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ApoA-I Facilitates ABCA1 Recycle/Accumulation to Cell Surface by Inhibiting Its Intracellular Degradation and Increases HDL Generation

Rui Lu, Reijiro Arakawa, Chisato Ito-Osumi, Noriyuki Iwamoto, Shinji Yokoyama

Objective—Calpain-mediated proteolysis is one of the major regulatory factors for activity of ATP-binding cassette transporter (ABC) A1. Helical apolipoproteins protect ABCA1 against this degradation and increase generation of HDL. We investigated the mechanism for this reaction focusing on roles of endocytotic internalization of ABCA1.

Methods and Results—Surface ABCA1 was labeled with biotin and traced for its internalization and degradation. ABCA1 in the cell surface was internalized within 10 minutes regardless of the presence of apoA-I. ABCA1 was intracellularly degraded and was protected against this only when exposed to extracellular apoA-I before its endocytosis. Consequently, recycle of ABCA1 to the surface was enhanced, and surface ABCA1 was increased by apoA-I. Direct inhibition of ABCA1 endocytosis led to decrease of its degradation and increase of surface ABCA1. Generation of HDL increased in parallel with surface ABCA1.

Conclusion—Surface ABCA1 is internalized and degraded, and apoA-I interferes with only the latter step to recycle ABCA1 to the surface. Increase of surface ABCA1 results in the increase of generation of HDL. **(*Arterioscler Thromb Vasc Biol.* 2008;28:1820-1824)**

Key Words: ABCA1 ■ calpain ■ HDL ■ endocytosis ■ apoA-I ■ cholesterol

High density lipoprotein (HDL) is biogenerated with helical apolipoproteins and cellular lipid being mediated by the membrane protein ATP-binding cassette transporter (ABC) A1.[1] Helical apolipoprotein, mainly apolipoprotein A-I (apoA-I), is delivered and liberated by plasma HDL to somatic cells that do not synthesize apolipoproteins for biogenesis of new HDL,[2] whereas apoA-I is likely secreted from hepatocytes in a free form and interacts with its own ABCA1 in an autocrine manner.[3] HDL particles are formed with apolipoprotein and membrane phospholipid, and cholesterol is integrated into this particle being dependent on various cellular factors.[4,5] ABCA1 expression is upregulated mainly by the liver X receptor as sensing a cellular oxysterol level,[6] but it is also under the regulation by sterol regulatory element binding protein 2 in the liver perhaps to properly maintain whole body cholesterol homeostasis.[7] Interestingly, it is downregulated by activator protein 2α,[8] but its physiological role is unknown. On the other hand, ABCA1 is rapidly degraded by calpain and it seems an important regulation system for its activity of generation of HDL.[9] This proteolytic degradation is retarded when ABCA1 interacting with helical apolipoproteins[9,10] suggesting that this is a positive feedback system for HDL biogenesis. This must be a steady state ongoing in vivo in most of the cells that are chronically exposed to helical apolipoproteins such as apoA-I, although this view is yet to be proven for its relevance. It was proposed that ABCA1 is internalized by endocytosis and seems recycled,[11,12] and HDL biogenesis is associated with such endocytotic reactions.[13–16] Involvement of adaptor proteins is also suggested in the endocytosis and degradation of ABCA1.[17,18] Deletion of PEST sequence of ABCA1 inhibited its endocytosis, degradation by calpain, and HDL biogenesis, suggesting that endocytosis of ABCA1 is a key process for these all.[19] However, there are other views that HDL biogenesis takes place rather in the cell surface[20–22] than in the endosomes, where ABCA1 is entrapped. Most of these studies were carried out with ABCA1 transfected and overexpressed. In this work, we attempted to understand this complicated process by labeling the endogenous ABCA1 in cell surface and tracing it.

Materials and Methods

Cell Culture and ApoA-I

BALB/3T3 clone A31 cells were maintained and incubated in 5% Eagle minimum essential medium (MEM) with low glucose and 30% Ham F-12 (Wako) with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2.[23] THP1 cells were differentiated to macrophages by incubating in 10% FCS in the presence of 3.2×10^{-7} mol/L of phorbol 12-myristate 13-acetate (PMA; Wako)

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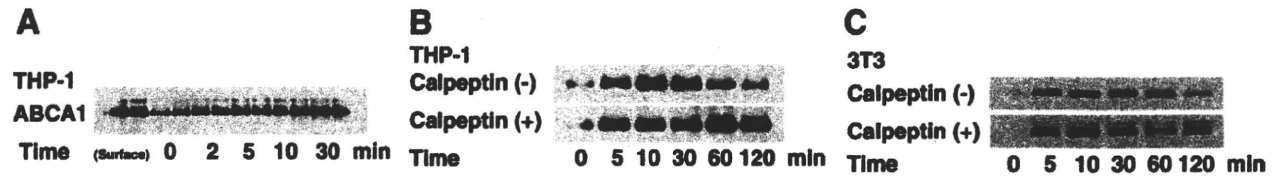

Figure 1. Internalization of ABCA1. A, Cell surface protein of the differentiated THP-1 cells was pulse-labeled with sulfo-SS-biotin. After further incubation at 37°C for the indicated time, surface biotin was cleaved to detect ABCA1 internalized. Biotinylated protein was selectively adsorbed by streptavidine-agarose and analyzed by Western blotting for ABCA1. Surface ABCA1 indicates the samples just after the biotinylation. B, Internalized-ABCA1 was detected in the presence or absence of 100 μmol/L of calpeptin in the same condition as in A. C, Internalization of ABCA1 was analyzed in BALB/3T3 cells by the same procedure as described above.

for 24 hours.[23] Human apoA-I was isolated from human plasma HDL as described previously.[24] It was used at 10 μg/mL in the medium for all the experiments unless otherwise specified.

Labeling and Tracing ABCA1

Cell surface proteins were biotinylated with sulfosuccinimidyl 2-(biotinamido)-rthyl-1, 3-dithiopropionate (sulfo-SS-biotin) (Pierce) for 1 hour at 4°C according to the methods previously reported.[25] After quenching the reaction, cells were washed and lysed, and the membrane fraction was prepared as described previously.[9] Biotinylated membrane proteins were isolated by incubating with streptavidin-agarose beads (Sigma) at 4°C for 1 hour.[26] After recovering the beads, proteins bound to the beads were eluted by incubating in the sample buffer for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzed for Western immunoblotting by using specific antibody against ABCA1 as previously described.[9] To trace the labeled surface ABCA1 for internalization, biotinylation of the cell surface proteins was cleaved by incubating the cells with 50 mmol/L reduced glutathione (Sigma) in pH 7.8 three times for 20 minutes,[26] and the remaining biotinylated ABCA1 was analyzed as above as the internalized portion. Intracellular ABCA1 degradation was measured as the time-dependent decrease of biotinylated ABCA1 after the surface biotin was cleaved after the incubation of the biotinylated cells for 1 hour at 37°C. To examine recycle of ABCA1, intracellular ABCA1 was prelabeled as above. At the various period of the incubation, the cell surface biotin was cleaved again and the remaining biotinylated ABCA1 was analyzed and compared with the biotinylated ABCA1 without the second cleavage to estimate the resurfaced ABCA1.

Cellular Lipid Release

Cellular lipid release by apoA-I was measured as described elsewhere. After incubation of the cells with apoA-I for the indicated time, concentration of cholesterol and choline-phospholipid in the medium were evaluated by enzymatic measurement.[27]

Quantification of Western Blotting Results

The bands were digitally scanned by using an EPSON GT-X700 and analyzed with Adobe Photoshop software.

Results

Figure 1A shows time-dependent internalization of ABCA1 in THP-1 cells. Most of the biotinylated ABCA1 in the surface was recovered as the internalized protein after incubating the cells at 37°C longer than 10 minutes, by cleaving the surface biotinylation with glutathione after the incubation. The internalized ABCA1 apparently decreased after 30 minutes of incubation, and this decrease was inhibited by a calpain inhibitor, calpeptin, both in THP-1 cells and BALB/3T3 cells (Figure 1B and 1C). ABCA1 was thus shown degraded by calpain after the internalization.

Degradation of ABCA1 by calpain was shown inhibited by helical apolipoproteins, such as apoA-I.[9] Therefore, the internalization of ABCA1 was examined in the presence of apoA-I. After 10 minutes of the incubation, most of the surface-labeled ABCA1 was internalized regardless of the presence of apoA-I (Figure 2A). This process was not modified any further even by the presence of calpeptin, indicating that ABCA1 was protected by apoA-I against the calpain-mediated degradation (Figure 2B). To investigate whether ABCA1 is "preprotected" by apoA-I before its internalization or extracellular apoA-I protects ABCA1 even after it is internalized, degradation of the internalized ABCA1 was examined for timing of adding apoA-I (Figure 2C). When apoA-I was present in the medium for the period before the internalization of the prebiotinylated surface ABCA1, degradation of ABCA1 was retarded (apoA-I (+)). However, when apoA-I was added to the medium after the prelabeled ABCA1 was internalized, the degradation was not much retarded (Chased). This result indicates that the protective effect of apoA-I on ABCA1 against its degradation is achieved before ABCA1 is internalized, and not by cell–apoA-I interaction to cause a distant effect on the internalized ABCA1.

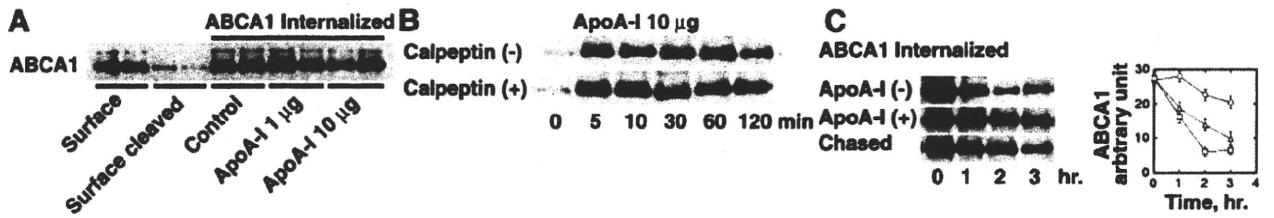

Figure 2. ABCA1 internalization and the effect of apoA-I in BALB/3T3 cells. A, ABCA1 internalization in the presence of apoA-I for 10 minutes at 37°C. "Surface" ABCA1, immediately after the labeling. "Surface cleaved", after biotin cleavage before internalization. B, ABCA1 internalization with apoA-I with and without calpeptin. C, Retardation of ABCA1 internalization by apoA-I. Cells were biotinylated and incubated for 1 hour at 37°C, and surface biotinylation was cleaved. Biotinylated ABCA1 was analyzed by Western blotting. ApoA-I (−) indicates without apoA-I throughout the incubation (squares); ApoA-I(+), with apoA-I in the preinternalization period (circles); Chased, apoA-I was added after the surface biotin cleavage (triangles).

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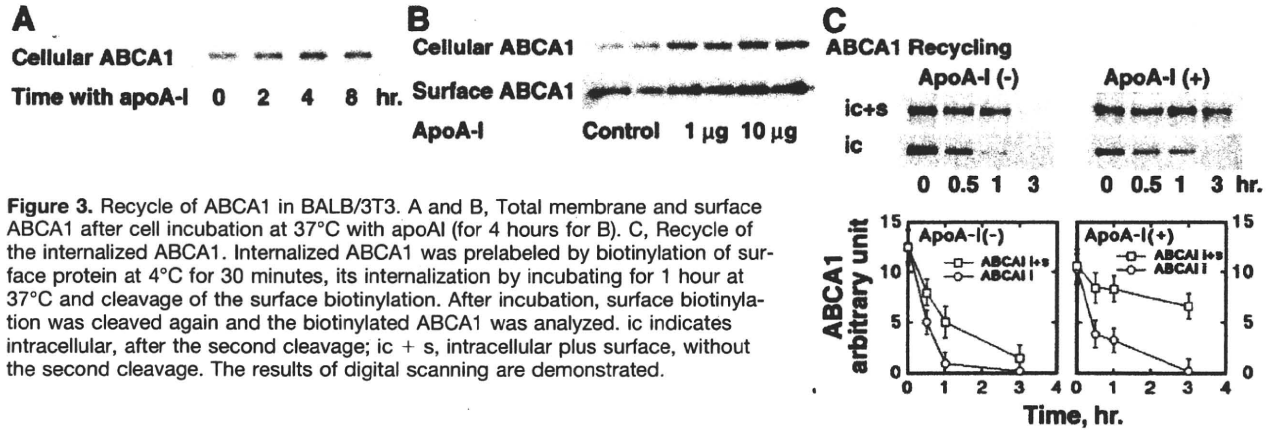

Figure 3. Recycle of ABCA1 in BALB/3T3. A and B, Total membrane and surface ABCA1 after cell incubation at 37°C with apoAI (for 4 hours for B). C, Recycle of the internalized ABCA1. Internalized ABCA1 was prelabeled by biotinylation of surface protein at 4°C for 30 minutes, its internalization by incubating for 1 hour at 37°C and cleavage of the surface biotinylation. After incubation, surface biotinylation was cleaved again and the biotinylated ABCA1 was analyzed. ic indicates intracellular, after the second cleavage; ic + s, intracellular plus surface, without the second cleavage. The results of digital scanning are demonstrated.

ABCA1 in the whole cell membrane increased up to 4 hours of the incubation, and this increase was parallel between the whole cell and cell surface (Figure 3A and 3B). To examine the mechanism for this increase of the surface ABCA1, recycle to the surface of the internalized ABCA1 was examined. After the prelabeled ABCA1 was internalized and surface biotinylation was cleaved, the cells were further incubated for certain periods of time and the surface biotinylation was cleaved again to assess the recycled ABCA1 to the surface (Figure 3C). In the absence of apoA-I, the internalized ABCA1 rapidly disappeared, and only its small portion was found recycled to the surface. In the presence of apoA-I, clearance of the internalized ABCA1 was substantially retarded as presented above, and a large portion of it was found recycled to the surface. Thus, apoA-I increased recycling of ABCA1 apparently by blocking the intracellular calpain-mediated degradation.

To examine whether internalization of ABCA1 is mandatory for the HDL biogenesis reaction, clathrin-mediated endocytosis was inhibited by cytochalasin D.[28,29] ABCA1 in the cell was decreased within 60 minutes in the absence of helical apolipoproteins when its synthesis was inhibited by cycloheximide (Figure 4A). When the endocytosis was inhibited by cytochalasin D, ABCA1 did not decrease. The increase of cellular ABCA1 by cytochalasin D was shown attributable to its increase in the cell surface (Figure 4B) as its endocytosis was strongly inhibited (Figure 4C).

Finally, generation of HDL was evaluated by measuring release of cellular phospholipid and cholesterol by apoA-I[5,30] when the endocytosis of ABCA1 was inhibited and its amount in the cell surface was increased. As shown in Figure 5, releases of phospholipid and cholesterol were both increased by this treatment. As apoA-I by itself increases surface ABCA1 by increasing its recycling, the increment of the HDL biogenesis should not be to the same extent as the increase of surface ABCA1 by cytochalasin D in the absence of apoA-I. This was in fact demonstrated in Figure 5B. The relative increase of the surface ABCA1 by cytochalasin D was to a less extent in the presence of apoA-I because the surface ABCA1 is already increased by apoA-I even in the absence of cytochalasin D. The increase of ABCA1 by cytochalasin D in the presence of apoA-I was parallel to the increase of lipid release by apoA-I (Figure 5C).

Discussion

In summary, we have shown that: (1) ABCA1 is rapidly degraded intracellularly by calpain after its clathrin-mediated endocytotic internalization in the absence of helical apolipoprotein; (2) Helical apolipoproteins, represented by apoA-I, protect ABCA1 from the degradation, not by inhibiting the internalization but by inhibiting the intracellular proteolysis; (3) This inhibition is achieved by preexposure of ABCA1 to extracellular apoA-I before the internalization and not by the exposure of the cells after ABCA1 is internalized;

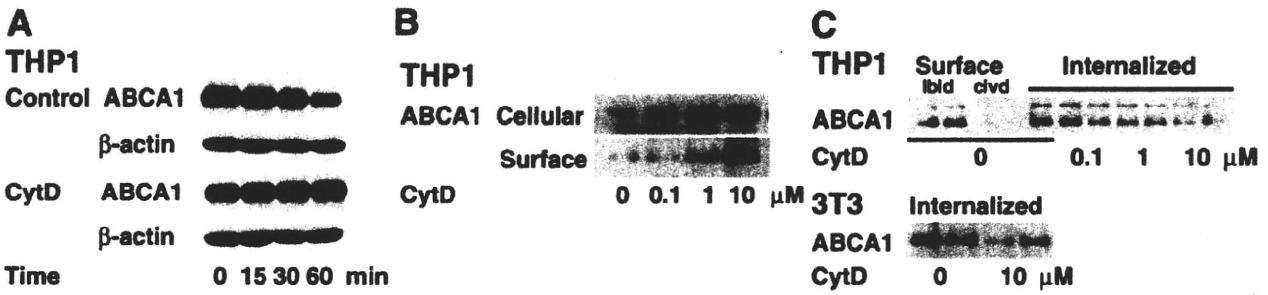

Figure 4. Effects of cytochalasin D on ABCA1 in differentiated THP-1 cells. A, Cells were incubated with 10 μmol/L cytochalasin D at 37°C and total membrane ABCA1 was analyzed. B, ABCA1 in total membrane and cell surface after incubation with cytochalasin D at 37°C for 30 minutes. C, ABCA1 internalization in the presence of cytochalasin D. Surface lbld indicates the biotinylated ABCA1 immediately after the labeling; Surface clvd, biotinylated ABCA1 after cleavage of surface biotin before the incubation at 37°C; Internalized, ABCA1 after the incubation at 37°C for 10 minutes and cleavage of the surface biotin. ABCA1 in BALB/3T3 cells were also analyzed for the internalization by the incubation for 10 minutes at 37°C.

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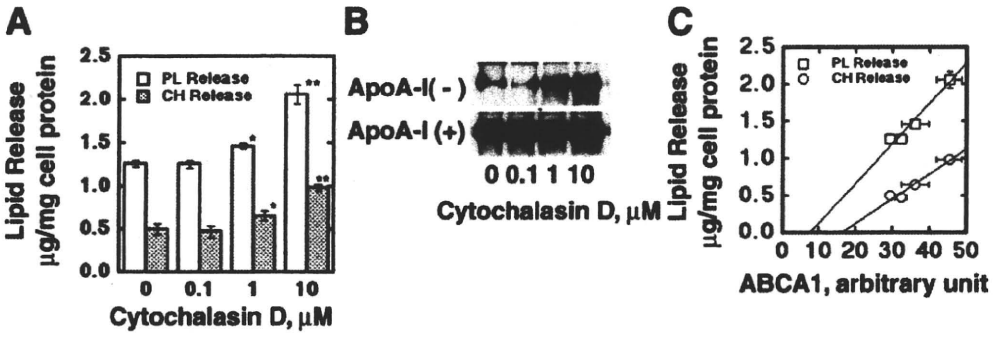

Figure 5. Effect of cytochalasin D on the apoAI-mediated HDL biogenesis. A, Differentiated THP-1 cells were incubated with apoA-I for 6 hours, and release of cholesterol and phospholipids was measured. The effect of cytochalasin D was examined in a dose-dependent manner. The data represent mean± SE for 3 measurements as $^{*}P<0.05$ and $^{**}P<0.01$ from the control. B, ABCA1 protein in cell surface in the absence and presence of apoA-I for 6 hours. C, The results of B were digitally scanned, and the lipid release data were plotted against the surface ABCA1.

(4) ABCA1 that escaped from the intracellular proteolysis is recycled to the cell surface, and apoA-I therefore enhances this process to increase cell surface ABCA1; (5) Generation of HDL is directly proportional to the surface ABCA1 level. The results are summarized in supplemental Figure I (available online at http://atvb.ahajournals.org).

It is well recognized that activity of ABCA1 is a rate-limiting factor for biogenesis of HDL and therefore plasma HDL concentration in vivo.[1] Expression of the gene has been shown to regulate it in vitro and in vivo,[6–8] but the degradation of ABCA1 protein seems an important regulatory factor for its activity as a posttranslational regulation at the cellular level,[9,10] whose physiological relevance, however, is yet to be proven.

We used THP-1 cells and BALB/3T3 fibroblasts as models for generation of HDL. HDL biogenesis in vivo is largely in the liver and intestine,[31] but any peripheral cells must carry on the HDL biogenesis reaction for their cholesterol homeostasis.[1] Indeed, it was proposed that peripheral tissue may be a significant source of plasma HDL in human.[32] Therefore, the use of these cells is justified to investigate mechanism for HDL biogenesis by the ABCA1/apolipoprotein system. ABCA1 seems stabilized in hepatocytes in an autocrine mechanism by a large amount of apoA-I produced and secreted by themselves, and the effects of additional apoA-I may not be apparent.[3]

When HDL generation is ongoing, helical apolipoproteins interact with ABCA1 before its internalization and make ABCA1 resistant to the calpain-mediated degradation. Consequently, a large portion of ABCA1 is recycled to the surface without degradation for further HDL generation. This view is consistent with most of the previous findings that apoA-I/ABCA1 complex recycles and apoA-I may be released by exocytosis during the HDL generation reactions.[11,12] Most of the cells in the body are chronically exposed to HDL, which liberates apoA-I for generation of HDL.[2] Therefore, in the physiological environment in vivo, ABCA1 seems protected from the degradation and its clearance rate should be rather slow. Recently, 2 independent articles proposed that HDL biogenesis by ABCA1 mainly takes place on cell surface rather than in the endosomes by tracing the labeled apoA-I in the presence of transfected ABCA1.[21,22] The conclusion in the present work is consistent with these proposals and may not agree with the view that HDL biogenesis occurs intracellularly.[13–16]

In the steady state of HDL generation, ABCA1 should be increased in cell surface from the baseline condition without apolipoprotein. It is therefore of interest whether there is a room for further increase of surface ABCA1 and consequently for the increase of HDL biogenesis by inhibiting the endocytotic internalization of ABCA1. Inhibition of the endocytosis by cytochalasin D increased surface ABCA1×50 to 60% as well as HDL biogenesis in parallel (Figure 5C).

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Disclosures

Shinji Yokoyama is involved in establishment of a Venture Company, HYKES Laboratories.

References

1. Yokoyama S. Assembly of high-density lipoprotein. *Arterioscler Thromb Vasc Biol.* 2006;26:20–27.
2. Okuhira K, Tsujita M, Yamauchi Y, Abe-Dohmae S, Kato K, Handa T, Yokoyama S. Potential involvement of dissociated apoA-I in the ABCA1-dependent cellular lipid release by HDL. *J Lipid Res.* 2004;45:645–652.
3. Tsujita M, Wu CA, Abe-Dohmae S, Usui S, Okazaki M, Yokoyama S. On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway. *J Lipid Res.* 2005;46:154–162.
4. Yamauchi Y, Abe-Dohmae S, Yokoyama S. Differential regulation of apolipoprotein A-I/ATP binding cassette transporter A1-mediated cholesterol and phospholipid release. *Biochim Biophys Acta.* 2002; 1585:1–10.
5. Hayashi M, Abe-Dohmae S, Okazaki M, Ueda K, Yokoyama S. Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7. *J Lipid Res.* 2005;46:1703–1711.
6. Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, Tontonoz P. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. *Proc Natl Acad Sci U S A.* 2000; 97:12097–12102.
7. Tamehiro N, Shigemoto-Mogami Y, Kakeya T, Okuhira K, Suzuki K, Sato R, Nagao T, Nishimaki-Mogami T. Sterol regulatory element-binding protein-2- and liver X receptor-driven dual promoter regulation of hepatic ABC transporter A1 gene expression: mechanism underlying the unique response to cellular cholesterol status. *J Biol Chem.* 2007;282: 21090–21099.
8. Iwamoto N, Abe-Dohmae S, Ayaori M, Tanaka N, Kusuhara M, Ohsuzu F, Yokoyama S. ATP-binding cassette transporter A1 gene transcription is downregulated by activator protein 2alpha. Doxazosin inhibits activator

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protein 2alpha and increases high-density lipoprotein biogenesis independent of alpha1-adrenoceptor blockade. *Circ Res.* 2007;101:156–165.
9. Arakawa R, Yokoyama S. Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. *J Biol Chem.* 2002;277:22426–22429.
10. Arakawa R, Hayashi M, Remaley AT, Brewer BH Jr, Yamauchi Y, Yokoyama S. Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. *J Biol Chem.* 2004;279:6217–6220.
11. Neufeld EB, Remaley AT, Demosky SJ, Stonik JA, Cooney AM, Comly M, Dwyer NK, Zhang M, Blanchette-Mackie J, Santamarina-Fojo S, Brewer HB Jr. Cellular localization and trafficking of the human ABCA1 transporter. *J Biol Chem.* 2001;276:27584–27590.
12. Neufeld EB, Stonik JA, Demosky SJ Jr, Knapper CL, Combs CA, Cooney A, Comly M, Dwyer N, Blanchette-Mackie J, Remaley AT, Santamarina-Fojo S, Brewer HB Jr. The ABCA1 transporter modulates late endocytic trafficking: insights from the correction of the genetic defect in Tangier disease. *J Biol Chem.* 2004;279:15571–15578.
13. Takahashi Y, Smith JD. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc Natl Acad Sci U S A.* 1999;96:11358–11363.
14. Chen W, Sun Y, Welch C, Gorelik A, Leventhal AR, Tabas I, Tall AR. Preferential ATP-binding cassette transporter A1-mediated cholesterol efflux from late endosomes/lysosomes. *J Biol Chem.* 2001;276:43564–43569.
15. Smith JD, Waelde C, Horwitz A, Zheng P. Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J Biol Chem.* 2002;277:17797–17803.
16. Hassan HH, Bailey D, Lee DY, Iatan I, Hafiane A, Ruel I, Krimbou L, Genest J. Quantitative analysis of ABCA1-dependent compartmentalization and trafficking of apolipoprotein A-I: Implications for determining cellular kinetics of nascent HDL biogenesis. *J Biol Chem.* 2008;283:11164–11175.
17. Munehira Y, Ohnishi T, Kawamoto S, Furuya A, Shitara K, Imamura M, Yokota T, Takeda S, Amachi T, Matsuo M, Kioka N, Ueda K. a1-syntrophin modulates turnover of ABCA1. *J Biol Chem.* 2004;279:15091–15095.
18. Okuhira K, Fitzgerald ML, Sarracino DA, Manning JJ, Bell SA, Goss JL, Freeman MW. Purification of ATP-binding cassette transporter A1 and associated binding proteins reveals the importance of beta1-syntrophin in cholesterol efflux. *J Biol Chem.* 2005;280:39653–39664.
19. Chen W, Wang N, Tall AR. A PEST deletion mutant of ABCA1 shows impaired identification and defective cholesterol efflux from late endosomes. *J Biol Chem.* 2005;280:29277–29281.
20. Witting SR, Maiorano JN, Davidson WS. Ceramide enhances cholesterol efflux to apolipoprotein A-I by increasing the cell surface presence of ATP-binding cassette transporter A1. *J Biol Chem.* 2003;278:40121–40127.
21. Faulkner LE, Panagotopulos SE, Johnson JD, Woollett LA, Hui DY, Witting SR, Maiorano JN, Davidson WS. An analysis of the role of a retroendocytosis pathway in ATP-binding cassette transporter (ABCA1) - mediated cholesterol efflux from macrophages. *J Lipid Res.* 2008;49:1322–1332.
22. Denis M, Landry YD, Aha X. ATP-binding cassette A1-mediated lipidation of apolipoprotein A-I occurs at the plasma membrane and not in the endocytotic compartment. *J Biol Chem.* 2008;283:16178–16186.
23. Arakawa R, Tamehiro N, Nishimaki-Mogami T, Ueda K, Yokoyama S Fenofibric acid, an active form of fenofibrate, increases apolipoprotein A-I-mediated high-density lipoprotein biogenesis by enhancing transcription of ATP-binding cassette transporter A1 gene in a liver X receptor-dependent manner. *Arterioscler Thromb Vasc Biol.* 2005;25:1193–1197.
24. Yokoyama S, Tajima S, Yamamoto A. The process of dissolving apolipoprotein A-I in an aqueous buffer. *J Biochem (Tokyo).* 1982;91:1267–1272.
25. von Boxberg Y, Wütz R, Schwarz U. Use of the biotin-avidin system for labelling, isolation and characterization of neural cell-surface proteins. *Eur J Biochem.* 1990;190:249–256.
26. Vagin O, Turdikulova S, Yakubov I, Sachs G. Use of the H,K-ATPase beta subunit to identify multiple sorting pathways for plasma membrane delivery in polarized cells. *J Biol Chem.* 2005;280:14741–14754.
27. Abe-Dohmae S, Suzuki S, Wada Y, Aburatani H, Vance DE, Yokoyama S. Characterization of apolipoprotein-mediated HDL generation induced by cAMP in a murine macrophage cell line. *Biochemistry.* 2000;39:11092–11099.
28. Jackman MR, Shurety W, Ellis JA, Luzio JP. Inhibition of apical but not basolateral endocytosis of ricin and folate in Caco-2 cells by cytochalasin D. *J Cell Sci.* 1994;107: 2547–2556.
29. Szaszi K, Paulsen A, Szabo EZ, Numata M, Grinstein S, Orlowski J. Clathrin-mediated endocytosis and recycling of the neuron-specific Na+/H+ exchanger NHE5 isoform. Regulation by phosphatidylinositol 3′-kinase and the actin cytoskeleton. *J Biol Chem.* 2002;277:42623–42632.
30. Hara H, Yokoyama S. Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J Biol Chem.* 1991;266:3080–3086.
31. Timmins JM, Lee JY, Boudyguina E, Kluckman KD, Brunham LR, Mulya A, Gebre AK, Coutinho JM, Colvin PL, Smith TL, Hayden MR, Maeda N, Parks JS. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest.* 2005;115:1333–1342.
32. Nanjee MN, Cooke CJ, Olszewski WL, Miller NE. Lipid and apolipoprotein concentrations in prenodal leg lymph of fasted humans. Associations with plasma concentrations in normal subjects, lipoprotein lipase deficiency, and LCAT deficiency. *J Lipid Res.* 2000;41:1317–1327.

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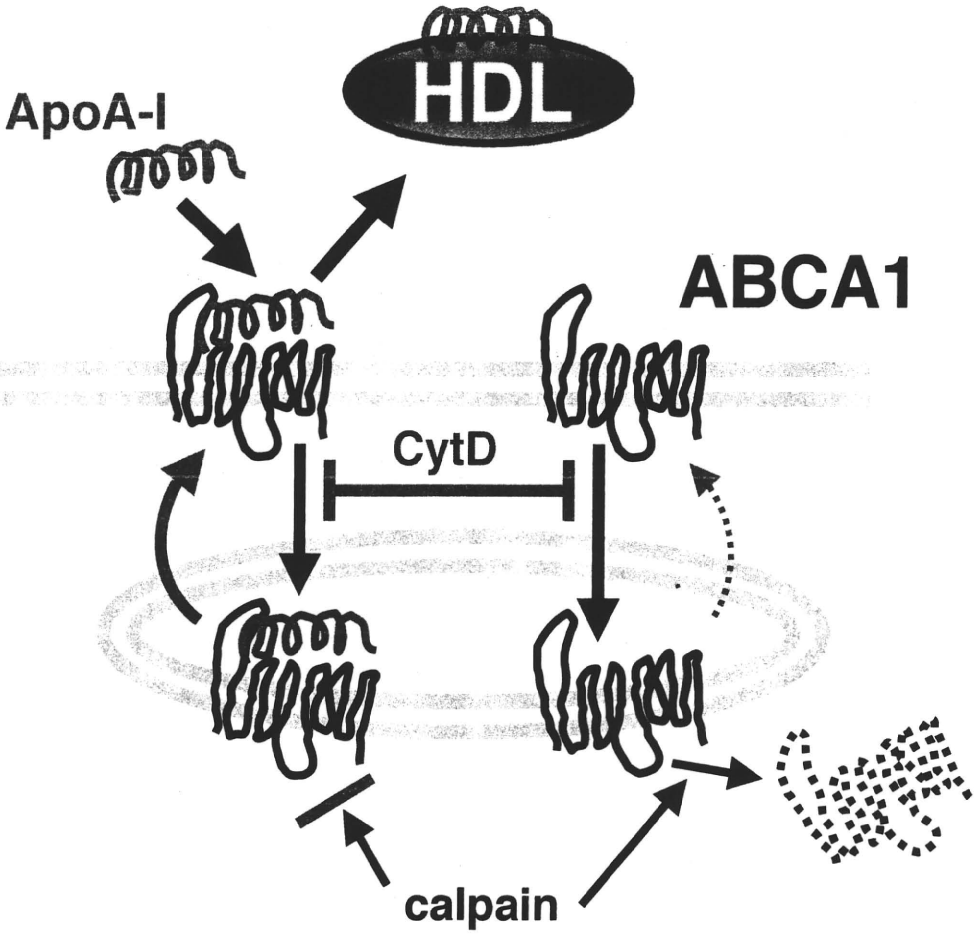

Supplementary Figure I. Schematic summary of the results. In the absence of apoA-I (right), ABCA1 is internalized and degraded by calpain and only very limited amount of ABCA1 could be recycled to the surface. Inhibition of calpain may lead to more recycle of ABCA1 to the surface. In the presence of apoA-I (left), ABCA1 is pre-protected by apoA-I in the surface against the intracellular calpain-mediated proteolysis. ABCA1 is therefore recycled to the surface. Inhibition of ABCA1 internalization by cytochalasin D (CytD) results in the increase of surface ABCA1. Surface ABCA1 is parallel to generation of HDL by apoA-I.

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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

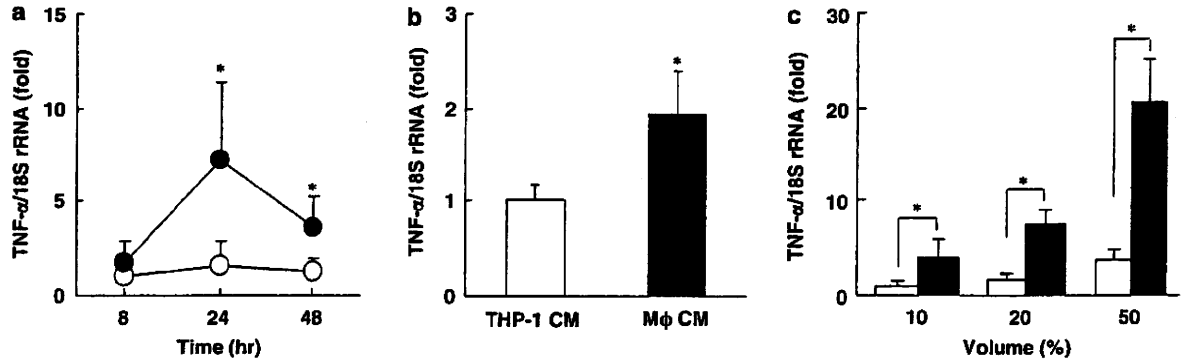

Figure 1 Mϕ-secreted factors increased the expression of tumor necrosis factor-α (TNF-α) in 3T3-L1 adipocytes. (a) Time course of the TNF-α expression in 3T3-L1 adipocytes co-cultured either with THP-1 cells (open circle) or Mϕ (filled circle). 3T3-L1 adipocytes were first seeded on the well bottom,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

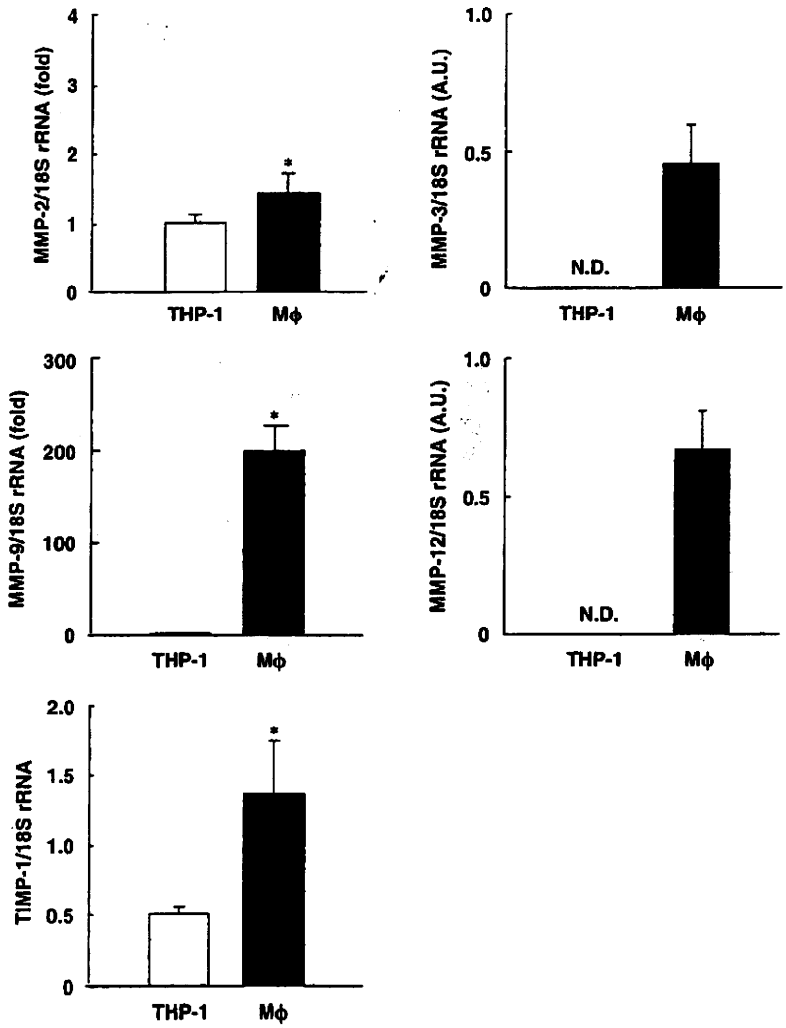

Figure 2 The expression of matrix metalloproteinase (MMP) family genes was induced in Mϕ. THP-1 cells were differentiated into Mϕ by incubating PMA for 72 h. MMP-2, MMP-3, MMP-9, MMP-12 and TIMP-1 mRNA levels were analyzed using quantitative real-time RT–PCR. Relative ratios of mRNA levels in Mϕ to those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

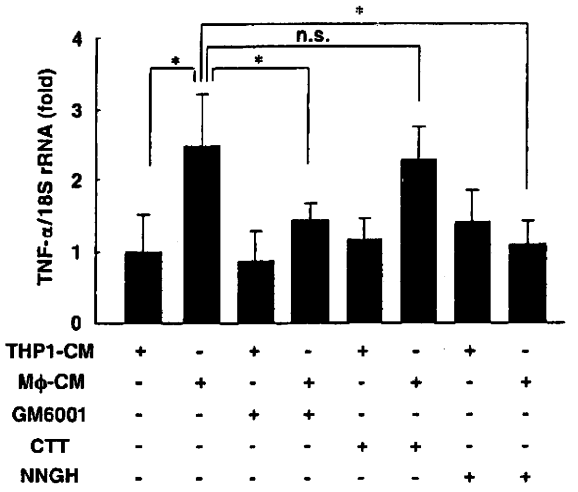

Figure 3 Effect of the inhibition of matrix metalloproteinase (MMP) activity on Mϕ-CM-induced tumor necrosis factor-α (TNF-α) gene expression in adipocytes. Serum-starved 3T3-L1 adipocytes were treated with THP-1-CM or Mϕ-CM in the absence or presence of 10 μM GM6001, 85 μM CTT or 60 μM NNGH for 4 h.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

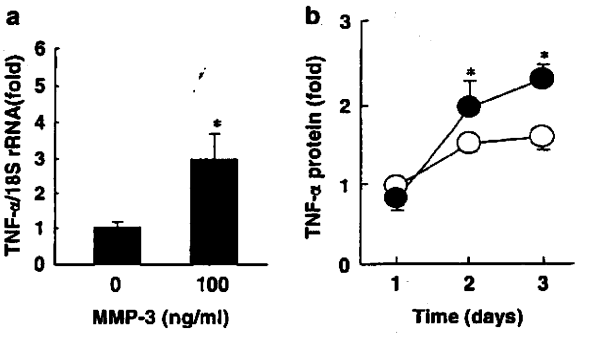

Figure 4 Matrix metalloproteinase (MMP)-3 induced the tumor necrosis factor-α (TNF-α) mRNA and protein expression in 3T3-L1 adipocytes. (a) Serum-starved 3T3-L1 adipocytes were treated with the active form of MMP-3 (100 ng ml^{-1}) for 8 h. The TNF-α mRNA levels were analyzed using quantitative real-time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

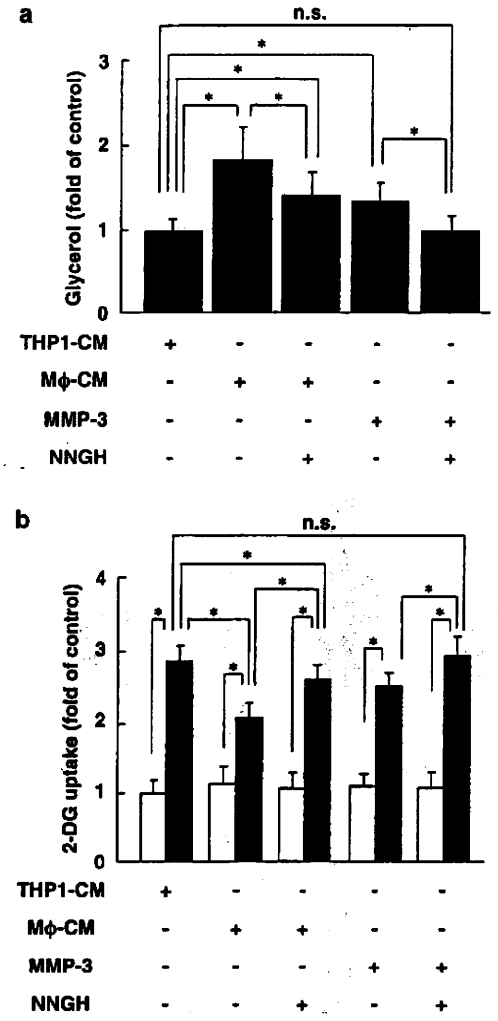

Figure 5 Effect of matrix metalloproteinase (MMP)-3 on lipolysis and insulin-induced glucose incorporation in 3T3-L1 adipocytes. (a) Glycerol release was measured in the media of 3T3-L1 adipocytes after treatment with 50%THP1-CM, 50% Mϕ-CM or 100 μg ml^{-1} human MMP-3, in the absence or presence of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

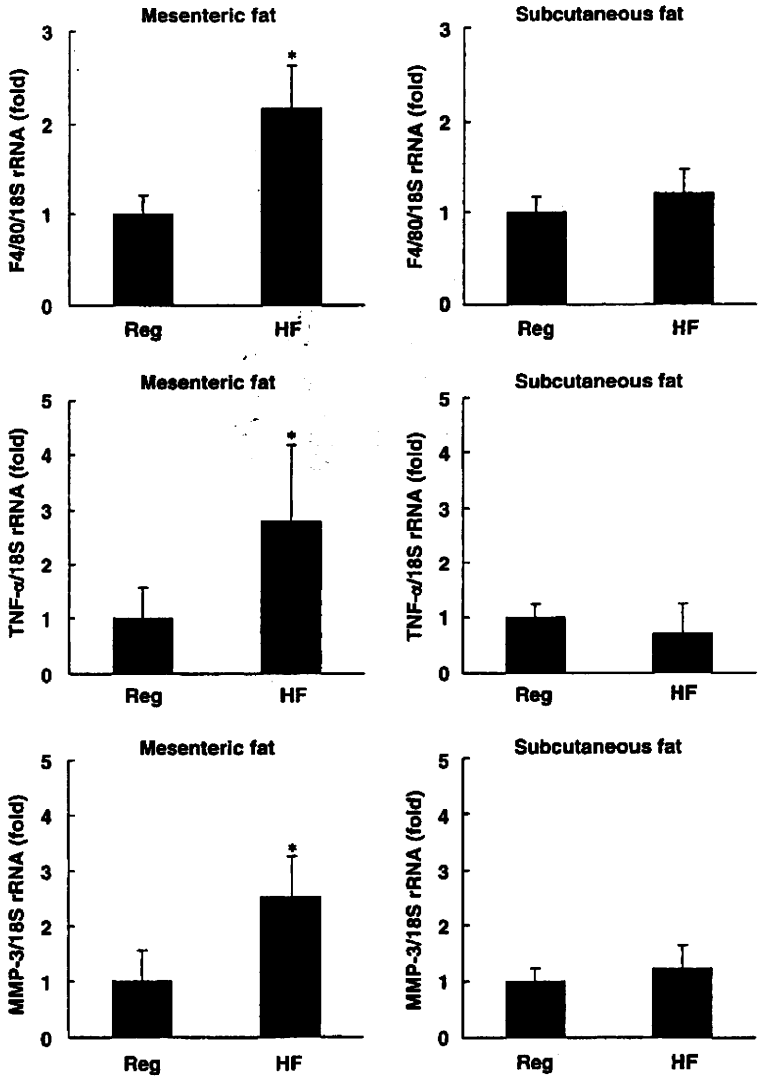

Figure 6 High fat induces F4/80, tumor necrosis factor-α (TNF-α) and matrix metalloproteinase (MMP)-3 mRNA expression in mesenteric fat. Mesenteric or subcutaneous fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. F4/80, TNF-α and MMP-3 mRNA levels were analyzed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

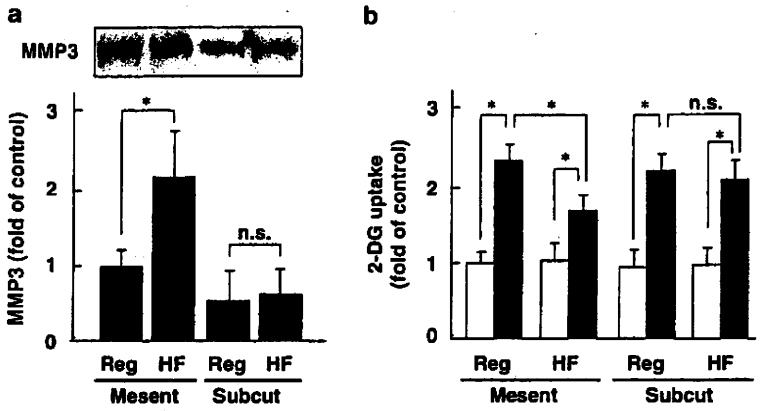

Figure 7 High fat induces matrix metalloproteinase (MMP)-3 protein expression, and reduces insulin-induced glucose incorporation of adipocytes in mesenteric fat. Mesenteric (Mesent) or subcutaneous (Subcut) fat tissues were prepared from C57BL/6 mice fed regular chow (Reg) or high-fat (HF) diet. (a)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

1 Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. *J Clin Endocrinol Metab* 2004; 89: 2595–2600.
2 Garg A. Regional adiposity and insulin resistance. *J Clin Endocrinol Metab* 2004; 89: 4206–4210.
3 Matsuzawa Y. Adiponectin: identification, physiology and clinical relevance in metabolic and vascular disease. *Atheroscler Suppl* 2005; 6: 7–14.
4 Pittas AG, Joseph NA, Greenberg AS. Adipocytokines and insulin resistance. *J Clin Endocrinol Metab* 2004; 89: 447–452.
5 Shibasaki M, Takahashi K, Itou T, Miyazawa S, Ito M, Kobayashi J *et al*. Alterations of insulin sensitivity by the implantation of 3T3-L1 cells in nude mice. A role for TNF-α? *Diabetologia* 2002; 45: 518–526.
6 Unoki H, Bujo H, Shibasaki M, Saito Y. Increased matrix metalloproteinase-3 mRNA expression in visceral fat in mice implanted with cultured preadipocytes. *Biochem Biophys Res Commun* 2006; 350: 392–398.
7 Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115: 1111–1119.
8 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; 112: 1796–1808.
9 Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ *et al*. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; 112: 1821–1830.
10 Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N *et al*. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 2006; 281: 26602–26614.
11 Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R *et al*. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 2006; 116: 1494–1505.
12 Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α. *Arterioscler Thromb Vasc Biol* 2005; 25: 2062–2068.
13 Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006; 116: 3015–3025.
14 Murakami K, Bujo H, Unoki H, Saito Y. High fat intake induces a population of adipocytes to co-express TLR2 and TNFα in mice with insulin resistance. *Biochem Biophys Res Commun* 2007; 354: 727–734.
15 Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A *et al*. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J Biol Chem* 2007; 282: 35279–35292.
16 Murakami K, Bujo H, Unoki H, Saito Y, Murakami K, Bujo H *et al*. Effect of PPARα activation of macrophages on the secretion of inflammatory cytokines in cultured adipocytes. *Eur J Pharmacol* 2007; 561: 206–213.
17 Hirata T, Unoki H, Bujo H, Ueno K, Saito Y. Activation of diacylglycerol O-acyltransferase 1 gene results in increased tumor necrosis factor-α gene expression in 3T3-L1 adipocytes. *FEBS Lett* 2006; 580: 5117–5121.
18 Shibasaki M, Bujo H, Takahashi K, Murakami K, Unoki H, Saito Y. Catalytically inactive lipoprotein lipase overexpression increases insulin sensitivity in mice. *Horm Metab Res* 2006; 38: 491–496.

19 Ohwaki K, Bujo H, Jiang M, Yamazaki H, Schneider WJ, Saito Y. A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates formation of lipid-laden macrophages. *Arterioscler Thromb Vasc Biol* 2007; 27: 1050–1056.
20 Khazen W, M'bika JP, Tomkiewicz C, Benelli C, Chany C, Achour A *et al.* Expression of macrophage-selective markers in human and rodent adipocytes. *FEBS Lett* 2005; 579: 5631–5634.
21 Cowherd RM, Lyle RE, McGehee Jr RE. Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* 1999; 10: 3–10.
22 Ishikawa K, Takahashi K, Bujo H, Hashimoto N, Yagui K, Saito Y. Subcutaneous fat modulates insulin sensitivity in mice by regulating TNF-α expression in visceral fat. *Horm Metab Res* 2006; 38: 631–638.
23 Bouloumié A, Sengenès C, Portolan G, Galitzky J, Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001; 50: 2080–2086.
24 Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E *et al.* Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 2003; 278: 11888–11899.
25 Maquoi E, Demeulemeester D, Voros G, Collen D, Lijnen HR. Enhanced nutritionally induced adipose tissue development in mice with stromelysin-1 gene inactivation. *Thromb Haemost* 2003; 89: 696–704.
26 Lijnen HR, Van HB, Frederix L, Rio MC, Collen D. Adipocyte hypertrophy in stromelysin-3 deficient mice with nutritionally induced obesity. *Thromb Haemost* 2002; 87: 530–535.
27 Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* 2006; 125: 577–591.
28 Traurig MT, Permana PA, Nair S, Kobes S, Bogardus C, Baier LJ. Differential expression of matrix metalloproteinase 3 (MMP3) in preadipocytes/stromal vascular cells from nonobese nondiabetic versus obese nondiabetic Pima Indians. *Diabetes* 2006; 55: 3160–3165.
29 Croissandeau G, Chretien M, Mbikay M. Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes. *Biochem J* 2002; 364: 739–746.
30 Morimoto Y, Nishikawa K, Ohashi M. KB-R7785, a novel matrix metalloproteinase inhibitor, exerts its antidiabetic effect by inhibiting tumor necrosis factor-alpha production. *Life Sci* 1997; 61: 795–803.
31 Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ *et al.* A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. *FASEB J* 2007; 21: 179–187.
32 Si-Tayeb K, Monvoisin A, Mazzocco C, Lepreux S, Decossas M, Cubel G *et al.* Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis. *Am J Pathol* 2006; 169: 1390–1401.

Opinion

Proposed Guidelines for Hypertriglyceridemia in Japan with Non-HDL Cholesterol as the Second Target

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The Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases, proposing management for LDL cholesterol as the primary target, have successfully contributed to the prevention of cardiovascular events; however, recently, the impact of hypertriglyceridemia as an additional cardiovascular risk has become understood, especially in light of the rise in obesity, metabolic syndrome, and diabetes in the Japanese population. Rather than waiting to obtain conclusive domestic data confirming that hypertriglyceridemia is a cardiovascular risk factor and that its management is efficacious, we propose guidelines for hypertriglyceridemia using non-HDL cholesterol as a second target.

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Key words; Hyperlipidemia, Dyslipidemia, Triglycerides, HDL cholesterol, LDL cholesterol

Introduction

Many prospective epidemiological studies have indicated a positive relationship between serum triglyceride (TG) levels and the incidence of coronary heart disease (CHD)[1, 2]. TG-rich lipoproteins such as remnant lipoproteins and small dense LDL particles are increased in hypertriglyceridemia and have been established to be atherogenic by numerous clinical and experimental studies[3-6]; however, classification of the plasma TG level as an independent risk factor for atherosclerosis has been controversial. This is partly because plasma TG levels are inversely intercorrelated by other well-established risk factors, such as low HDL cholesterol. To date, large scale trials for intervention targeting plasma TGs with TG reducing agents such as fibrates have not reached definitive conclusions about their effectiveness on primary endpoints, although fibrates have some impact on both primary and secondary prevention in small scale studies[7-9].

The precise estimation of plasma TGs as a cardiovascular risk is confounded by other risk factors, such as obesity, diabetes, hypertension and smoking. In addition, a cluster of metabolic risk factors, such as visceral obesity and insulin resistance with hypertriglyceridemia, referred to as metabolic syndrome, indicates that plasma TG concentrations are tightly linked to other strong risk factors for CHD. Thus, patients with elevated TGs are at increased risk for CHD, although greater risk cannot be independently explained by TGs. Meanwhile, recent meta-analyses suggested that plasma TGs could be an independent factor for CHD[1, 2]. Supportively, many experimental studies indicated that triglyceride-rich lipoproteins as well as LDL are atherogenic. Taken together, these data suggest that hypertriglyceridemia should be regarded as a semi-independent risk factor and should be included as a clinical target for the prevention of CHD. Considering the increasing prevalence of obesity, metabolic syndrome, and diabetes in this country, guidelines specialized for patients with hypertriglyceridemia need to be immediately established. In this study, we propose new guidelines for Japanese patients with hypertriglyceridemia

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Table 1. Plasma lipid profile of severe and mild type IIb hyperlipidemic patients sub-grouped by non-HDL cholesterol level

Male	severe type IIb non-HDLc >190 mg/dL	mild type IIb non-HDLc <190 mg/dL	p
n	51	54	
Total Cholesterol	270±41.8	234±40.3	0.001
Triglycerides	347±286	236±110	0.031
HDL Cholesterol	42.4±8.0	54.9±15.2	0.000
LDL Cholesterol	159±51.6	135±38.1	0.029
non-HDL Cholesterol	228±41.6	182±39.1	0.000

Female	severe type IIb non-HDLc >180 mg/dL	mild type IIb non-HDLc <180 mg/dL	p
n	52	48	
Total Cholesterol	265±29.6	231±20.2	0.000
Triglycerides	242±120	218±56	0.1
HDL Cholesterol	47.3±14.1	63.2±19.5	0.000
LDL Cholesterol	175±40.4	125±17.9	0.000
non-HDL Cholesterol	224±30.2	168±14.9	0.000

Subjects were patients who visited the outpatient clinic of the Endocrinology and Metabolism Unit of Tsukuba University Hospital on a regular basis (monthly or bimonthly) as described in Materials and Methods. Data are the means±SD (mg/dL).

using non-HDL as a secondary target after the goal for LDL cholesterol as the primary target is achieved.

Materials and Methods

A total of 1,124 patients in Tsukuba University hospital in 2006 were consecutively included in the study (**Table 1**). Patients with severe illness were excluded. Plasma total cholesterol (TC), LDL-C, TG, HDL-C, glucose and HbA1c in either the fasted or fed state were determined enzymatically with the Hitachi 7070. Plasma HDL-C concentration was measured by a direct method using polyethylene-glycoso-pretreated enzymes. We calculated LDL-C concentration with Friedewald's formula (TC-TG/5-HDL-C) when TG was less than 400 mg/dL. Plasma non-HDL-C concentration was calculated as TC−HDL-C. One hundred and five male and 100 female patients were diagnosed with Type IIb hyperlipidemia (TC>220 mg/dL and TG>150 mg/dL). They were subcategorized into two groups according to their non-HDL cholesterol level (**Table 1**).

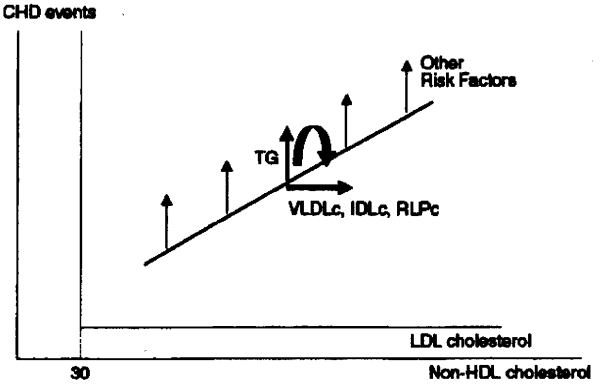

Fig. 1. Rationale for usage of non-HDL cholesterol: impact of TG and other risk factors on correlation between LDL-cholesterol CHD event.

nonHDL cholesterol = Total cholesterol − HDL cholesterol = VLDL cholesterol + IDL cholesterol (remnant lipoprotein cholesterol) + LDL cholesterol (Friedewald formula).
VLDL cholesterol + IDL cholesterol (RLP cholesterol) = TG/5
The risk of hypertriglyceridemia is approximated to VLDL, IDL, and RLP cholesterol estimated as TG/5, and incorporated into non-HDLC. The difference between non-HDL cholesterol and LDL cholesterol on X-axis was set up at 30 mg/dL based upon the data from Fig. 2.

Results and Discussion

Advantage of Non-HDL Cholesterol as a Marker for Hypertriglyceridemia

LDL cholesterol has been established as the most potent predictor of CHD and is currently the primary target for treatment and prevention. Other risk factors, including TG, diabetes, obesity, and metabolic syndrome, do not directly elevate plasma LDL cholesterol, but could enhance the risk of LDL cholesterol by shifting up the curve, as depicted in **Fig. 1**. To evaluate and manage the risk of hypertriglyceridemia, the TG level must be interpolated into the risk of plasma cholesterol. In patients with high TGs, most VLDL cholesterol resides in the smaller (remnant) VLDL fraction. Cholesterol of remnant lipoproteins (VLDL and IDL), which is concomitantly increased by elevation of plasma TG is an appropriate surrogate marker of hypertriglyceridemia. TG-rich remnant lipoproteins have been established as atherogenic lipoproteins[4, 5]. Thus, RLPc, a commercially available laboratory test for remnant lipoprotein cholesterol, could be a suitable marker for the atherogenicity of hypertriglyceridemia; however, this test is expensive and is not practical for use as a routine parameter. In contrast, non-HDL cholesterol, defined as total cholesterol − HDL cholesterol, is easily calculated, and represents the sum-

mation of VLDL/IDL (remnant) cholesterol and LDL cholesterol. It reflects the risks for all apoB-containing lipoproteins and could be an excellent marker for atherogenic lipoproteins. Plasma TG itself is not an appropriate marker for CHD risk due to its internal and dietary variability. In contrast, non-HDL cholesterol is not affected by dietary states and has much less daily variability than TG.

Predictive Power of Non-HDL Cholesterol

Non-HDL cholesterol reflects the risks of both hypertrigyceridemia and LDL-cholesterol[10, 11]. Several studies have indicated that non-HDL cholesterol is better than LDL cholesterol in its predictive power of cardiovascular diseases, indicating that VLDL cholesterol could contribute to CVD[12]. Non-HDL cholesterol is also a useful marker in a variety of subpopulations: men, the elderly, and patients with high-risk diseases such as diabetes and end-stage renal disease[13-16]. Our current clinical data from patients with type IIb hyperlipidemia also support the usefulness of non-HDL cholesterol (**Table 1**). In our outpatient clinic, 70% of patients had diabetes and roughly 10% were type IIb hyperlipidemia (cholesterol > 220 mg/dL and TG > 150 mg/dL). These type IIb hyperlipidemic patients were equally divided into two sub-groups: severe (non-HDL cholesterol levels ≥ 190 mg/dL for male patients and 180 mg/dL for female patients) and mild < 190 mg/dL for male patients and 180 mg/dL for female patients. When the severe and mild IIb groups were compared, total, LDL, HDL cholesterol, and TG levels were significantly different among these two groups for both genders, except for serum triglyceride in females (**Table 1**). These data indicate that non-HDL cholesterol is an excellent marker representing all the components of dyslipidemia. The usefulness of non-HDL cholesterol rather than low-density lipoprotein cholesterol as a tool for lipoprotein cholesterol screening and assessment of risk and therapy has been already recognized in the USA[17, 18]. Another candidate marker for both remnant and LDL cholesterol is plasma apoB level[19]. ApoB is a direct marker for the particle number of apoB-containing lipoproteins and reflects risks of both remnants and LDL. Non-HDL cholesterol is highly correlated with apoB, and should replace this specialized and expensive laboratory test despite some reports indicating that apoB is better than non-HDL cholesterol for the predictive power of CHD[13, 20].

However, according to the Friedewald formula, the TG risk in non-HDL cholesterol represents only one fifth of TG levels as remnant cholesterol, and thus, the contribution of the risk is relatively weak compared to that of LDL cholesterol. Our previous data indicated that the correlation of non-HDL cholesterol to LDL cholesterol was much stronger than that to the TG level (**Fig. 2**)[21]. It should be noted that non-HDL cholesterol is not a specific marker for hypertriglyceridemia. Rather, non-HDL cholesterol should be regarded as a general single marker for both hypercholesterolemia and/or hypertriglyceridemia.

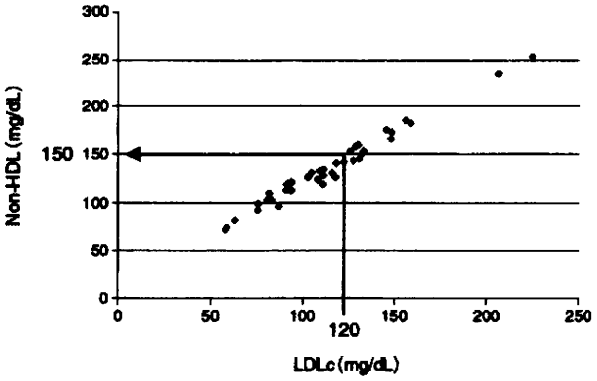

Fig. 2. Distribution of non-HDL cholesterol vs. calculated LDL cholesterol in normolipidemic patients.

Non-HDL cholesterol and LDL cholesterol calculated from Friedewald formula were highly correlated. Subjects were from the outpatient clinic of Tsukuba University Hospital[21].

Proposed Guidelines for Hypertriglyceridemia

Based upon these considerations, we propose guidelines for hypertriglyceridemia in Japanese patients using non-HDL cholesterol as a secondary target, as shown in **Table 2**. This is an extended version of the 2007 edition of the Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases in which LDL cholesterol is the primary marker and target. It is essentially similar to the AHA-ATPIII guidelines for hyperTG in USA[22]. ATPIII recommends using non-HDL cholesterol as a secondary target when plasma TG is greater than 200 mg/dL because VLDL cholesterol is not significantly accumulated if TG is less than 200 mg/dL[23]. We do not have enough clinical data for Japanese on the relationship between TG and VLDL cholesterol to provide the appropriate TG level where the use of a non-HDL marker should be considered. Currently, we recommend using non-HDL for patients with hypertriglyceridemia (TG > than 150 mg/dL). Even for patients with hypertriglyceridemia, the primary target is still LDL cholesterol. In the 2007 JAS guidelines, goals of LDL for the secondary prevention group and the primary prevention group with category I, II, and III are 100, 120, 140, and 160 mg/