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

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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]

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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

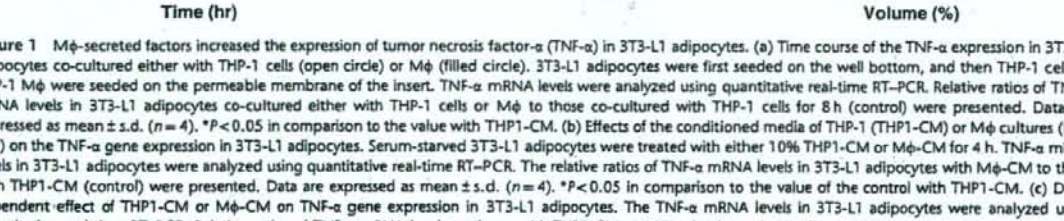

Figure 1 Mϕ-secreted factors increased the expression of tumor necrosis factor-α (TNF-α) in 3T3-L1 adipocytes. (a) Time course of the TNF-α expression in 3T3-L1 adipocytes co-cultured either with THP-1 cells (open circle) or Mϕ (filled circle). 3T3-L1 adipocytes were first seeded on the well bottom, 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

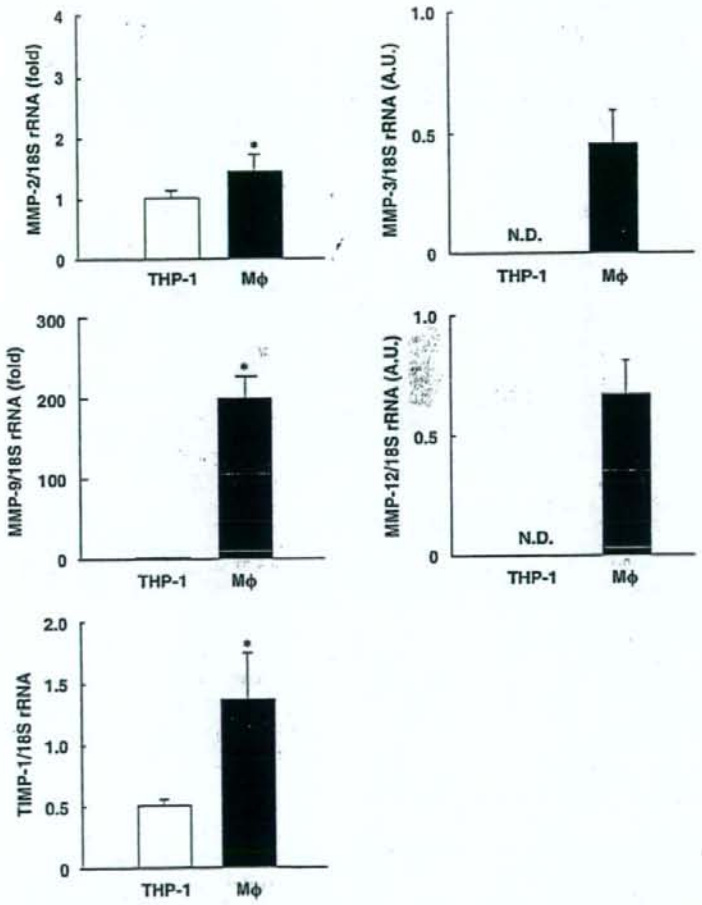

Figure 2 The expression of matrix metalloproteinase (MMP) family genes was induced in Mϕ. THP-1 cells were differentiated into Mϕ by incubating PMA for 72 h. MMP-2, MMP-3, MMP-9, MMP-12 and TIMP-1 mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of mRNA levels in Mϕ to those 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.

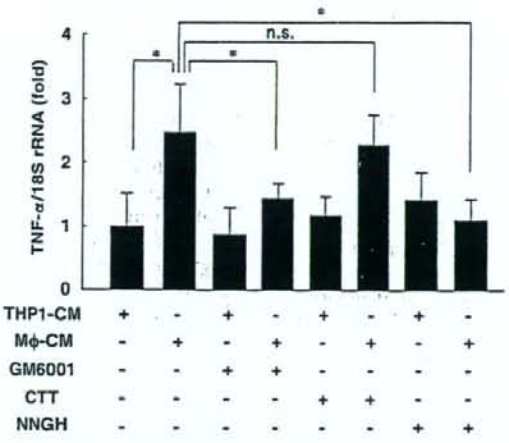

Figure 3 Effect of the inhibition of matrix metalloproteinase (MMP) activity on Mϕ-CM-induced tumor necrosis factor-α (TNF-α) gene expression in adipocytes. Serum-starved 3T3-L1 adipocytes were treated with THP-1-CM or Mϕ-CM in the absence or presence of 10 μM GM6001, 85 μM CTT or 60 μM NNGH for 4 h. 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.

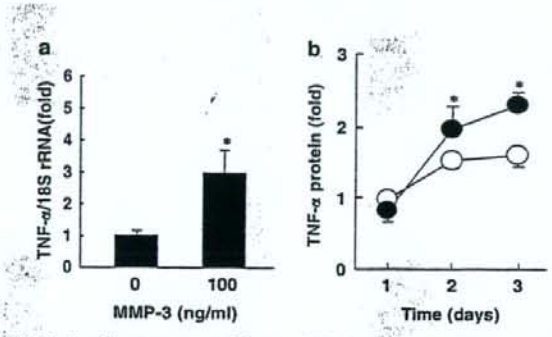

Figure 4 Matrix metalloproteinase (MMP)-3 induced the tumor necrosis factor-α (TNF-α) mRNA and protein expression in 3T3-L1 adipocytes. (a) Serum-starved 3T3-L1 adipocytes were treated with the active form of MMP-3 (100 ng ml^{-1}) for 8 h. The TNF-α mRNA levels were analyzed using quantitative real-time 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

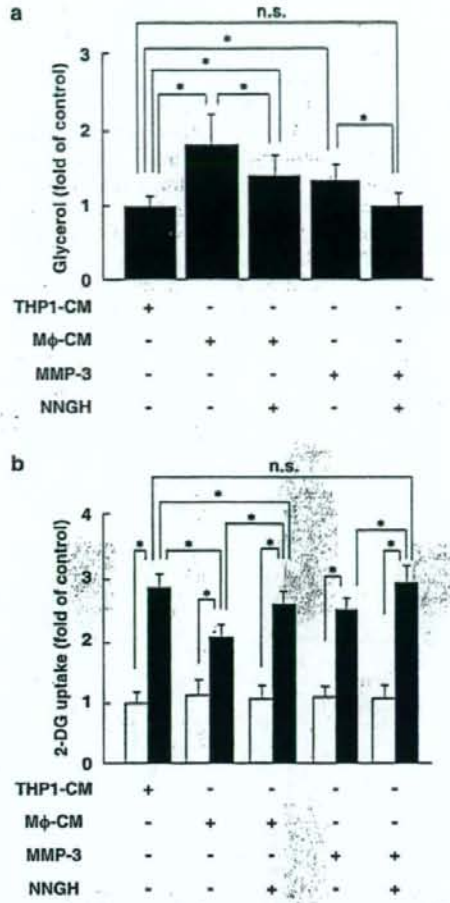

Figure 5 Effect of matrix metalloproteinase (MMP)-3 on lipolysis and insulin-induced glucose incorporation in 3T3-L1 adipocytes. (a) Glycerol release was measured in the media of 3T3-L1 adipocytes after treatment with 50%THP1-CM, 50% Mϕ-CM or 100 μg ml^{-1} human MMP-3, in the absence or presence of 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

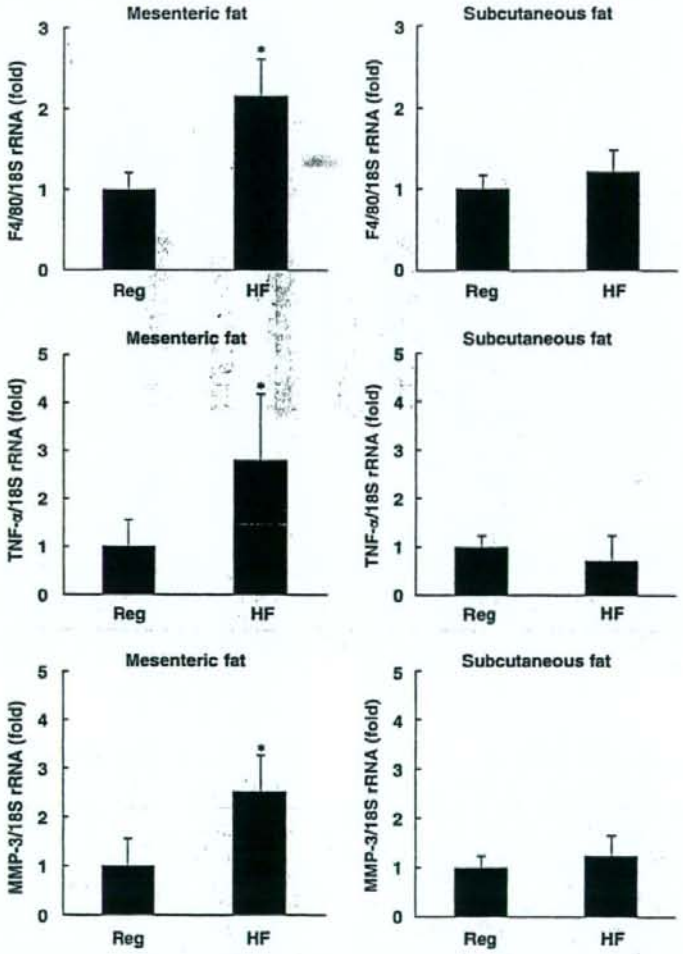

Figure 6 High fat induces F4/80, tumor necrosis factor-α (TNF-α) and matrix metalloproteinase (MMP)-3 mRNA expression in mesenteric fat. Mesenteric or subcutaneous fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. F4/80, TNF-α and MMP-3 mRNA levels were analyzed 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.

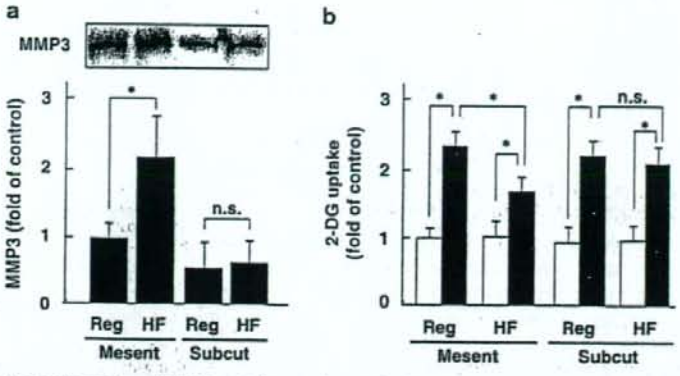

Figure 7 High fat induces matrix metalloproteinase (MMP)-3 protein expression, and reduces insulin-induced glucose incorporation of adipocytes in mesenteric fat. Mesenteric (Mesent) or subcutaneous (Subcut) fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. (a) 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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REVIEW

The role of fat topology in the risk of disease

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Clustering of multiple risk factors such as impaired glucose metabolism, lipid disorders and hypertension has been shown to be the major background of atherosclerotic diseases, and disease entities such as the metabolic syndrome represent a highly atherogenic state. Although these common risks may generally co-exist by accident in one individual, clustering of multiple risk factors in the metabolic syndrome does not occur by accident, and there should be a key player for the syndrome. In 1983, we reported the method for fat analysis using computed tomography scan, which enables us to analyze intra-abdominal visceral adiposity as well as subcutaneous fat. Visceral fat accumulation has been shown to cause impaired glucose metabolism, lipid disorders, and hypertension, and therefore it is considered to be a key player in the metabolic syndrome. To clarify the mechanism by which visceral fat accumulation causes a variety of metabolic and vascular diseases, we studied the molecular characteristics of adipose tissue and adipocytes by investigating expressed genes in visceral and subcutaneous adipocytes and revealed that adipocytes, especially visceral adipocytes, secrete a variety of bioactive substances, the so-called adipocytokines. We showed that visceral fat accumulation causes abnormalities in adipocytokine secretion, such as hypersecretion of plasminogen activator inhibitor 1, which is related to thrombogenic vascular diseases. More importantly, we discovered an important benign adipocytokine named adiponectin, which protects against the development of diabetes mellitus, hypertension, inflammation, and atherosclerotic vascular diseases. Plasma levels of adiponectin decreased in individuals with visceral fat accumulation, and hypoadiponectinemia caused by visceral fat accumulation might be one of the major causes of metabolic syndrome.
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Keywords: visceral fat; metabolic syndrome; adipocytokines; adiponectin; aquaporin adipose; hypoadiponectinemia

Introduction

As recently as 30 years ago, individuals with massive obesity were very rare in Japan and had few metabolic and cardiovascular complications, yet I noticed that mildly obese individuals who gained 2–3 kg sometimes suffered marked metabolic and circulatory disorders. This fact suggests that the extent of body fat accumulation is not necessarily a determinant for morbidity of obesity.

Professor Vague was a pioneer of this concept. He said 60 years ago that although women normally have twice the fat levels as men, they live longer, with less morbidity from the metabolic complications of obesity. He proposed classifying android-type excess weight as high-risk obesity and gynoid type as low-risk obesity.[1]

Figure 1 shows two classical paintings that suggest the important role of obesity in morbidity. The obese woman in the left panel must be ill, because this picture was taken from an illustrated book titled 'Yamai-Zoshi' (which means 'Disease Scrolls') published 800 years ago in Japan. The difference in fat topology between this woman and the girl painted by Renoir shown in the right panel is remarkable.

In the early 1980s, Professor Björntorp[2] proposed classifying obesity into central obesity and peripheral obesity, and Professor Kissebah[3] proposed the concept of upper- and lower-body obesity, based on the waist–hip ratio, with upper-body obesity having a higher occurrence of obesity-related diseases.

Current perspective favors another viewpoint. Our group reported a method for fat analysis using computed tomography (CT) scan in 1983,[4] and then proposed classifying obesity into visceral fat obesity and subcutaneous fat obesity.[5] In 1990, the International Symposium on Regional Fat Distribution and Morbidity was held in Osaka as a Satellite Symposium of the 6th International Congress on Obesity held in Kobe, Japan. This symposium, in which Professors Tarui, Bouchard, Kissebah and Björntorp served as Chairs and myself as Secretary General, may have been the first consensus meeting on the importance of intra-abdominal visceral fat with respect to obesity-related diseases.

In this review, I would like to introduce the history of the concept of visceral fat syndrome and also discuss the mechanisms of a variety of cardiometabolic complications caused by visceral fat accumulation.

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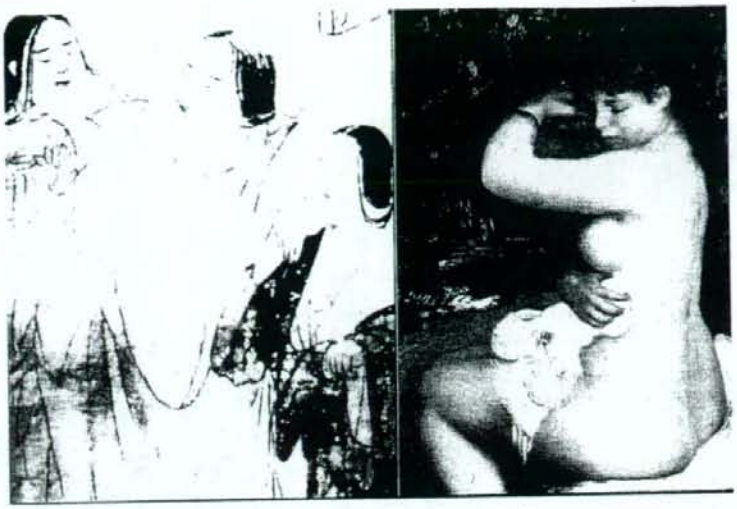

Figure 1 Classical arts present both typical cases of abdominal obesity (left) and gluteal obesity (right).

Visceral fat analysis and its clinical significance

Figure 2 shows the first report of the method for the analysis of regional fat volume using CT scan published in 1983.[4] In this method, a human body is divided into 11 cylindrical shapes, and 11 computed tomographic sections are scanned at the middle of each segment to obtain the area of fat tissue. The volume of each cylindrical segment is calculated by multiplying a cross-sectional area of fat tissue by the height of each part. We had originally determined fat volume by the integration of CT area of multi-cut scans for abdominal subcutaneous fat and visceral fat, but multiplication of single-cut CT area at the umbilicus level by the length of abdomen turned out to be closely correlated to the volume gained by integrated multi-cut area as the gold standard. Finally, the fat area of a single CT scan at the umbiliculus level has been used as the indicator for determining subcutaneous and visceral adiposity in the abdomen.

From the analysis of abdominal adipose tissue, we noticed a remarkable variation in fat distribution between the subcutaneous and intra-abdominal cavities as shown in Figure 3. Individuals with increased visceral adiposity were shown to have more obesity-related complications by subsequent studies. Fat areas from CT images had been determined by a very primitive manual identification in the beginning, but currently are developed with very sophisticated software named 'Fat Scan,' which can detect areas with adipose tissue immediately (Figure 4).[4]

Using this method, we reported that visceral fat accumulation closely correlated to the impairment of glucose and lipid metabolism in 1987[6] and to hypertension in 1990.[7] Professor Despres[8] also measured intra-abdominal adiposity by CT scan and found that an increase in visceral fat caused the abnormalities in glucose and lipoprotein metabolism. Since then, many clinical studies on the correlation between visceral adiposity and disease have been performed all over the world. Figure 5 shows our group's findings on the relationship of visceral fat accumulation with the development of many common diseases. It is especially significant that visceral fat accumulation directly correlates to the development of cardiovascular disease.[9] Visceral fat accumulation is sometimes seen not only in obese individuals but also in non-obese individuals. Subjects with a visceral fat area more than $100\,cm^2$ have been shown to have more obesity-related diseases than obese individuals without visceral fat accumulation.[10] From these clinical studies, we proposed a disease entity named 'visceral fat syndrome,' which is similar to the syndrome X proposed by Professor Reaven[11] and the deadly quartet proposed by Professor Kaplan.[12] The concept of the metabolic syndrome has become well established as a highly atherogenic state in which insulin resistance, hyperglycemia, hypertension and lipid disorders cluster.[13] In this syndrome, visceral fat has been recognized to play a key role in the development of a variety of diseases including cardiovascular disease. Therefore, visceral fat syndrome closely resembles metabolic syndrome.

Factors inducing visceral fat accumulation

Sex hormones might be one of the factors determining body fat distribution. Visceral fat accumulation is more predominant in males than in females when compared among age-matched individuals with similar body mass index. Earlier

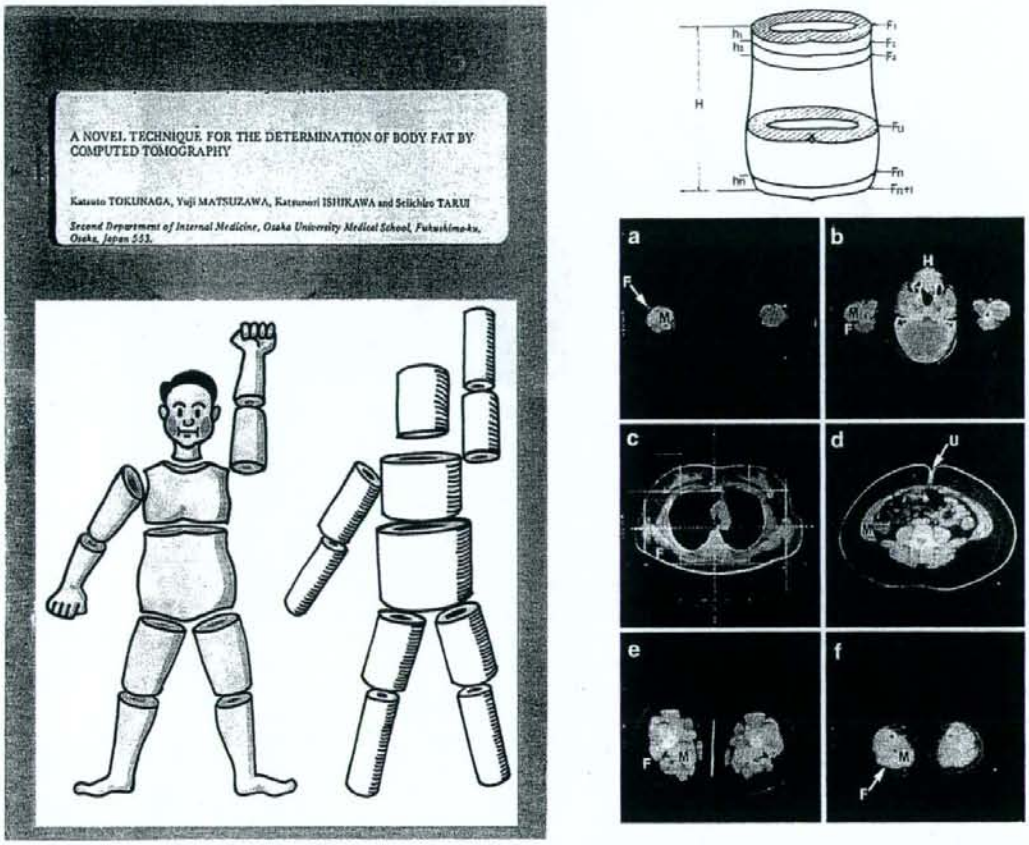

Figure 2 Our first report of fat analysis using computed tomography. Adapted from Tokunaga *et al.*[4]

studies have shown a negative correlation between plasma levels of sex hormone-binding globulin and waist–hip ratio in females, indicating that an active form of testosterone is an important determinant of visceral adiposity in females. In contrast, in males, low testosterone levels were reported to relate to visceral adiposity.

Aging is also an important factor in accumulation of visceral fat. A close linear correlation between age and visceral fat volume was shown in male subjects in a cross-sectional study in 1557 obese individuals of varying ages.[14] Although this correlation was also present in female obese subjects, the slope was gentle in premenopausal individuals, but suddenly became steep in menopausal individuals, compared with that of males.

Among dietary factors, high sucrose intake is a candidate for promoting visceral fat accumulation. High sucrose loading has been known to cause the increase in mesenteric fat in both human and animal models.[15]

Physical exercise has been suggested to prevent and reduce visceral fat accumulation. We analyzed fat distribution in Japanese Sumo wrestlers to investigate the effects of physical exercise on visceral adiposity. Sumo wrestlers eat a high-energy diet (7000–10 000 kcal) every day to gain weight, but at the same time they perform strenuous physical training daily. Although they show marked obesity and have markedly high waist circumference, the average ratio of visceral-to-subcutaneous fat is 0.25 in young Sumo wrestlers, which is comparable to subcutaneous obesity, and their glucose and lipid levels remain normal. A typical CT image of abdominal fat in Sumo wrestlers is shown in Figure 6 with metabolic parameters, showing very little intra-abdominal visceral fat with developed muscularity and increased subcutaneous fat with almost normal lipid and glucose metabolism.[16] The incidence of diabetes mellitus may increase in retired wrestlers who do not continue physical exercise, if they continue heavy caloric intake. Physical

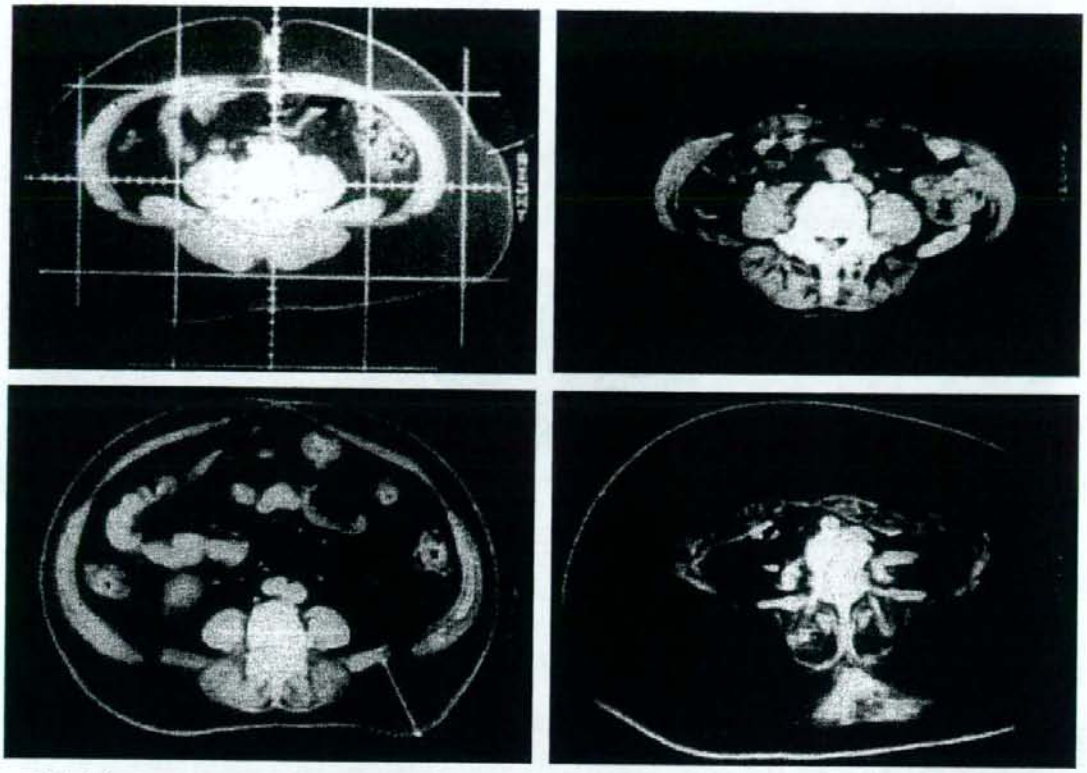

Figure 3 Marked variation of distribution between subcutaneous fat and intra-abdominal visceral fat in obese women. Adapted from Tokunaga *et al.*[4]

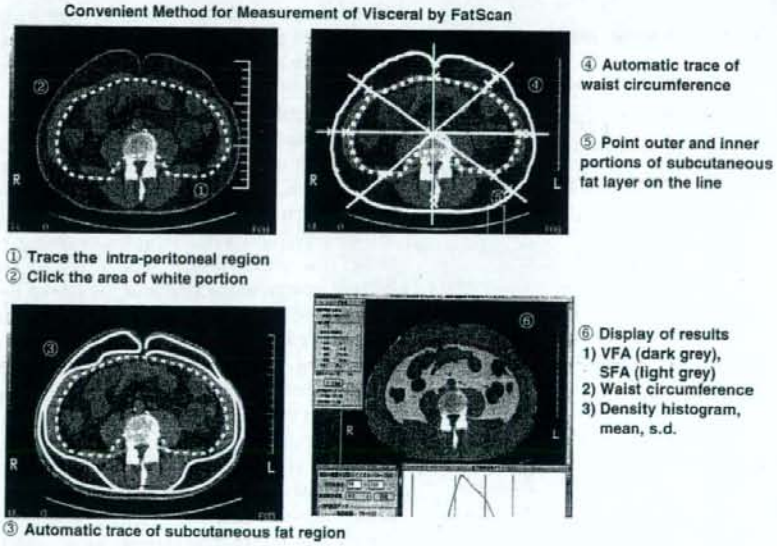

Figure 4 Analysis of abdominal subcutaneous fat area and visceral fat area by newly developed software, FatScan.

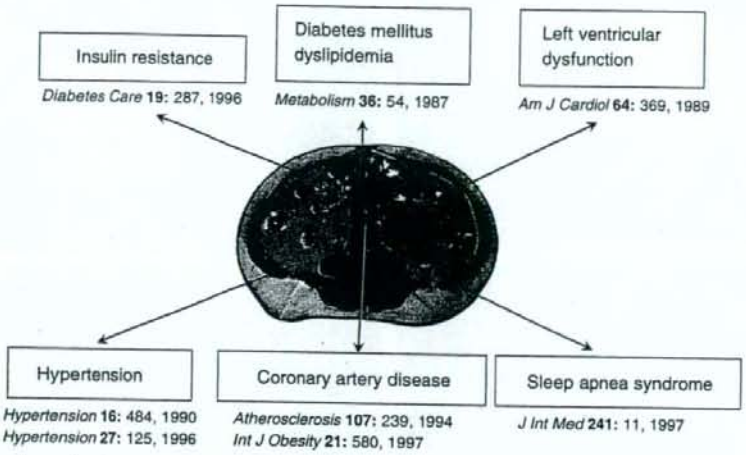

Figure 5 Metabolic and cardiovascular diseases caused by visceral fat accumulation.

Metabolic profiles in young Sumo wrestlers

	Sumo wrestlers	Control
BMI (kg/m^2)	36 ± 6	23 ± 4
TC (mg per 100 ml)	160 ± 29	189 ± 17
TG (mg per 100 ml)	105 ± 13	79 ± 44
FPG (mg per 100 ml)	95 ± 21	92 ± 5
V/S	0.25 ± 0.13	

Matsuzawa, Y. *et al Proc Soc Exp Biol Med* **200:** 197, 1992

Figure 6 CT image of abdominal fat and metabolic profiles of Japanese professional Sumo wrestlers.

exercise is not only useful in preventing visceral fat accumulation but also in reducing visceral adiposity. It is noteworthy that subcutaneous adiposity is rather insensitive and not markedly altered in this condition.

There may be genetic factors for visceral adiposity, as visceral obesities sometimes cluster in the same family. A non-conservative missense mutation in the β3-adrenergic receptor gene was suggested as a candidate for a genetic factor of visceral obesity.[17]

Why does visceral fat play a key role in the development of multiple risks and cardiovascular disease?

To answer this question, we have been investigating adipose tissue biology from two different approaches: (a) increased influx of free fatty acids (FFA) and glycerol from accumulated visceral fat to the liver, and (b) increased influx of FFA in the liver causing enhanced lipoprotein formation and secretion.

Increased influx of FFA and glycerol from accumulated visceral fat to the liver

Visceral fat is characterized by enhanced lipolysis and augmented plasma FFA flux to the portal circulation, which is connected directly to the liver. Insulin resistance has been shown to be exacerbated by an increased supply of FFA to peripheral tissue and the liver. *In vitro* studies have shown that palmitate exposure caused dose-dependent reduction in cell-surface insulin receptor binding of isolated hepatocytes and, further, was associated with a proportionally diminished receptor-mediated internalization and with decreased intracellular and total receptor-mediated insulin degradation. This phenomenon may contribute to the reduced hepatic insulin extraction and peripheral hyperinsulinemia.

Increased FFA influx in the liver from accumulated visceral fat may contribute to the enhancement of lipoprotein secretion as well as lipoprotein formation. Recent studies indicate that microsomal triglyceride transfer protein plays a key role in the lipoprotein assembly of apolipoprotein B and lipids to form very-low-density lipoprotein in the liver. This process is considered to be a limiting factor for very-low-density lipoprotein secretion from the liver to plasma. Our group reported that FFA enhance mRNA expression of microsomal triglyceride transfer protein in the liver, which suggests that increased FFA influx to the liver from visceral fat may contribute increased very-low-density lipoprotein formation and secretion, and results in hyperlipidemia.[18]

In contrast to the observation on FFA metabolism, the fate of glycerol, another metabolite of triglyceride lipolysis, has not been well investigated.

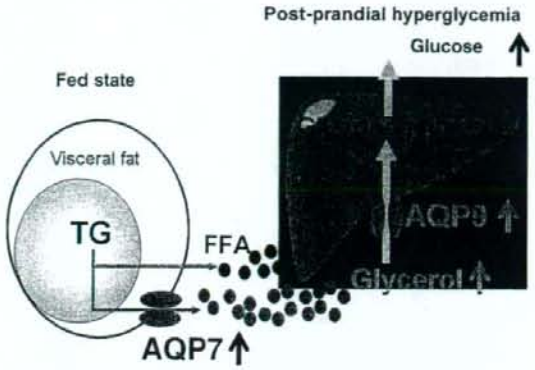

Figure 7 Dysregulation of aquaporin adipose or aquaporin 7, glycerol transporter may contribute hyperglycemia in visceral obesity.[20]

We have been investigating biological and genetic characteristics of adipose tissue comparing subcutaneous fat with visceral fat as well as adipose tissue, or adipocytes with other mesenchymal cells to develop a comprehensive analysis of expressed genes in adipocytes. This research line, called the Body Map Project, compares the profile of expressed genes in adipocytes with the expressed genes in other mesenchymal cells.[19] In this research, we discovered an adipose tissue-specific water channel named aquaporin adipose or aquaporin 7.[20] Aquaporin adipose has been found to play an important role in membrane transport of glycerol. Aquaporin adipose mRNA is sensitive to feeding and fasting, in that fasting remarkably enhances the expression and feeding suppresses it. This regulation causes increased glycerol release from adipose tissue during fasting in order to supply glycerol from visceral fat to the liver to maintain hepatic glucose production. Insulin has a major role in the suppression of aquaporin mRNA at feeding.[21] However, mRNA expression of aquaporin adipose in visceral fat is enhanced in obese model animals, such as the db/db mouse, and the suppression by feeding does not occur, resulting in the increase of portal glycerol influx to the liver even at feeding stage (Figure 7). From these results, it can be speculated that the physiological roles of aquaporin adipose in maintaining blood glucose levels, thus preventing hypoglycemia in the fasting stage by transporting glycerol, which is one of the products of lipolysis in visceral adipose tissue, to the liver. However, in visceral obesity, suppression system by insulin at feeding stage is impaired and excess glycerol influx to the liver may cause hyperglycemia, especially at post-prandial stage.

The concept of adipocytokines

In the Body Map Project, we analyzed the profile of expressed gene groups classified by their function or subcellular localization. As shown in Figure 8, adipocytes abundantly express the genes encoding secretory proteins, most of which are important bioactive substances such as

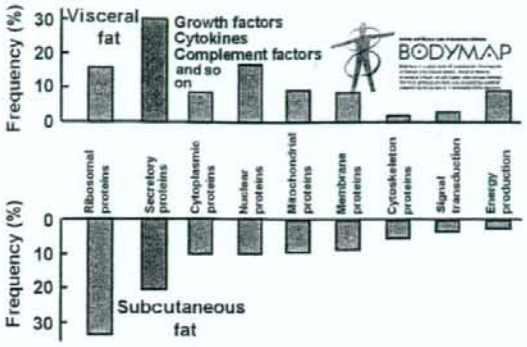

Figure 8 Gene expression profile of human adipose tissues.

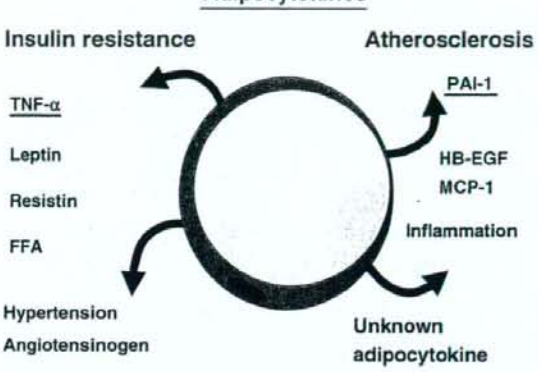

Figure 9 Representative adipocytokines related to pathophysiology and of pathogenesis of metabolic syndrome.

growth factors, cytokines and complements.[19] We named these adipose tissue-derived bioactive substances as adipocytokines (or adipokines).[22] Figure 9 shows representative adipocytokines that are related to pathophysiology and pathogenesis of metabolic syndrome. The secretion of these adipocytokines increases in obesity, especially with visceral fat accumulation. For example, plasma levels of plasminogen activator inhibitor 1 have been shown to correlate to visceral adiposity in humans. Animal experiments using ventromedial hypothalamus-lesioned rats revealed that mRNA levels of plasminogen activator inhibitor 1 in visceral fat, but not in subcutaneous fat, were enhanced in accordance with the development of obesity.[23] These data suggest that increased plasminogen activator inhibitor 1 levels caused by visceral fat accumulation may be one cause of vascular disease in the metabolic syndrome.

Discovery of adiponectin

In the Body Map Project, we identified several novel adipocytokines in addition to known bioactive substances.

Among them, adiponectin may be the most important, because this collagen-like protein has potent preventive properties against various common diseases. Adiponectin was expressed most abundantly among whole adipose tissue-expressed genes, and so we named the gene as *'adipose most abundant gene transcript-1' (apM-1)*.[24] The protein encoded by *apM-1* was named 'adiponectin' because this collagen-like protein has a characteristic of matrix protein such as fibronectin. The adiponectin gene expresses only in adipose tissue. It has collagen repeat and C1q-like globular structure, and in plasma is present in a very unique structure with a polymerized bouquet-like form. Just after the discovery of adiponectin from human adipose tissue, we developed an assay system, which has shown that adiponectin plasma levels are highly significant for a variety of diseases. It should be noted that plasma levels of adiponectin are extremely high, amounting to 10–15 $\mu g\,ml^{-1}$ in normal individuals, which far exceeds the typical levels of hormones or cytokines. Its plasma levels are strongly negatively correlated to visceral adiposity, but not to subcutaneous adiposity.[25]

The mechanism by which plasma levels are reduced in individuals with visceral fat accumulation is not yet clarified. However, co-culture with visceral fat inhibits adiponectin secretion from subcutaneous adipocytes. This finding suggests that some inhibiting factors for adiponectin synthesis or secretion are secreted from visceral adipose tissue. Tumor necrosis factor-α (TNF-α) was reported to be a strong inhibitor of the adiponectin promoter activity. The negative correlation between visceral adiposity and adiponectin levels might be explained by the increased secretion of TNF-α from accumulated visceral fat as at least one mechanism.[26]

Plasma adiponectin concentrations are lower in people who have type 2 diabetes mellitus than in body mass index-matched controls. The plasma concentrations have been shown to correlate strongly with insulin sensitivity, which suggests that low plasma concentrations are related to insulin resistance. In a study of Pima Indians, individuals with high levels of adiponectin were less likely to develop type 2 diabetes than those with low concentrations. High adiponectin concentration was, therefore, a notable protective factor against development of type 2 diabetes.[27]

Plasma levels of adiponectin are also decreased in hypertensive humans, irrespective of the presence of insulin resistance. Endothelium-dependent vasoreactivity is impaired in people with hypoadiponectinemia, which might be at least one mechanism of hypertension in visceral obesity.

Most importantly, plasma concentrations of adiponectin are lower in people with coronary heart disease than in controls, even when body mass index and age are matched. The Kaplan–Meier analysis in Italian individuals with renal insufficiency showed that those with high adiponectin concentrations were free from cardiovascular death for a longer time than other groups.[28] A case–control study performed in Japan showed that the group with the plasma

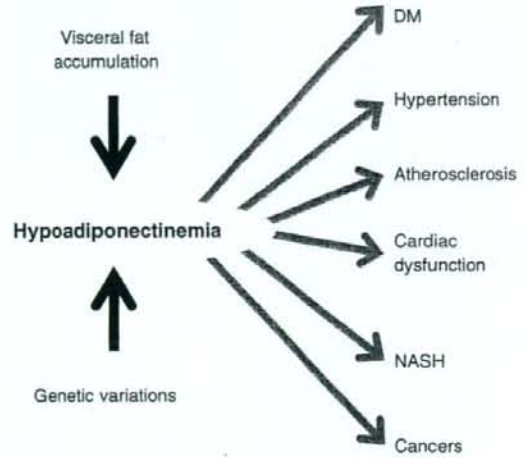

Figure 10 Hypoadiponectinemia and a variety of diseases.

levels $<4\,\mu g\,ml^{-1}$ has been shown to have an increased risk of coronary artery disease and multiple metabolic risk factors, which indicates that hypoadiponectinemia is a key factor in the metabolic syndrome.[29] A prospective study also confirmed that high adiponectin concentrations are associated with reduced risk of acute myocardial infarction in men. In addition to hypoadiponectinemia accompanied by visceral fat accumulation, genetic hypoadiponectinemia caused by a missense mutation has been reported, which also exhibits the clinical phenotype of metabolic syndrome[30] (Figure 10).

These clinical evidences show that hypoadiponectinemia is a strong risk factor for cardiovascular disease.[30]

Adiponectin as a potent anti-inflammation adipocytokine

As already mentioned, adiponectin has multiple functions for prevention of metabolic diseases and cardiovascular diseases. More recently, adiponectin was shown to prevent liver fibrosis and some kinds of cancer such as endometrial cancer, breast cancer, liver cancer and colon cancer.[31] In addition, recent evidences support its strong anti-inflammatory function. We first reported that adiponectin suppresses the production of the potent pro-inflammatory cytokine TNF-α, in macrophages.[32] Treatment of cultured macrophages with adiponectin significantly inhibits their phagocytic activity and their lipopolysaccharide-induced production of TNF-α. Suppression of phagocytosis by adiponectin is mediated by one of the complement C1q receptors, C1qRp, because this function was completely abrogated by the addition of an anti-C1qRp monoclonal antibody. These observations suggest that adiponectin is an important negative regulator in immune and inflammation systems, indicating that it may be involved in controlling inflammatory responses through its inhibitory functions.

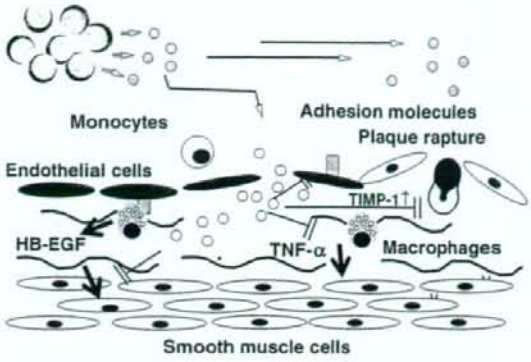

Figure 11 Mechanism of antiatherogenicity of adiponectin.

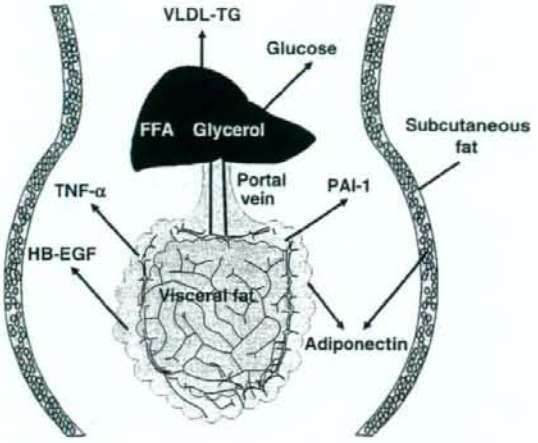

Figure 12 Visceral fat accumulation causes hyperlipidemia and hyperglycemia through FFA and glycerol and also dysregulation of adipocytokine secretion.

In the process of development of atherosclerosis, macrophages play crucial roles in plaque formation but adiponectin attenuates cholesterol ester accumulation in them. The adiponectin-treated macrophages contained fewer lipid droplets stained by oil red O. Furthermore, adiponectin suppresses the expression of the class A macrophage scavenger receptor at both mRNA and protein levels by northern and immunoblot analyses, respectively, without affecting the expression of CD36, which was qualified by flow cytometry.[33] Adiponectin and TNF-α interactions are numerous. Adiponectin inhibited the TNF-α-induced mRNA expression of monocyte adhesion molecules without affecting the interaction between TNF-α and its receptors in human aortic endothelial cells. Adiponectin suppressed the TNF-α-induced inducible nuclear factor kappaB (IκB) α phosphorylation and subsequent nuclear factor-κB activation without affecting other TNF-α-mediated signals, including Jun N-terminal kinase, p38 kinase and Akt kinase. This inhibitory effect of adiponectin is accompanied by cAMP accumulation and is blocked by either adenylate cyclase inhibitor or protein kinase A inhibitor.[34] These observations suggest that adiponectin, which is naturally present at high levels in the blood stream, modulates the inflammatory response of both macrophages and endothelial cells through cross-talk between cAMP–protein kinase A and nuclear factor-κB signaling pathways. The anti-inflammatory function of adiponectin may prevent atherogenic cell phenomena, such as monocyte adhesion to endothelial cells, differentiation of monocytes to macrophages and, finally, foam cell formation.[35]

Current studies have shown that adiponectin also induces various anti-inflammatory cytokines, such as interleukin-10 (IL-10) or IL-1 receptor antagonists, and IL-10 is a biomarker for risk in acute coronary syndrome.[36] Acute coronary syndrome determines the prognosis of cardiovascular disease, in which plaque vulnerability is the important determinant of plaque rupture. In the process of plaque rupture, matrix metalloproteinase secreted by macrophages is considered to increase plaque vulnerability.Tissue inhibitor of metalloproteinase (TIMP) appears to protect against plaque rapture by inhibition of matrix metalloproteinase activity. Adiponectin increases the expression of mRNA and protein production of TIMP in macrophages.[37] Before the induction of TIMP formation and secretion, adiponectin has been shown to induce IL-10 synthesis in macrophages, suggesting that adiponectin induces TIMP formation and secretion through the induction of IL-10 synthesis in an autocrine manner in macrophages, thus possibly inhibiting matrix metalloproteinase activity.[34]

These functions may result in the prevention of acute coronary syndrome. As shown earlier, adiponectin is