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Familial massive tendon xanthomatosis with decreased high-density lipoprotein–mediated cholesterol efflux

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Abstract

We experienced a family with novel massive tendon xanthomatosis which can be excluded from known disease causing xanthomatosis. The proband was a 58-year-old man who had necrosis in his massive Achilles tendon xanthoma. Three of 5 brothers including him and his nephew had the same clinical phenotype. The systemic tendon xanthomatosis became apparent around 30 years of their age. The proband and his elder brother had mild elevations of serum total cholesterol level (251 and 228 mg/dL, respectively). The low-density lipoprotein receptor activity of the proband's lymphocytes was normal. Neither plant sterol nor cholestanol level was increased in the proband's plasma. Magnetic resonance image of the proband's Achilles tendon demonstrated a massive expansion of the soft tissue with salami sausage–like appearance in his heels (50 mm in thickness). The physiological function of macrophages (MΦ) from the patients was investigated to clarify the mechanism for the formation of xanthomatosis. There was no significant difference in the uptake of oxidized low-density lipoprotein between the proband's MΦ and the control. High-density lipoprotein 3–mediated cholesterol efflux from the patients' MΦ (n = 2) was significantly reduced compared with the controls (n = 3), whereas there was no difference in apolipoprotein (apo) A-I–mediated cholesterol efflux between the patients' MΦ and the controls. Furthermore, there was no reduction of the messenger RNA levels of ATP-binding cassette transporter 1 (ABCA1), which is involved in apo A-I–mediated cholesterol efflux, in the proband's MΦ compared with the control. The present study demonstrates that the mechanism for the formation of novel familial massive tendon xanthomatosis may be, at least in part, associated with decreased high-density lipoprotein 3, but not free apo A-I–mediated cholesterol efflux from MΦ in vivo.
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1. Introduction

Macrophages (MΦ) are known to have a variety of physiological activities in vivo such as phagocytosis and immunologic defense to pathogens to maintain homeostasis in the body. Any dysfunction of MΦ may cause serious damages in the hosts such as increased susceptibility to some infections and atherosclerotic cardiovascular diseases. From the viewpoint of the development of atherosclerosis, MΦ express scavenger receptors and possess the ability to transform into foam cells in vivo. It is of great importance to elucidate the mechanism for the foam cell formation because it would provide some clues for establishing a novel strategy for treatment of atherosclerotic cardiovascular diseases.

Several disorders are known to manifest systemic xanthomatosis. It is needless to say that patients with homozygous familial hypercholesterolemia are characterized by the presence of massive xanthomatosis and premature coronary heart disease. Cerebrotendinous xanthomatosis is a rare autosomal recessive disorder, of which primary defect is the mutations in the 27-α hydroxylase gene, and is characterized by accumulation of cholestanol in plasma [1]. Patients with β-sitosterolemia also have tendon xanthomas, of which primary defect is the gene mutation of ATP-binding cassette transporter, subfamily G (ABCG5 and/or ABCG8) [2]. Necrobiotic xanthogranuloma is 1 of the diseases with systemic skin xanthomatosis [3]. We have reported that peripheral monocytes from a patient with necrobiotic xantho-

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granuloma have gained an ability to take up lipoprotein-derived lipids [4].

We recently experienced a family with massive tendon xanthomatosis which can be excluded from known diseases causing xanthomatosis as described above. We investigated the physiological function of the MΦ from the patients to clarify the mechanism for the formation of xanthomatosis. In the present study, we demonstrate that the impaired high-density lipoprotein 3 (HDL3)–mediated, but not free apolipoprotein (apo) A-I–mediated, cholesterol efflux from their MΦ may be related to the appearance of xanthomatosis observed in this case.

2. Materials and methods

2.1. Isolation and modification of lipoproteins

Blood samples were collected into tubes with or without EDTA-2Na (final concentration, 1 mg/mL) after 12 hours of fasting. After plasma or serum was recovered by centrifugation at 3000 rpm at 4°C, protease inhibitors were added at the following final concentrations: aprotinin, 100 KIU/mL; benzamidine, 2 mmol/L; and phenylmethylsulfonyl fluoride, 2 mmol/L. The concentrations of serum total cholesterol, triglyceride, and HDL cholesterol were determined by enzymatic methods, using commercial kits (Kyowa Medex Co, Tokyo, Japan). The concentrations of serum apo A-I and apo B were determined by an immunoturbidity method, using commercial antibodies (Daiichi Pure Chemicals, Tokyo, Japan). Serum lipoproteins were separated by preparative sequential ultracentrifugation (Beckman, type 70.1Ti rotor, Palo Alto, Calif). Serum was adjusted to appropriate densities with NaBr. Each lipoprotein was fractionated as follows: low-density lipoprotein (LDL; $1.019 < d < 1.063$ g/mL) and HDL3 ($1.125 < d < 1.210$ g/mL).

Oxidized LDL was prepared by dialyzing the samples of native LDL in 5 μmol/L $CuSO_4$ at 37°C for 24 hours as previously described [4]. Oxidized LDL was extensively dialyzed against 0.15 mol/L NaCl containing 1 mmol/L EDTA (pH 7.4).

2.2. Analysis of plasma sterols

Plasma sterol concentrations at fasting were determined by high-performance liquid chromatography method [5].

2.3. Cell culture

Human monocytes were isolated from peripheral venous blood by density gradient centrifugation using NycoPrep (NYCOMED PHARMA AS, Oslo, Norway), with a density of 1.068 g/mL. After the isolation, the cells were resuspended in RPMI-1640 (Gibco Laboratories, Grand Island, NY). Cells were incubated on 6-well plastic plates (Primaria brand, Falcon Labware, Becton Dickinson and Co, Lincoln Park, NJ) with 5% CO_2 at 37°C in RPMI-1640 medium containing 10% human type AB serum (vol/vol), penicillin (100 IU/mL), and streptomycin (100 μg/mL). One hour after the incubation, nonadherent cells were removed by washing with the same fresh medium. The medium was changed twice a week, and the cells obtained after 7 days were used as human monocyte–derived MΦ.

2.4. LDL receptor activity in human lymphocytes

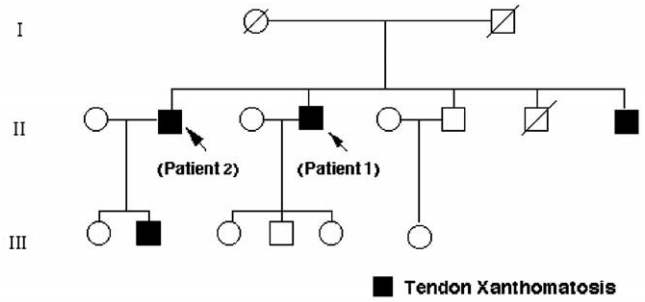
Human mononuclear cells were isolated from peripheral venous blood by density gradient centrifugation using NycoPrep (NYCOMED PHARMA AS), with a density of 1.077 g/mL. The monocytes adhered to the dishes, and only the lymphocytes that remained in suspension were collected and cultured. To up-regulate the LDL receptor, the mononuclear cells were incubated for 72 hours on the dish with serum-free RPMI-1640 medium. Thereafter, the lymphocytes were resuspended in Dulbecco's phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) at cell density of 2 to 4×10^6 cells/mL. For the assay, 100 μL of the cell suspension was incubated at 37°C for 2 hours in the plastic tube with DiI-LDL (Molecular Probes Inc, Eugene, Ore). After the incubation with DiI-LDL, the cells were washed twice with PBS and resuspended in 300 μL of PBS for flow cytometric analysis.

2.5. Effect of oxidized LDL on cholesteryl ester accumulation in human monocyte–derived MΦ

Human monocyte–derived MΦ (7 days' culture) were incubated for 24 hours at 37°C on plastic plates (10^5 cells/well) with oxidized LDL (50 μg/mL). The cellular lipids were extracted by incubation of the cells with 0.5 mL of hexane/isopropanol (3:2, vol/vol) for 30 minutes at room temperature. After saving the supernatant, cellular lipids were reextracted with 0.5 mL of the same solvent and collected into the same tubes [6]. The cholesterol content of the combined supernatant was determined by the method of Heider and Boyett [7]. Briefly, the lipid extract was dried under a nitrogen flush and dissolved in 180 μL of isopropanol. Next, 30 μL of the supernatant was added to 0.4 mL of an enzyme mixture containing 0.16 IU/mL cholesterol oxidase for determining free cholesterol. To evaluate total cholesterol, the whole extract was added to 0.4 mL of an enzyme mixture containing both cholesterol oxidase (0.16 IU/mL) and cholesterol esterase (60 IU/mL). The reaction mixtures for measuring free cholesterol and those for total cholesterol were incubated at 37°C for 1 hour and 2 hours, respectively, followed by the addition of 0.81 mL of 0.5 N NaOH to terminate the reaction. Fluorescence intensity was measured with excitation at 310 nm and emission at 407 nm. The mass of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. The cellular proteins were dissolved in 1.0 mL of 0.1 N NaOH, and their concentrations were measured by method of Lowry et al [8].

2.6. Cholesterol efflux from human monocyte–derived MΦ

Human monocyte–derived MΦ were incubated for 48 hours in RPMI-1640 containing 10% human type AB serum supplemented with 1 μCi/mL of [1,2-^{3}H] cholesterol



	Patient 1	Patient 2	Reference range
Sex	M	M	
Age (y)	58	60	
Total cholesterol	245	228	120 - 220 mg/dL
HDL cholesterol	36	45	40 - 80 mg/dL
Triglyceride	92	93	<150 mg/dL

Fig. 1. Pedigree of the proband's family and their lipid profiles. Upper panel shows the pedigree of the proband's family (the proband: patient 1, his elder brother: patient 2). Table in the lower panel presents their lipid profiles.

and 2.0 μg/mL of an inhibitor of acyl-coenzyme A/cholesterol acyltransferase (F-1394, Fuji Rebio, Japan) in 6-well plastic plates. After 48 hours' incubation, the cells were washed 5 times with PBS. Cholesterol efflux studies were carried out with RPMI-1640 plus 0.1% BSA in the presence of 100 μg/mL protein of HDL3 isolated from a healthy control subject's serum at 37°C for 12 hours or 10 μg/mL protein of apo A-I purchased from Sigma Chemical Co for 6 hours. Cellular cholesterol efflux was determined as the percentage of radioactivity of [^{3}H]-cholesterol in the efflux medium to total cell–labeled radioactivity. The radioactivity was determined by liquid scintillation counting. The background for the efflux was in the presence of 0.1% BSA alone. Specific HDL3 or apo A-I–mediated cholesterol efflux was calculated by subtracting the background from total cellular cholesterol efflux.

2.7. Semiquantitative reverse transcription–coupled polymerase chain reaction

Total cellular RNA from human monocyte–derived MΦ was isolated using Trizol reagent (Gibco BRL). The RNA levels of ATP-binding cassette transporter 1 (ABCA1) in MΦ were assessed by semiquantitative RT-PCR (Ready-To-Go RT-PCR Beads, Amersham, Piscataway, NJ). Two micrograms of total RNA of MΦ was used for RT-PCR analysis. After heating at 42°C for 30 minutes, PCR was

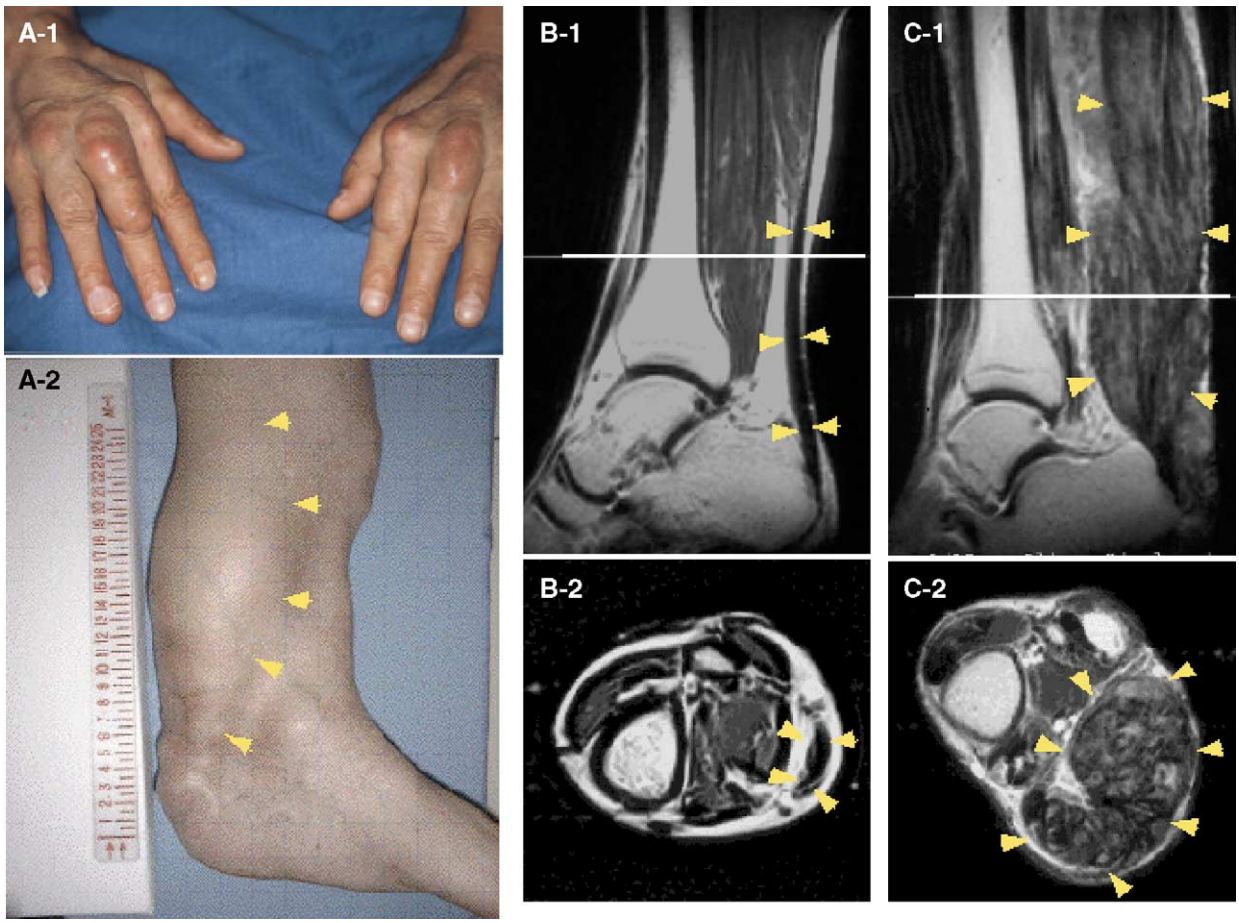


Fig. 2. Massive tendon xanthomatosis on the proband's extremities. A, Massive xanthomas observed on the proband's hands (A-1) and his left lower limb (A-2). B, C, Magnetic resonance images of the lower limb of healthy control subject (B-1 and B-2) and the proband (C-1 and C-2). B-1 and C-1 show longitudinal images. B-2 and C-2 show cross-sectional images at the level of white bars. Arrowheads in each figure show the margin of Achilles tendon.

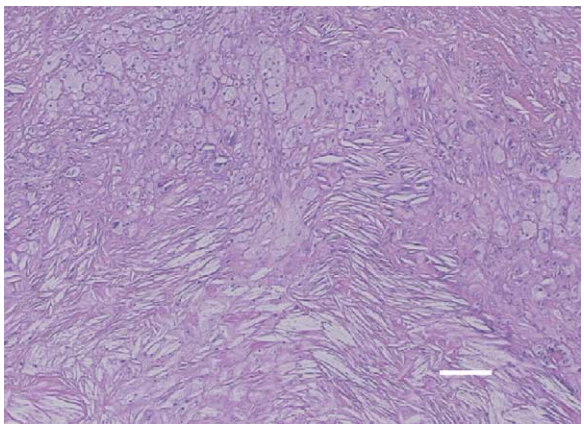


Fig. 3. Pathological analysis of the biopsy specimen from the proband's Achilles tendon xanthoma. Many foam cells and cholesterol crystals can be observed (hematoxylin and eosin stain). Scale bar = 100 μm.

conducted: 95°C for 60 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60 seconds. To monitor that PCR was in the exponential phase, each gene was amplified for 25, 30, and 35 cycles, respectively. Amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served to normalize input RNA amounts. The primers for ABCA1 (GenBank accession no. AJ012376): 5′-GTTGGAAAGATTCTCTATACACCTGA-3′ (forward primer) and 5′-CGTCAGCACTCTGAT-GATGGCCTG-3′ (reverse primer). The primers for GAPDH (GenBank accession no. J02642): 5′-CCCTTCATTGACCT-CAACTACAT-3′ (forward primer) and 5′-ACGATAC-CAAAGTTGTCATGGAT-3′ (reverse primer).

2.8. Statistical analysis

Results were expressed as the mean values ± SD. The significance of difference between the mean values of various parameters was determined by the Student *t* test and analysis of variance. A level of $P < .05$ was accepted as statistically significant.

All subjects gave informed consent to their participation in this study, and the analysis protocol was approved by the ethics committees of Osaka University.

3. Results

In 1996, a 58-year-old man was admitted to our hospital because of severe infection with methicillin-resistant *Staphylococcus aureus* and necrosis of Achilles tendon xanthomas. On admission, he had normal constitution and was not obese (height, 165 cm; weight, 57 kg). He was born in Saga Prefecture, Kyushu Island, Japan, in 1938. Three of 5 brothers including him and his nephew showed similar tendon xanthomas (Fig. 1). Neither mental retardation nor cerebellar ataxia was observed in any family member. Massive and systemic tendon xanthomatosis became manifest around 30 years of their ages. Routine biochemical analysis showed that the proband (patient 1) and his elder brother (patient 2) had mild elevations of total cholesterol (251 and 228 mg/dL, respectively, normal range 120-220 mg/dL). Patient 1 had mild reductions of HDL cholesterol (36 mg/dL, normal range 40-80 mg/dL) and apo A-I (81 mg/dL, normal range 95-170 mg/dL). The serum level of apo B was slightly increased in patient 1 (148 mg/dL, normal range 45-125 mg/dL), The concentrations of their serum triglyceride were normal. The patients had the apo E-IV/III phenotype. Although ultrasonography presented the mild calcifications in the aortic valve of patient 1, atherosclerotic lesions such as plaques, calcifications, and stenosis in his carotid arteries were not observed. He did not have any evidence of coronary heart disease, and his exercise electrocardiogram was normal. Known disorders causing similar xanthoma such as cerebrotendinous xanthomatosis, β-sitosterolemia, and familial hypercholesterolemia were excluded by the following analyses. We could not observe the elevation of plant sterol or cholestanol in plasma of patient 1 (cholestanol 0.6 mg/dL, normal range 0.1-0.6 mg/dL; β-sitosterol 1.8 mg/dL, normal range <2.0 mg/dL) [9]. The activity of LDL receptor in the patients' lymphocytes was normal.

Fig. 2A shows the photographs of xanthomas of patient 1 on his hands and lower extremities. As shown in Fig. 2B and C, magnetic resonance image showed that Achilles tendon of a control subject was demonstrated as a clear region with homogeneous weak intensity, whereas that of the patient was expressed as a huge expansion of soft tissue with "salami sausage"–like heterogeneous intensities (50 mm in thickness). Pathological analysis of biopsy specimen from his Achilles tendon xanthoma revealed the extreme accumulation of foam cells and cholesterol crystals (Fig. 3).

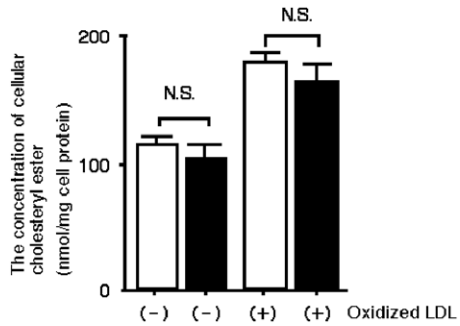


Fig. 4. Effect of oxidized LDL on the cellular cholesteryl ester concentration of human monocyte–derived MΦ. Monocytes were isolated by density gradient centrifugation method from peripheral venous blood of a healthy subject and the proband. Monocyte-derived MΦ were incubated on plastic plates (10^5 cells/well) for 24 hours with RPMI-1640 containing oxidized LDL (50 μg/mL). After incubation, the cellular lipids were extracted by incubation of the cells with hexane/isopropanol (3:2, vol/vol). The cholesterol content was determined by the method of Heider and Boyett [7]. The cellular proteins were dissolved in 1.0 mL of 0.1 N NaOH, and their concentrations were measured by the method of Lowry et al [8]. Open bars, the control MΦ; closed bars, the proband's MΦ. Each data represent the mean ± SD of triplicate determinations.

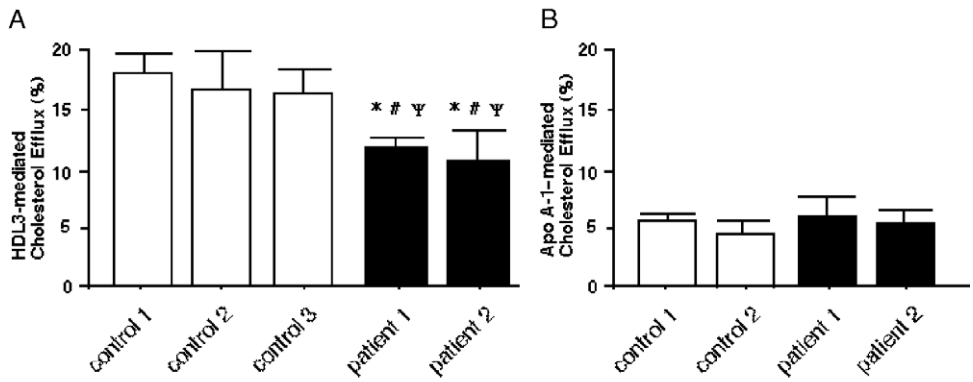


Fig. 5. HDL3 and apo A-I–mediated cholesterol efflux from human monocyte–derived MΦ. Macrophages from the proband (patient 1), his elder brother (patient 2), and 3 controls were labeled for 24 hours by incubation with RPMI-1640 containing 10% human type AB serum, [1,2-^{3}H]-cholesterol (1 μCi/mL), and an acyl-coenzyme A/cholesterol acyltransferase inhibitor (2 μg/mL, F-1394, Fuji Rebio, Japan). After 48 hours' incubation, the cells were washed 5 times with PBS; cholesterol efflux studies were carried out with RPMI-1640 plus 0.1% BSA in the presence of 100 μg/mL protein of HDL3 at 37°C for 12 hours (A) or 10 μg/mL protein of apo A-I for 6 hours (B). Cellular cholesterol efflux is the ratio of counts in the medium to the sum of radioactive counts in the medium plus the cell fraction. Open bars, the controls' MΦ; closed bars, the patients' MΦ. Each data represent the mean ± SD of triplicate determinations. *, #, Ψ: $P < .05$ versus the controls, respectively.

We performed cell biologic analyses of the patients' MΦ. To clarify the mechanism of foam cell formation, we investigated the effect of oxidized LDL on the cellular cholesteryl ester concentration of MΦ. There was no significant difference in the cholesteryl ester accumulation between the MΦ of patient 1 and the control, suggesting that the uptake of oxidized LDL by his MΦ was not increased compared with that of the control (Fig. 4). Next, we examined HDL3-mediated and apo A-I–mediated cholesterol efflux from MΦ. HDL3-mediated cholesterol efflux from the patients' MΦ (n = 2) was significantly reduced compared with the controls (n = 3) (Fig. 5A), whereas there was no significant difference in apo A-I–mediated cholesterol efflux between the patients' MΦ and the controls (n = 2) (Fig. 5B). These data suggested that the formation of massive tendon xanthomatosis may be partly attributed to the decreased HDL3-mediated cholesterol efflux from MΦ.

Finally, we investigated the gene expressions of ABCA1, which promotes apo A-I–mediated cholesterol efflux from MΦ , by RT-PCR method (Fig. 6). However, there was no significant reduction in the messenger RNA levels of ABCA1 in the MΦ of patient 1 compared with the MΦ of the control.

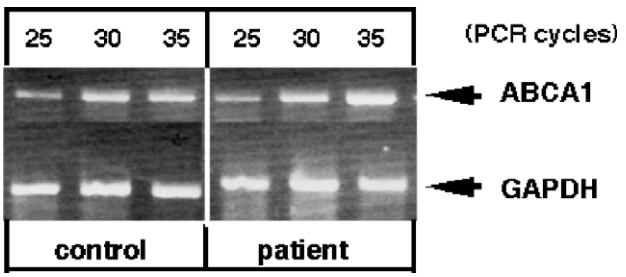


Fig. 6. Expression of ABCA1 mRNA in the proband's monocyte-derived MΦ. ABCA1 mRNA levels of ABCA1 in the MΦ of the proband (patient 1) were assessed by semiquantitative RT-PCR as described in Materials and methods. To monitor that PCR was in the exponential phase, each gene was amplified for 25, 30, and 35 cycles. Amplification of the housekeeping gene GAPDH served to normalize input RNA amounts.

4. Discussion

The present study demonstrated a novel familial massive tendon xanthomatosis associated with the impaired HDL3-mediated cholesterol efflux from the patients' MΦ. Cholesterol efflux mediated by HDL3/apo A-I is the initial step of reverse transport of cholesterol from the peripheral tissues to the liver in vivo. Reverse cholesterol transport plays an important role in the prevention of atherosclerosis formation. The impaired cholesterol efflux leads to accumulating lipoprotein-derived lipids in various peripheral tissues. Therefore, we speculate that the massive deposition of lipids in the patient's tendons may be, at least in part, related to the reduction of HDL3-mediated cholesterol efflux from his cells.

A rapid progress has been made to identify the various molecules such as ATP-binding cassette transporters (ABC), which are responsible for cellular efflux pump. It is known that the human ABC proteins are classified into many subfamilies [10]. However, their detailed functions in cells have not been clarified enough. The relationship between the mutations of various ABC transporters and clinical phenotypes has been noticed. For example, pseudoxanthoma elasticum, of which primary defect is the mutation of ABC, subfamily C (ABCC6), is characterized by the appearance of systemic eruptive xanthomas associated with the deposition and calcification of elastic fibers in the skin [11,12]. Adrenoleukodystrophy caused by the mutation of ABC, subfamily D (ABCD1), which is a cellular pump of very long-chain fatty acids, is characterized by the accumulation of unbranched saturated fatty acids in the cholesteryl ester or sphingolipids of brain [13]. β-Sitosterolemia is caused by the mutation of ABC, subfamily G (ABCG5 and/

or ABCG8), which may be involved in the transport of plant sterols such as sitosterol in the epithelial cells of small intestine. Recently, it has been reported by some laboratories that a familial HDL deficiency including Tangier disease, of which primary defect is the gene mutation of ATP-binding cassette transporter-1 (ABCA1), is characterized by the impaired apo A-I–mediated cholesterol efflux in the cells from the patients [14-16] and that ABCA1 interacts with lipid-poor apo A-I, but not HDL [17]. Our patients have no typical phenotype of orange-color tonsil, hepatosplenomegaly, and peripheral neuropathy as observed in the patients with Tangier disease, and their plasma HDL cholesterol and apo A-I levels were almost normal. Although we investigated the gene expressions of ABCA1 of MΦ by RT-PCR method (Fig. 6), there was no reduction of the messenger RNA levels of ABCA1 in the MΦ of patient 1 compared with the control. Furthermore, only HDL3-mediated, but not apo A-I–mediated, cholesterol efflux was reduced in the MΦ of the patients. Therefore, although we did not investigate ABCA1 gene mutation in our patients, ABCA1 heterozygous or homozygous deficiency was excluded.

It is well known that an important mediator of HDL metabolism is scavenger receptor class B type I (SR-BI) [18]. SR-BI is abundantly expressed in liver and steroidogenic tissues, where it mediates the selective uptake of cholesteryl esters from HDL [19]. We previously reported that SR-BI is also expressed in human monocyte–derived MΦ which are closely related to formation of atherosclerosis and xanthomatosis [20]. In MΦ, SR-BI is involved in the bidirectional flux (efflux and selective uptake) of cholesterol and phospholipids between HDL and the cell membrane [21]. Although, in this study, SR-BI messenger RNA levels of our patients' MΦ were not decreased compared with those of the controls (data not shown), the abnormal expression of SR-BI cell surface protein by some gene mutations might reduce the interaction with HDL and be involved in the abnormal HDL-mediated cholesterol efflux or selective uptake of cholesteryl ester from HDL in the patients' MΦ . This issue needs to be investigated in the future studies.

Furthermore, we reported that the expression of Cdc42, a member of Rho GTPases, which is involved in intracellular lipid transport, regulation of gene transcription, and actin-filament reorganization [22-24], was decreased in the cells from the patients with Tangier disease [25]. Although we analyzed the expression level of Cdc42 protein of MΦ, the reduction of Cdc42 expression was not observed in the MΦ of patient 1 (data not shown).

It is well known that both HDL3-mediated and apo A-I–mediated cholesterol efflux is decreased in the cells from the patients with Tangier disease [26-28], whereas in the patients, MΦ were characterized by the reduction of HDL3-mediated, but not apo A-I–mediated, cholesterol efflux in this study. It is yet to be determined why only HDL3-mediated cholesterol efflux was decreased in the patients' MΦ. For 1 of the possible explanations for the reductions of only HDL-mediated cholesterol efflux observed in the patients' MΦ, the abnormality of other responsible receptor for cholesterol efflux, to which HDL particles rather than free apo A-I bind specifically, for example, ABCG1 and/or ABCG4, might explain this observation [29]. This issue needs to be clarified in future studies.

In 1983, Vega et al [30] reported a tendon xanthomatosis with overproduction of apo B. They showed that the patient had severe bilateral xanthomas of Achilles tendons and small lesions on patellar tendons with a marked elevation of plasma very low-density lipoprotein–apo B level and speculated that the patient's xanthomatosis might be the result of an overproduction of apo B possibly combined with a defect in HDL. However, the plasma apo B level of our patient 1 (the proband) was almost normal. Therefore, the xanthomatosis associated with overproduction of apo B was excluded in our case.

The mechanism for the above abnormal cell biologic functions remains to be elucidated. However, if our findings from in vitro analyses could be applied to in vivo conditions, such speculation is easily accepted that the unique activities of MΦ we found in the present study might be involved in the accumulation of foam cells and subsequent development of xanthomatosis. Taken together, this family has a novel clinical entity of familial massive tendon xanthomatosis.

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