50 ng ml^{-1} PMA; Sigma, St Louis, MO, USA) for 3 days (at day 0), and were incubated for 3 more days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% bovine serum albumin (BSA). At day 3, the culture media were collected, centrifuged and stored as Mϕ-CM. Control CM were prepared by incubating the THP-1 cells with DMEM supplemented with 2% BSA for 3 days (THP1-CM). Mϕ-CM and THP1-CM were stored at −80 °C until use. The differentiation of THP1 to mature Mϕ was evaluated by the quantification of CD11b and CD68 mRNA levels using real-time PCR. The differentiated macrophages with CD11b and CD68 mRNA levels of more than two fold greater than those in THP-1 were used for further experiments. Co-culture of adipocytes and Mϕ was performed using transwell inserts with 0.4-μm porous membrane (Becton Dickinson, Franklin Lakes, NJ, USA) to separate adipocytes from Mϕ. To determine the role of Mϕ-secreted factors on adipocyte responses, serum-starved 3T3-L1 adipocytes were incubated with Mϕ-CM or THP1-CM ranging from 10 to 50% of the final volume, for the indicated time periods. To evaluate the effects of MMP inhibition on Mϕ-CM, Mϕ-CM was treated with a broad-spectrum MMP inhibitor GM-6001, a specific peptide inhibitor of the gelatinases MMP-2 and -9, CTTHWGFTLC-decapeptide (CTT) or an MMP-3 inhibitor, NNGH (Calbiochem, San Diego, CA, USA) prior to the addition to adipocytes.

RNA preparation and quantitative real-time RT–PCR

Total RNA was isolated from cultured cells, and quantitative real-time reverse transcription (RT)–PCR was performed with an ABI 7000 sequence detection system using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression Assay Mix (PE Applied Biosystems, Foster City, CA, USA) described previously.[17] The quantification of a given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to 18S rRNA.

Enzyme-linked immunosorbent assay

Serum-starved 3T3-L1 adipocytes were incubated with 100 μg ml^{-1} human MMP-3 (Sigma) for 1–3 days, and the culture medium was assayed for mouse TNF-α using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions as described previously.[16]

Glycerol release measurement

Differentiated 3T3-L1 adipocytes were incubated with DMEM supplemented with 1% FFA-free BSA for 2 days, and then treated with same medium with Mϕ-CM at 50% of volume, THP1-CM at 50% of volume or human MMP-3 at 100 μg ml^{-1}, in the absence or presence of 60 μM NNGH for 6 h. The concentrations of glycerol in the media were determined using a free glycerol determination kit (Sigma) following the manufacturer's protocol.

2-Deoxyglucose uptake assay

Differentiated 3T3-L1 adipocytes were preincubated in serum-starved DMEM with 50% Mϕ-CM, 50% THP1-CM or human MMP-3 at 100 μg ml^{-1}, in the absence or presence of 60 μM NNGH for 6 h. Single adipocytes were prepared from mesenteric or subcutaneous fat of mice, fed with high-fat or regular diet as described.[14] The cells were incubated in DMEM without serum for 2 h at 37 °C, and then either treated or not treated with 100 nM insulin for 15 min at 37 °C, as described previously.[18] After stimulation, 10 μM 2-[^{3}H]deoxyglucose was added and incubated for 5 min. Glucose uptake was stopped by the addition of ice-cold Krebs-Ringer HEPES buffer with 5 μM cytochalasin B and

25 mM glucose. The cells were washed three times with ice-cold Krebs-Ringer HEPES buffer with 25 mM glucose, and the ^{3}H-labeled radioactivity was counted using a scintillation counter (LS-6500; Beckman Coulter Inc., Fullerton, CA, USA).

Animals and animal care

Male C57BL/6J mice (Charles River, Wilmington, MA, USA) were rendered insulin resistant by feeding a high-fat diet consisting of 20% protein, 20% carbohydrate and 60% fat (Research Diet, New Brunswick, NJ, USA) starting at 8 weeks of age for 2 weeks as described previously.[14] Control mice were fed a standard diet consisting of 4.5% fat (Research Diet). Mesenteric and subcutaneous fat tissue specimens were resected, and total RNA was isolated as described previously.[14] All applicable institutional and governmental regulations concerning the ethical use of animal were followed during this research. All animal care and procedures were approved by the Animal Care Committee of Chiba University School of Medicine as described previously.

Western blot analysis

Membranes from fat tissue specimens were prepared and solubilized in solubilization buffer (200 mM Tris-maleate, pH 6.5, 2 mM $CaCl_2$, 0.5 mM PMSF, 2.5 mM leupeptin and 1% Triton X-100) as previously described.[19] The protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). For immunoblotting, equal amounts of membrane protein, protein extracted from pelleted beads, or concentrated media were separated by 10% SDS–PAGE after heating to 95 °C for 5 min under reducing conditions, and transferred to a nitrocellulose membrane. The blots were incubated with antibody against MMP-3 (SC-6839, 1:100 dilution), followed by peroxidase-conjugated anti-goat IgG, and then they were developed using the ECL detection reagents (Amersham Pharmacia, Piscataway, NJ, USA). The signals were quantified by densitometric scanning using the NIH image software program.

Statistical analysis

Results are presented as mean ± s.d. 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

Effects of Mϕ-CM on TNF-α gene expression in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated with Mϕ in the transwell system to evaluate the interactions between Mϕ and adipocytes. A co-culture of adipocytes and Mϕ revealed significant induction of TNF-α gene in adipocytes relative to the control culture at 24 h (Figure 1a). The extent of changes in TNF-α mRNA expression was dependent on the number of Mϕ (data not shown). The role of Mϕ factors on TNF-α gene in adipocytes was investigated by incubating 3T3-L1 adipocytes with Mϕ-CM for 4 h. Consistent with the results in the transwell system, Mϕ-CM significantly induced expression of TNF-α mRNA in adipocytes (Figure 1b). The

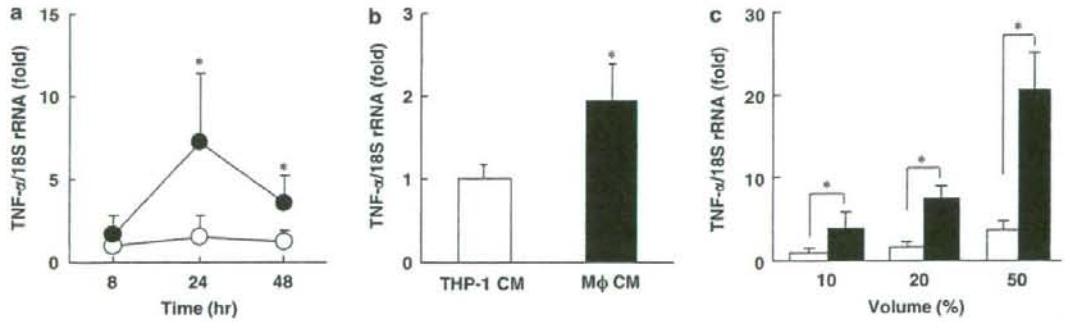

Figure 1 Mϕ-secreted factors increased the expression of tumor necrosis factor-α (TNF-α) in 3T3-L1 adipocytes. (a) Time course of the TNF-α expression in 3T3-L1 adipocytes co-cultured either with THP-1 cells (open circle) or Mϕ (filled circle). 3T3-L1 adipocytes were first seeded on the well bottom, and then THP-1 cells or THP-1 Mϕ were seeded on the permeable membrane of the insert. TNF-α mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of TNF-α mRNA levels in 3T3-L1 adipocytes co-cultured either with THP-1 cells or Mϕ to those co-cultured with THP-1 cells for 8 h (control) were presented. Data are expressed as mean ± s.d. ($n=4$). *$P<0.05$ in comparison to the value with THP1-CM. (b) Effects of the conditioned media of THP-1 (THP1-CM) or Mϕ cultures (Mϕ-CM) on the TNF-α gene expression in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were treated with either 10% THP1-CM or Mϕ-CM for 4 h. TNF-α mRNA levels in 3T3-L1 adipocytes were analyzed using quantitative real-time RT–PCR. The relative ratios of TNF-α mRNA levels in 3T3-L1 adipocytes with Mϕ-CM to those with THP1-CM (control) were presented. Data are expressed as mean ± s.d. ($n=4$). *$P<0.05$ in comparison to the value of the control with THP1-CM. (c) Dose-dependent effect of THP1-CM or Mϕ-CM on TNF-α gene expression in 3T3-L1 adipocytes. The TNF-α mRNA levels in 3T3-L1 adipocytes were analyzed using quantitative real-time RT–PCR. Relative ratios of TNF-α mRNA levels to those with THP1-CM at 10% of volume (control) were presented. Data are expressed as mean ± s.d. ($n=4$). *$P<0.05$.

induction of mRNA for TNF-α was 1.9-fold after 4 h of incubation with Mϕ-CM in comparison to that in control. Mϕ-CM dose-dependently increased TNF-α mRNA expression at the concentrations from 10 to 50% (Figure 1c). There were no obvious changes in the morphology of the adipocytes, and there was no apparent toxicity with either Mϕ-CM or THP1-CM (data not shown).

Role of Mϕ-derived factors in induction of TNF-α mRNA in adipocytes

The expression of the MMP-3 gene is one of most induced genes in accumulated visceral fat tissues, and MMP-3 induces the TNF-α secretion from adipocytes.[6] To explore the molecular mechanisms of the above observed interaction between Mϕ and adipocytes, the role of MMP secreted from Mϕ in the induction of TNF-α mRNA was investigated in adipocytes. The expression of MMP genes significantly increased in Mϕ in comparison to those in THP-1 cells (Figure 2). Among them, MMP-9 was most induced gene in Mϕ (199-fold). The expression of MMP-3 and -12 genes was hardly detected in THP-1 cells. These results raise the possibility that Mϕ-secreted MMP enhances the expression of the TNF-α gene in 3T3-L1 adipocytes in co-culture system. Mϕ-CM treated with various types of MMP inhibitors was added to 3T3-L1 adipocytes to examine the changes of TNF-α gene expression in 3T3-L1 adipocytes (Figure 3). GM6001, a broad-spectrum MMP inhibitor, markedly altered

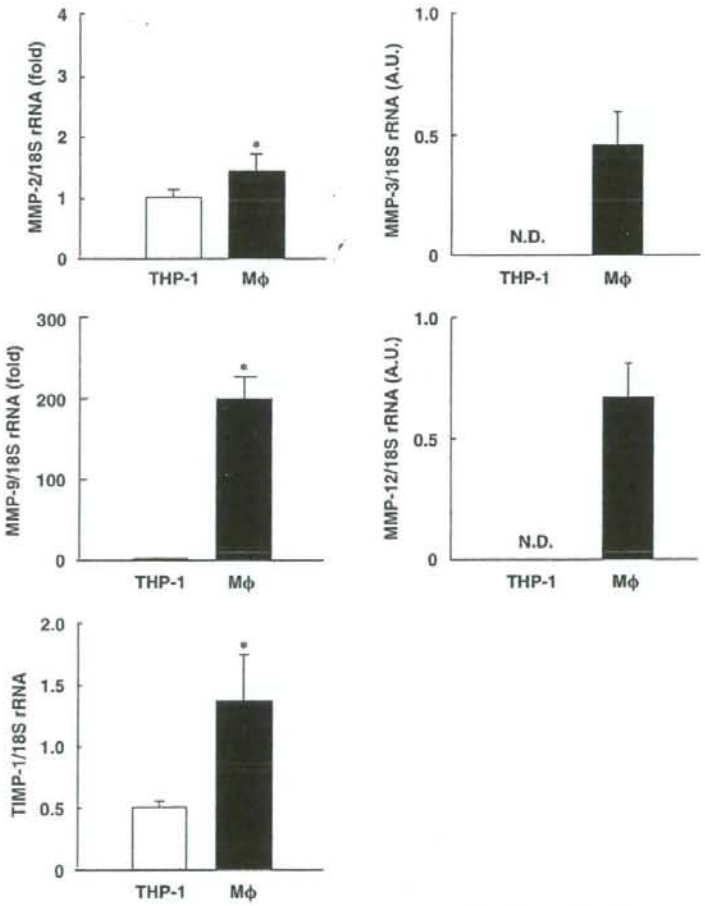

Figure 2 The expression of matrix metalloproteinase (MMP) family genes was induced in Mϕ. THP-1 cells were differentiated into Mϕ by incubating PMA for 72 h. MMP-2, MMP-3, MMP-9, MMP-12 and TIMP-1 mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of mRNA levels in Mϕ to those in THP-1 cells (control) or absolute mRNA levels were presented. Data are expressed as mean ± s.d. ($n = 6$). *$P < 0.05$ in comparison to the value of the control with THP-1 cells. N.D., not detected.

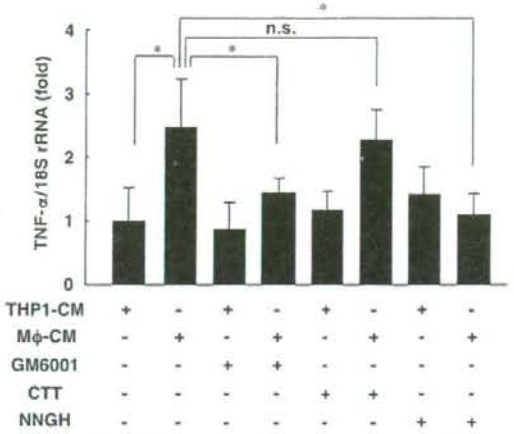

Figure 3 Effect of the inhibition of matrix metalloproteinase (MMP) activity on Mϕ-CM-induced tumor necrosis factor-α (TNF-α) gene expression in adipocytes. Serum-starved 3T3-L1 adipocytes were treated with THP-1-CM or Mϕ-CM in the absence or presence of 10 μM GM6001, 85 μM CTT or 60 μM NNGH for 4 h. the TNF-α mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of the TNF-α mRNA levels in 3T3-L1 adipocytes to those with THP1-CM (control) were presented. Data are expressed as mean ± s.d. ($n = 4$). *$P < 0.05$. n.s., not significant.

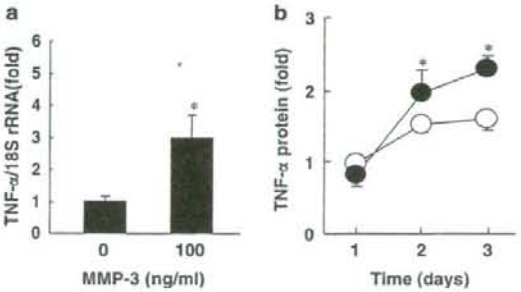

Figure 4 Matrix metalloproteinase (MMP)-3 induced the tumor necrosis factor-α (TNF-α) mRNA and protein expression in 3T3-L1 adipocytes. (**a**) Serum-starved 3T3-L1 adipocytes were treated with the active form of MMP-3 (100 ng ml^{-1}) for 8 h. The TNF-α mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of the TNF-α mRNA levels to those without MMP-3 (control) were presented. Data are expressed as mean ± s.d. ($n = 6$). *$P < 0.05$ compared to the value of the control. (**b**) Serum-starved 3T3-L1 adipocytes were treated with MMP-3 for 1–3 days. TNF-α protein concentrations in conditioned media were analyzed using an ELISA. Relative ratios of TNF-α concentration with those in the absence of MMP-3 for a day (control) were presented. Data are expressed as mean ± s.d. ($n = 6$). *$P < 0.05$ in comparison to the value without MMP-3.

the stimulatory effects of Mϕ-CM on the gene expression of TNF-α (−42%). The gelatinases inhibitor, CTT and an MMP-3 inhibitor, NNGH were used to determine the role of the gelatinases (MMP-2 and -9) and MMP-3 on the TNF-α gene expression in 3T3-L1 adipocytes. The stimulatory effect of Mϕ-CM on the TNF-α gene expression was not significantly inhibited by CTT treatment. In contrast, the induction of TNF-α by Mϕ-CM was markedly inhibited by NNGH treatment (−56%), suggesting an important role for MMP-3 in the adipocyte function. To determine if MMP-3 is the soluble mediator causing TNF-α induction in adipocytes, 3T3-L1 adipocytes were treated with activated MMP-3, and TNF-α mRNA expression and release were measured. MMP-3 treatment significantly increased TNF-α mRNA expression by 3.2-fold (Figure 4a), and the increases were also detected after 50–200 ng ml^{-1} MMP-3 treatments for 8 h (data not shown). Figure 4b shows that MMP-3 treatment (100 ng ml^{-1}) increased TNF-α secretion in a time-dependent manner.

Active MMP-3 induces lipolysis, and reduces insulin-induced glucose incorporation in 3T3-L1 adipocytes

In order to determine the role of Mϕ-derived MMP-3 in the functional changes of adipocytes to induce the TNF-α mRNA expression in adipocytes, the effect of MMP-3 on the lipolysis of 3T3-L1 adipocyte was analyzed (Figure 5a). The glycerol release was significantly increased in the media of

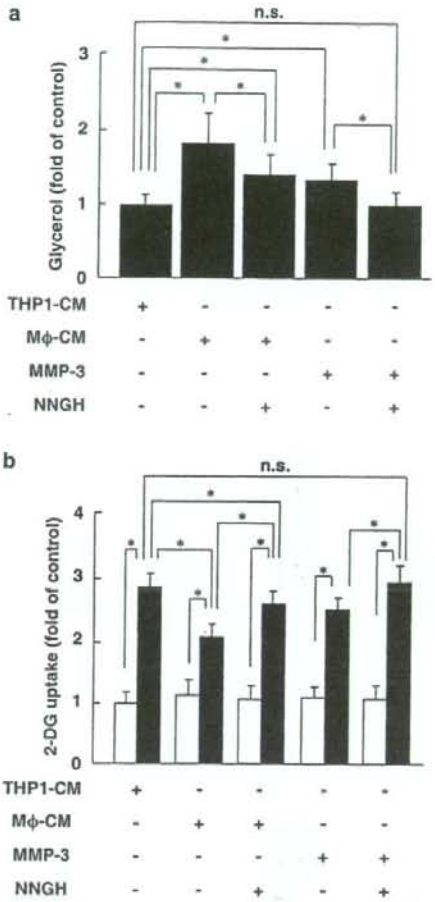

Figure 5 Effect of matrix metalloproteinase (MMP)-3 on lipolysis and insulin-induced glucose incorporation in 3T3-L1 adipocytes. (a) Glycerol release was measured in the media of 3T3-L1 adipocytes after treatment with 50%THP1-CM, 50% Mϕ-CM or 100 μg ml^{-1} human MMP-3, in the absence or presence of 60 μM NNGH for 6 h. The relative ratios of glycerol content with those treated with THP1-CM (control) were presented. Data are expressed as mean ± s.d. ($n=8$). *$P<0.05$. n.s., not significant. (b) 2-DG uptake was measured in 3T3-L1 adipocytes in the absence (open column) or presence (closed column) of 100 nM insulin for 15 min after treatment with either 50% THP1-CM, 50% Mϕ-CM or 100 μg ml^{-1} human MMP-3, in the absence or presence of 60 μM NNGH, for 6 h. Relative ratios of 2-DG contents with those treated with THP1-CM in the absence of insulin are indicated. Data are expressed as mean ± s.d. ($n=6$). *$P<0.05$. n.s., not significant.

3T3-L1 adipocytes incubated with Mϕ-CM, in comparison to those incubated with THP1-CM. The increase in glycerol release observed in the cells incubated with Mϕ-CM was inhibited by 38% in the presence of NNGH. The glycerol release in the media of 3T3-L1 adipocytes incubated with MMP-3 was also significantly increased, in comparison to those incubated with THP1-CM. The increased release was almost abolished by the NNGH treatment. Next, the effect of MMP-3 on the insulin-induced glucose incorporation into 3T3-L1 adipocytes was analyzed (Figure 5b). The glucose uptake was significantly decreased in the media of 3T3-L1 adipocytes incubated with Mϕ-CM, in comparison to those incubated with THP1-CM. The decrease in glycerol release by the incubation 3T3-L1 cells with Mϕ-CM was recovered by 69% in the presence of NNGH. The glycerol release in the media of 3T3-L1 adipocytes incubated with MMP-3 was significantly decreased, in comparison to those incubated with THP1-CM, and that reduction thereafter almost completely recovered due to the NNGH treatment. Therefore, Mϕ-CM induces lipolysis, and reduces insulin-induced glucose uptake in 3T3-L1 adipocytes, possibly in part through the secretion of MMP-3.

High-fat intake induced the expression of MMP-3 in mesenteric fat tissues as well as the induction of F4/80 gene

To assess the expression of MMP-3 gene in adipose tissue Mϕ, the levels of MMP-3 mRNA were examined in mesenteric fat tissue from mice fed with high-fat diet in relation to the expression of the F4/80 gene, an Mϕ-specific antigen[15,20] (Figure 6). High-fat intake for 2 weeks significantly induced the expression level of F4/80 mRNA in mesenteric fat tissue by 2.2-fold in comparison to the level in the control mice. The levels of MMP-3 and TNF-α genes in mesenteric fat tissue were also significantly induced by 2.8- and 2.5-fold in the mice fed the high-fat diet compared in the control mice, respectively. The expression of F4/80, MMP-3 or TNF-α gene in subcutaneous fat tissues was not significantly different between the mice fed with regular chow and high-fat diet. The MMP-3 protein expression was analyzed in either visceral or subcutaneous fat tissue specimens (Figure 7a). The MMP-3 protein levels in visceral fat tissues, but not in subcutaneous fat, were significantly higher in the mice fed with a high-fat diet than those fed with regular chow. The insulin-induced glucose uptake activity in the adipocytes prepared from visceral fat tissues was significantly decreased in the mice fed with high-fat diet in comparison to those consuming regular chow (Figure 7b). These results indicate

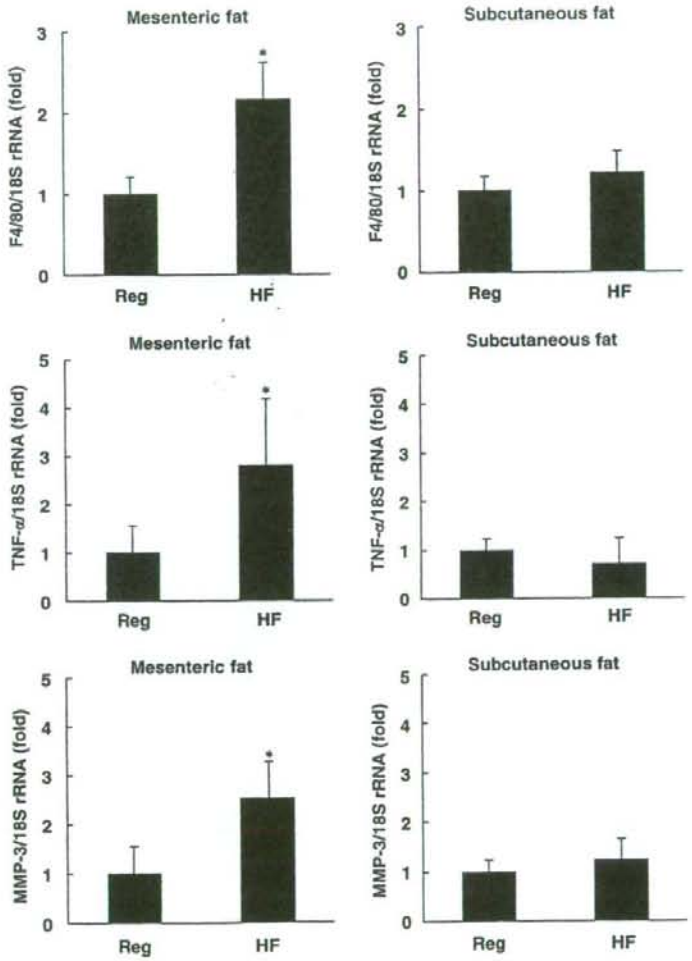

Figure 6 High fat induces F4/80, tumor necrosis factor-α (TNF-α) and matrix metalloproteinase (MMP)-3 mRNA expression in mesenteric fat. Mesenteric or subcutaneous fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. F4/80, TNF-α and MMP-3 mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of mRNA levels in mice fed high-fat to those fed regular chow were presented. Data are expressed as mean ± s.d. ($n=6$). *$P<0.05$ in comparison to the value of the control.

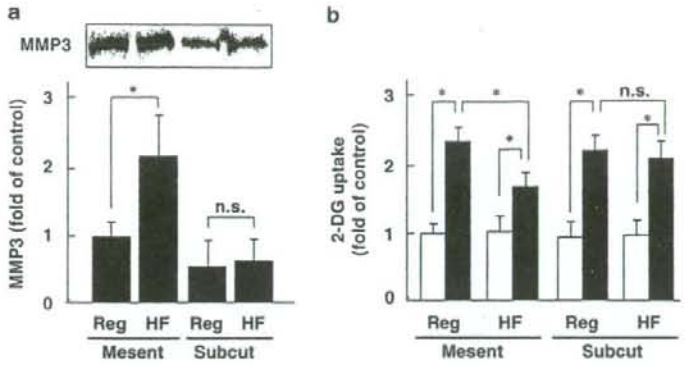

Figure 7 High fat induces matrix metalloproteinase (MMP)-3 protein expression, and reduces insulin-induced glucose incorporation of adipocytes in mesenteric fat. Mesenteric (Mesent) or subcutaneous (Subcut) fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. (a) The MMP-3 protein expression of tissue extracts were analyzed by western blot analysis using an antibody against MMP-3. Relative ratios of MMP-3 protein levels with those of mesenteric fat tissues of mice fed regular chow (control) are indicated. Data representative of three experiments are shown. Bars are expressed as mean ± s.d. ($n=6$). *$P<0.05$. n.s., not significant. (b) The 2-DG uptake was measured in single adipocytes prepared from fat tissues in the absence (open column) or presence (closed column) of 100 nM insulin for 15 min. The relative ratios of 2-DG contents with basal of cells prepared from mesenteric fat of mice fed regular chow are indicated. Data are expressed as mean ± s.d. ($n=6$). *$P<0.05$. n.s., not significant.

that high-fat intake causes Mϕ recruitment into visceral fat, and possibly leads to the induction of the MMP-3 and TNF-α expression, as well as the inhibition of glucose incorporation of adipocytes.

Discussion

The current study demonstrated that Mϕ-CM influences the expression of TNF-α from 3T3-L1 adipocytes. This induction of TNF-α is attenuated by an MMP-3 inhibitor, NNGH. The active form of MMP-3 showed the capability for the induction of lipolysis and the inhibition of the insulin-induced glucose uptake, as well as for the enhanced secretion of TNF-α. These findings suggest that MMP-3 thus plays a role in the modulation of the adipocyte function from Mϕ in adipose tissues.

Recent observations suggested that inflammatory conditions evoked in fat tissues recruit activated Mϕ, possibly enhancing and/or continuing the chronic process in fat tissues.[8,9] TNF-α is suspected to be one of the key players among many cytokines in the interactive modification of function in Mϕ and adipocytes.[4,7] Based on the results obtained herein using a culture system, infiltrating Mϕ may therefore modify the maturation process and secretion level of TNF-α in adipocytes in fat tissues. The expression of TNF-α is observed in 3T3-L1 preadipocytes, and declines gradually after the beginning of maturation in the presence of inducers.[21] The mice with transplanted cultured 3T3-L1 cells showed that the transplanted adipocytes in visceral space, and not subcutaneous space, secret TNF-α and the secreted molecules actually disturb the systemic insulin sensitivity, based on the decreased insulin action in tissues.[5] The induced expression of TNF-α is also observed in the adipocytes in visceral spaces of subcutaneously lipectomized mice.[22] Therefore, the adipocytes that accumulate in visceral space are potentially sensitive to induce the TNF-α gene expression in mice.

Recent studies have indicated that extracellular matrix (ECM) degradation is important for adipogenesis. MMPs are essential for proper matrix remodeling, a process that takes place during adipose tissue formation. Human mature adipocytes secret MMP-2 and -9 and their proteolytic activities are induced during differentiation of murine-cultured adipocytes.[23] mRNA levels for MMP-2, MMP-3, MMP-12, MMP-14, MMP-19 and TIMP-1 are strongly induced in obese adipose tissues in a genetic or a diet-induced model of obesity.[24] The treatment of cultured preadipocytes with either synthetic MMP inhibitors or neutralizing antibodies decreases differentiation.[22] These previous studies using cultured adipocytes suggest that MMP activity is required for adipocyte conversion. The body weight of MMP-3-deficient mice is increased in comparison to that of wild-type mice, as is the weight of the isolated subcutaneous and gonadal fat deposits.[25] MMP-11-deficient mice develop adipocyte hypertrophy in comparison to wild-type mice.[26] Furthermore, the membrane-anchored metalloproteinase, MT1-MMP, acts as a 3D-specific adipogenic factor that directs the dynamic adipocyte-ECM interactions critical to WAT development.[27] These studies using knockout models revealed critical roles of MMPs in fat tissue development and adipogenesis, and possibly also in fat accumulation accompanied with insulin resistance. A recent study reported that the MMP-3 expression levels are negatively correlated with percent body fat, and the MMP-3 gene variants are associated with both BMI and type 2 diabetes in Pima Indians.[28]

The mice with transplanted cultured 3T3-L1 cells showed that the transplanted adipocytes in the visceral space, and

not subcutaneous space, increased TNF-α gene expression.[5] A microarray analysis revealed that the MMP-3 gene expression is drastically induced in addition to TNF-α.[6] Therefore, the MMP-3 gene expression in visceral fat seems to be directly linked to cytokine expression in adipocytes. The current study showed that the active form of MMP-3 enhanced glycerol release, as well as TNF-α protein secretion, from 3T3-L1 adipocytes. The incubation of adipocytes with MMP-3 inhibited insulin-induced glucose uptake in adipocytes. Therefore, the induction of MMP-3 gene expression may modulate lipid and glucose metabolism in visceral adipocytes, leading to the induction of TNF-α secretion. The treatment of 3T3-L1 preadipocytes with the MMP inhibitor Ilomastat has been shown to prevent their differentiation into adipocytes.[29] The subcutaneous administration of MMP inhibitor KB-R7785 reduced the plasma glucose and insulin levels with a concomitant decrease in the TNF-α production in KK-A^{y} mice.[30] These observations indicate that Mϕ-MMP may thus play a functional role in the induction of TNF-α gene expression impairing insulin sensitivity in adipocytes.

Recently, MMP-3 has been shown to be a signaling molecule via the ERK pathway, followed by proinflammatory cytokine induction, and induce superoxide generation in microglia.[31] Moreover, activated MMP-3 is present in the nuclear compartment of malignant and nontransformed hepatocytes, and is associated with the onset of apoptosis.[32] These studies suggested a novel function of MMP-3 as a signaling molecule active for intracellular functions. The current results showed that high-fat intake induced a decrease in insulin-induced glucose incorporation in adipocytes, as well as an increase in Mϕ-infiltration and TNF-α expression in visceral fat tissue. Therefore, MMP-3 may affect the lipid metabolism of adipocytes through the ECM degradation and the activation of other extracellular and intracellular molecules leading to the lipolysis and glucose incorporation. Therefore, Mϕ-derived MMP-3 may modulate the secretion of TNF-α in adipocytes by modulating the lipid metabolism, which is tightly linked to visceral fat accumulation and systemic insulin resistance.

In conclusion, this study suggests that MMP-3 is important for the function of pathological link between Mϕ and adipocytes, which leads to insulin resistance in metabolic syndrome through the regulation of cytokine expression such as TNF-α. The further elucidation of the role of MMP-3 and its secretion from activated Mϕ and adipocytes is therefore expected to contribute to the elucidation of the unexpected relationship between chronic inflammation and disturbed insulin sensitivity in humans.

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Original Article

Long-Term Probucol Treatment Prevents Secondary Cardiovascular Events: a Cohort Study of Patients with Heterozygous Familial Hypercholesterolemia in Japan

Shizuya Yamashita[1], Hideaki Bujo[2], Hidenori Arai[3], Mariko Harada-Shiba[4], Shigeyuki Matsui[5], Masanori Fukushima[6], Yasushi Saito[7], Toru Kita[8], and Yuji Matsuzawa[9]

[1]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[2]Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba, Japan
[3]Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
[4]Department of Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan
[5]Department of Pharmacoepidemiology, School of Public Health, Kyoto University, Kyoto, Japan
[6]Translational Research Center, Kyoto University Hospital, Kyoto University Graduate School of Medicine, Kyoto, Japan
[7]Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba, Japan
[8]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
[9]Department of Internal Medicine, Sumitomo Hospital, Osaka, Japan

Aim: The POSITIVE study assessed whether long-term treatment with probucol, a potent anti-oxidant and cholesteryl ester transfer protein (CETP) activator, is associated with a lowered risk of cardiovascular events in a very high-risk population: familial hypercholesterolemia (FH).
Methods: The study cohort included 410 patients with heterozygous FH, diagnosed between 1984 and 1999 by cardiovascular and metabolic experts at fifteen centers. Traceable patients were screened using predefined eligibility criteria. The primary outcome measure for comparison between probucol exposure and non-exposure was the time to the first cardiovascular event involving hospitalization.
Results: Analysis revealed significant differences in baseline characteristics and follow-up treatment between exposure and non-exposure. An observed indication bias was the use of probucol in more severe FH at diagnosis, both for primary and secondary prevention. When the multivariate Cox regression procedure was used after adjustment for possible confounding factors, probucol lowered the risk (hazard ratio [HR], 0.13; 95% confidence interval [CI], 0.05–0.34) in secondary prevention ($n=74$) and was statistically significant ($p<0.001$), although not significant (HR, 1.5; 95% CI, 0.48–4.67; $p=0.49$) in primary prevention ($n=233$). Safety assessment found no specific difference between exposure and non-exposure.
Conclusion: Long-term probucol treatment may prevent secondary attack in a higher cardiovascular risk population of heterozygous FH.

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Key words; Atherosclerosis, Antioxidants, CETP activator, Dyslipidemia

Introduction

Cardiovascular (CV) diseases, including coronary heart disease and stroke, are the leading cause of death in Japan. Prevention of fatal CV events is therefore the final goal as well as the rationale of cholesterol-lowering therapy.

Probucol, a conventional cholesterol-lowering drug, originated with the report by Barnhart in 1970[1]. The drug has been used clinically in Japan since 1985. Nearly 60,000 Japanese patients still take probucol; western countries discontinued probucol use after

Address for correspondence: Shizuya Yamashita, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: shizu@imed2.med.osaka-u.ac.jp
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the original manufacturer's withdrawal notice to the United States FDA in 1995 after 18 year's use of the drug. Probucol's cholesterol-lowering mechanism has not yet been clearly established, but it is thought to increase catabolic excretion of cholesterol into bile[2]. Later studies[3-5] have described new mechanisms of probucol, including anti-atherogenic and anti-oxidant actions. Another controversial and anti-atherogenic feature of probucol is its paradoxical effect of lowering high-density lipoprotein cholesterol (HDL-C). This action reflects, most likely, its molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport by activation of cholesteryl ester transfer protein (CETP)[6-8] and class B type 1 scavenger receptor[9, 10]. Matsuzawa and his colleagues reported an observed close correlation between the extent of regression in Achilles' tendon xanthoma and probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia (FH)[11].

No large-scale, randomized, double blind comparative study has been conducted to justify the use of probucol in the prevention of CV events or diseases. however, clinical studies as well as pre-clinical data have been accumulating evidence of the clinical worth of probucol in arteriosclerotic diseases. Numerous clinical results, including a reduction in Achilles' tendon xanthoma thickness after long-term treatment for FH[12, 13], reduced rates of restenosis after angioplasty[14-16], and a decrease in carotid artery intima-media thickness[17, 18] support the therapeutic and preventative effects of probucol on arteriosclerotic lesions and plaque. To evaluate the risk and benefit of long-term probucol treatment, we conducted a cohort study to determine whether probucol treatment is associated with the risk reduction of CV events in patients with heterozygous FH, a very high-risk population.

Methods

Study Cohort

We registered patients with FH who received treatment between January 1, 1984 and December 31, 1999 at 15 centers specializing in CV and metabolic diseases, including FH, nationwide. Patients were traceable by medical recend and met the diagnostic criteria for heterozygous FH under the Japan Atherosclerosis Society Guidelines (2002) for the Diagnosis and Treatment of Atherosclerotic CV Diseases[19]. Definite heterozygous FH was defined as having at least two of the major features: total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or xanthoma tuberosum; reduced or abnormal receptor activity noted by LDL receptor analysis. Probable heterozygous FH was defined as having at least one each of the major (as above) and minor features: palpebral xanthoma; arcus juvenilis (< 50 years); juvenile (< 50 years) ischemic heart disease. For other eligibility criteria, we excluded patients with possible homozygous FH or with severe ventricular arrhythmias (polymorphic premature ventricular contractions). Possible homozygous FH was defined as having any one of the clinical features: defect of homozygous or hetero-polymeric LDL receptors confirmed by gene analysis; no LDLR activity observed by receptor analysis, severe elevation of plasma TC higher than 500 mg/dL; xanthoma or atherosclerotic vascular lesions including symptoms of juvenile ischemic heart disease; hypercholesterolemia confirmed in both parents; history of ischemic heart disease confirmed in both parents; or poor response to any 3-hydroxy-3methyl-glutaryl-coenzyme A reductase inhibitor (statin).

During the study period between June, 2004 and September, 2005, we collected anonymous case report forms with the patients' baseline data, including medical history, findings at clinical examination, medication data, and laboratory data. The investigators transcribed the data on to case report forms (identified by a code) from the stored medical charts of the patients. The observation period was the period for which each patient's clinical course could be traced. The longest observation period exceeded 20 years for patients on stable doses of probucol.

We required a sample size of 200 in both the probucol exposure and non-exposure groups, supposing a difference of 10% in the incidence of CV events for 5 years (15% in exposure and 25% in non-exposure). A least 400 subjects were needed to detect the difference with 80% power and a type I error of 5% at the 5% significance level with two-sided log-rank test based on normal approximation. The study protocol was approved through the process of ethics committee or institutional review board at each center.

Definitions and Endpoints

The primary outcome measure was the time to the first CV event, defined as acute myocardial infarction (MI), angina pectoris (AP), heart failure (HF), stroke, transient ischemic attack (TIA) or arteriosclerotic peripheral artery diseases (PAD) leading to hospitalization or death as well as sudden death within 24 hours of an observed intrinsic event. The obtained baseline data at the first visit of each patient included demographic characteristics: sex, date of diagnosis at the participant medical center, age, height, weight, and habits of smoking and drinking. Body mass index (BMI) was calculated as weight in kilograms divided

by the square of height in meters. The other collected characteristic factors at diagnosis were the presence of xanthoma and its location, prior CV event, onset date if any prior CV event, treatment for the event, and other possible risk factors for CV events, including the presence of hypertension, diabetes, ventricular arrhythmia, and PAD. We collected data on cholesterol-lowering therapy (with or without probucol) and other concomitant therapy with anti-platelet, antihypertensive or diabetic drugs. Dates of drug initiation, discontinuation, re-administration, and termination were entered as elemental information. Treatment period was defined as the length from initiation until medication termination, or until the occurrence of the defined CV event, whichever came first. A lipid profile of TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C, blood pressure, level of fasting blood sugar (FBS), hemoglobinA_{1c} (HbA_{1c}), and thickness of tendon xanthoma in both feet were variables of interest, seen as potential predictors of CV events. We obtained measurements of those variables on a yearly basis after each patient was diagnosed. LDL-C levels were calculated from TC and HDL-C measurements with the Friedewald formula in TG <400 mg/dL. For TG of 400 mg/dL and more than 400 mg/dL, the expression of 0.16 X TG was applied in stead of 0.2 X TG[20]. Most patients had fasted compliantly at periodic checkups of their lipid levels. We set a follow-up period of 10 years for the measurements.

Statistical Analyses

The primary objective of analysis was a comparison between probucol exposure and non-exposure to evaluate whether treatment with probucol (500 mg to 1,000 mg daily) for FH provided CV benefits. The analysis was based on intent-to-treat principles. The secondary objective was to assess whether changes in the lipid profile after probucol treatment predicted CV events in the cohort. Event-free survival, defined as the time from diagnosis to the first CV event, was determined as a response variable. Statistical analysis was performed to evaluate clinical outcomes separately for secondary and primary prevention groups; that is, patients with or without a history of CV events at diagnosis.

Baseline characteristics of each group were explored to detect risk factors for CV events because potential confounders, including indication bias, were anticipated. For baseline comparison, Wilcoxon's rank sum test and Fisher's exact test were used for continuous variables and categorical variables respectively. For detection of risk factors, univariate Cox proportional hazards regression with a baseline variable as covariate was used as a screening step to determine the relationship with CV events. Variables that achieved significance at the level of 20% in univariate analysis were subsequently included in a multivariate Cox proportional hazards regression using backward variable selection. Variables proving significant at the 10% significance level were selected as risk factors to be adjusted. Consequently, probucol treatment effect was evaluated using the multivariate Cox model with adjustment for the selected baseline variables. Finally, the other observed treatment factors: cholesterol-lowering drugs other than probucol, LDL-apheresis, anti-platelet drugs, anti-hypertensive drugs, and diabetic drugs were entered into that model to assess their effects.

For the association between changes in lipid profile after probucol treatment and the risk of CV events, pre-treatment values of TG, LDL-C, HDL-C as well as TC, and each lipid reduction ratio after treatment were used as covariates. Multivariate analyses of time from probucol start to the first CV event used multivariate Cox's proportional hazards models. Statistical analysis was peerformed with SAS version 8.2.

Results

Patient Characteristics

We collected data from the medical records of 541 patients, and excluded the data of 131 patients that did not meet eligibility predefined in the protocol.

The flow diagram (**Fig. 1**) gives reasons for the exclusion. A substantial fraction of probucol-exposed patients, 80.0% and 93.2%, took probucol within two years after diagnosis for in primary and secondary prevention groups, respectively. Baseline characteristics at diagnosis are given for each group (**Table 1**, **2**). The secondary prevention group (**Table 2**) had prior diseases of AP, MI, stroke, HF, and TIA. This group was found to have significant higher proportions of men (60.2%, $p<0.01$), smokers (50.0%, $p<0.01$), hypertension (40.9%, $p<0.001$) diabetes (15.9%, $p=0.02$), and older median age (52 years, $p=0.01$) than the primary prevention group. Moreover, the group tended to have hypo-HDL cholesterolemia of median 42 (20–90) mg/dL, and to receive combined treatments with anti-platelet drugs (56.8%), anti-hypertensive drugs (53.4%), and LDL-apheresis (14.8%).

Comparison between probucol-exposed and non-exposed groups revealed significant differences in some baseline characteristics and treatments, which showed a confounding indication that patients with more severe FH took probucol. For baseline characteristics, the exposed group for primary prevention had more

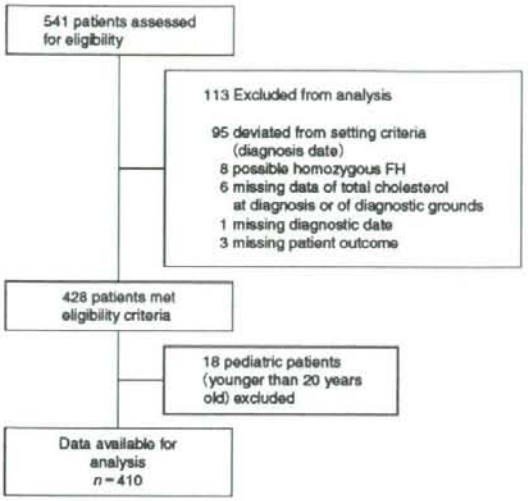

Fig. 1. Patient Flowchart.

We collected data from the medical records of 541 patients, and excluded the data of 131 patients who did not meet the eligibility predefined in the protocol. The flow diagram gives reasons for the exclusion.

palpebral xanthoma (13.4%, $p=0.05$), thicker median measurement of tendon xanthoma (12.5 mm, $p<0.01$), higher median HbA_{1c} (5.8%, $p=0.03$), and more use of antihypertensive drugs (25.3%, $p<0.01$). Their lipid profile was more severe with a higher median baseline TC (325 mg/dL, $p=0.001$), a higher median LDL-C level (253 mg/dL, $p<0.001$), and a lower HDL-C level (47 mg/dL, $p<0.001$) than the unexposed group. The exposed group for secondary prevention had a higher prevalence of post-MI (44.6%, $p<0.01$) than the unexposed group. Observed medications were also significantly different between the exposed and unexposed groups. The exposed group used anti-hypertensive drugs concomitantly at a higher rate (25.3% vs. 11.2%, $p<0.01$) for primary prevention.

Descriptive analysis of baseline characteristics and treatments during observation implies that in both primary and secondary prevention, the exposed groups tended to include patients with more severe FH at diagnosis. Arguably, patients considered more severe at diagnosis would receive more intensive treatment, including probucol.

Outcomes

We present the absolute number of CV events requiring hospitalization by prevention group with details of the events (**Table 3**). The incidence of CV events without consideration of confounding factors was 11.6% in the exposed group and 4.5% in the unexposed group for primary prevention. For secondary prevention, the incidence was 27.0% in the exposed group and 64.3% in the unexposed group. The event-free survival curve of the secondary prevention group is given (**Fig. 2**).

To identify risk factors for CV events, we determined the relationship between the incidence and every baseline variable using univariate Cox regression at a significant level of 20%. Variables proving significant at the 10% significance level in multivariate Cox regression were selected as risk factors to be adjusted. We estimated the effect of treatment after adjusting the selected risk factors. We calculated hazard ratios (HRs) with 95% confidence interval (CI) for binary variables, BMI ≥ 25 vs BMI < 25, drinking vs no drinking, for example, and the indicated HRs corresponded to a 1 standard deviation increase for continuous variables, including TC. Estimated results are given (**Table 4**).

In the primary prevention group, significant variables were BMI ≥ 25 (HR 1.86, 95% CI 0.87–3.98; $p=0.11$), drinking (HR 2.17, 95% CI 1.02–4.63; $p=0.05$), tendon xanthoma (HR 2.17, 95% CI 0.76–6.23; $p=0.15$), prior diseases other than CV events (HR 1.87, 95% CI 0.87–3.99; $p=0.11$), PAD (HR 5.23, 95% CI 0.70–39.2; $p=0.11$), diabetes (HR 2.27, 95% CI 0.79–6.50; $p=0.13$), TC (HR 1.37, 95% CI 0.99–1.89; $p=0.06$), HDL-C (HR 0.75, 95% CI 0.50–1.12, $p=0.16$), SBP (HR 1.48, 95% CI 1.00–2.18; $p=0.05$), and the thickness of tendon xanthoma (HR 1.50, 95% CI 1.06–2.14; $p=0.02$). Three of these variables, drinking, TC, and PAD were selected for adjustment at the 10% significance level as a result of a multivariate Cox regression with backward variable selection. After adjustment for these three baseline variables, we found no significant effect by probucol at the 5% significant level. The estimated hazard ratio of probucol use for CV events was 1.50 (95% CI 0.48–4.67; $p=0.49$).

In the secondary prevention group, significance variables were drinking (HR 1.74, 95% CI 0.80–3.79; $p=0.17$), presence of palpebral xanthoma (HR 5.34, 95% CI 2.26–12.61, $p<0.001$), TIA (HR 4.16, 95% CI 0.54–32.21; $p=0.17$), history of coronary artery bypass graft (HR 0.31, 95% CI 0.11–0.90; $p=0.03$), hypertension (HR 0.58, 95% CI 0.26–1.28; $p=0.18$), diabetes (HR 2.89, 95% CI 1.30–6.42; $p<0.01$), and fasting blood sugar (HR 1.31, 95% CI 0.91–1.89; $p=0.15$). Two of these variables, palpebral xanthoma and diabetes, were selected for adjustment at the 10% sig-

Table 1. Baseline characteristics of patients in primary prevention group[†]

Characteristics	Primary prevention No. (%) of patients			*p*
	All $n=322$	Exposed $n=233$ (72.4)	Unexposed $n=89$ (27.6)	
Age, mean (range)	49 (27-74)	50 (20-74)	47 (20-72)	0.18
Men, No. (%)	134 (41.6%)	96 (41.2%)	38 (42.7%)	0.90
BMI ≥25	71 (22.5%)	49 (21.4%)	22 (25.6%)	0.45
Smoker	99 (33.2%)	74 (34.1%)	25 (30.9%)	0.68
Drinker	124 (42.2%)	93 (43.7%)	31 (38.3%)	0.43
Xanthoma	259 (80.7%)	190 (81.9%)	69 (77.5%)	0.43
Tendon xanthoma	245 (76.3%)	181 (78.0%)	64 (71.9%)	0.30
Nodular xanthoma	28 (8.7%)	22 (9.5%)	6 (6.7%)	0.51
Palpebral xanthoma	36 (11.2%)	31 (13.4%)	5 (5.6%)	0.05
PAD	4 (1.2%)	1 (0.4%)	3 (3.4%)	0.07
Hypertension	54 (16.8%)	40 (17.2%)	14 (15.7%)	0.87
Diabetes	22 (6.9%)	17 (7.3%)	5 (5.6%)	0.81
Lipid profile, mg/dL				
TC[§]	320 (188-493)	325 (188-493)	307 (194-464)	0.001
TG[§]	120 (28-1289)	121 (34-1068)	120 (28-1289)	0.96
HDL-C[§]	49 (20-108)	47 (20-90)	52 (27-108)	<0.001
LDL-C[§]	244 (45-425)	253 (98-425)	223 (45-403)	<0.001
Blood Pressure, mmHg				
SBP[§]	129 (82-190)	128 (82-190)	131 (90-190)	0.57
DBP[§]	0 (48-120)	80 (48-120)	80 (56-120)	0.91
FBS (mg/dL)[§]	95 (63-276)	94 (63-140)	95 (81-276)	0.41
HbA_{1c} (%)[§]	5.7 (4.1-12.4)	5.8 (4.1-9.7)	5.3 (4.3-12.4)	0.03
Tendon xanthoma thickness (mm)[§]	12.1 (7.5-49.0)	12.5 (7.5-49.0)	10.5 (8.0-20.0)	<0.01
Treatment				
Cholesterol-lowering drugs (non-probucol)	302 (93.8%)	219 (94.0%)	83 (93.3%)	0.80
LDL-apheresis	7 (2.2%)	6 (2.6%)	1 (1.1%)	0.68
Anti-platelet drugs	49 (15.2%)	41 (17.6%)	8 (9.0%)	0.06
Anti-hypertensive drugs	69 (21.4%)	59 (25.3%)	10 (11.2%)	<0.01
Diabetic drugs	15 (4.7%)	12 (5.2%)	3 (3.4%)	0.37

[†]Continuous variables compared by Wilcoxon's rank sum test, distribution of categorical variables by Fisher's exact test. [§]Data are median (range). All data are number (%) unless otherwise indicated. Each percentage shown is related to the total number with measurement data. BMI, body mass index; PAD, peripheral artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA_{1c}, hemoglobin A_{1c}. LDL-C was calculated with the Friedewald formula.

nificance level as a result of multivariate Cox regression analysis using a backward variable selection. After adjustment for these two baseline variables, the hazard ratio of probucol use for CV events was estimated to be 0.13 (95% CI 0.05-0.34) and significant ($p<0.001$). In sensitivity analyses, we also obtained similar estimation results on probucol for various sets of baseline covariates for adjustment.

The lipid levels of TC, LDL-C and HDL-C were lowered after probucol treatment both in primary and secondary prevention. In the primary prevention group, the median (range) levels of TC, TG, LDL-C and HDL-C closest to before treatment were respectively 305 (165-493), 119 (35-1068), 228 (107-425) and 48 (25-96) mg/dL, and those at 10-year treatment were, respectively, 222 (141-371), 94 (43-335), 157 (91-311) and 39 (17-81) mg/dL. In the secondary prevention, the median levels of TC, TG, LDL-C and HDL-C closest to before atment were, respectively, 320 (191-469), 129 (37-636), 240 (117-381) and 44 (24-90) mg/dL, and those at 10-year treatment were, respectively, 211(135-305), 71 (48-475),