Decreased expression of a member of the Rho GTPase family, Cdc42Hs, in cells from Tangier disease – the small G protein may play a role in cholesterol efflux

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Abstract Cholesterol efflux (CE) is the initial and important step of reverse cholesterol transport (RCT), a major protective system against atherosclerosis. However, most of the molecular mechanism for CE still remains to be clarified. In the present study, cDNA subtraction revealed that the expression of a member of the Rho GTPase family, Cdc42Hs, was markedly decreased in both passaged fibroblasts and macrophages (Mϕ) from patients with Tangier disease (TD), a rare lipoprotein disorder with reduced CE. This small G protein is known to have many cell biological activities such as rearrangement of actin cytoskeleton and vesicular transport, however the association between this molecule and lipid transport has never been reported. We demonstrate that MDCK cells expressing the dominant negative form of Cdc42Hs had reduced CE, inversely ones expressing the dominant active form had increased CE. From these observations, we would like to raise a novel hypothesis that this type of small G protein may play a role in some steps of CE. To our knowledge, the present study is the first demonstration that the expression of this molecule is altered in cells from human disease.

Key words: Cdc42; Cholesterol efflux; Reverse cholesterol transport; Small G protein; Tangier disease

1. Introduction

Atherosclerotic cardiovascular disease is one of the major causes of death in the well-developed countries. Reverse cholesterol transport (RCT) is the formulated concept for the protective system against atherosclerosis [1,2]. In the RCT system, high density lipoprotein (HDL) particles are thought to play an important role as a shuttle carrying cholesterol from lipid-laden cells in the arterial walls to the liver, a terminal of RCT, where cholesterol is catabolized and secreted as a bile. The initial step of RCT is called 'cholesterol efflux (CE)', where small and lipid poor HDL particles remove cholesterol from the cells [3].

Enhancement of RCT, especially its initial step, CE, is one of the novel strategies for anti-atherosclerotic treatment, which would make it possible to regress and stabilize cholesterol-rich plaque in the atherosclerotic lesions. However, most of the molecular map for CE still remains to be drawn. Our purpose is to fill in such a large blank in the map for understanding the molecular mechanism for CE by patient- and disease-oriented researches [4].

Tangier disease (TD), a rare lipoprotein disorder, is thought to be a kind of model for the impairment of the first step of the RCT system by the following reasons [5]: (a) impaired CE from TD cells, (b) altered intracellular trafficking of HDL particles, and (c) hypercatabolism of HDL particles. Many laboratories including ours reported the premature atherosclerotic coronary artery disease in some patients with TD [6,7]. In the present study, we found that the expression of a member of the Rho GTPase family [8,9], Cdc42Hs, was decreased in cells from TD by cDNA subtraction technique.

2. Materials and methods

2.1. Patients

Cutaneous fibroblasts and monocyte-derived macrophages (Mϕ) were obtained from two unrelated TD patients after the informed consent: TD1 was a 56 years old male (plasma total cholesterol, 0.72 mmol/l; triglycerides, 2.60 mmol/l; HDL–cholesterol, 0.16 mmol/l). TD2 was a 60 years old male (plasma total cholesterol, 0.78 mmol/l; triglyceride, 2.0 mmol/l; HDL–cholesterol, 0.13 mmol/l). Both patients had typical clinical symptoms such as orange tonsils, hepatosplenomegaly, and increased levels of prepro apolipoprotein (Apo) AI in plasma. We have recently reported that TD1 had suffered from severe coronary heart diseases, demonstrated by coronary angiograms with intravascular ultrasoundgraphy [7]. It was reported that many of patients with TD have mutations in the ATP-binding cassette transporter-1 (ABCA1) gene [10–13]. The mutational analyses of ABCA1 gene in our patients have not been completed yet in TD1, however TD2 had a novel mutation in the ABCA1 gene (Inazu, A.

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Abbreviations: Apo, apolipoprotein; CE, cholesterol efflux; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoprotein; Mϕ, macrophages; RCT, reverse cholesterol transport; RT-PCR, reverse transcription-based polymerase chain reaction; SR-BI, scavenger receptor class B type I; TD, Tangier disease

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and Mabuchi, H. et al., personal communication). For cDNA subtraction, we have made pooled total RNA of Mϕ from 10 control subjects to minimize the difference of mRNA profiles between individuals. Control fibroblasts were obtained from age-matched healthy volunteers after obtaining informed consent.

2.2. Cell culture

Passaged fibroblasts and MDCK cells were cultured according to standard conditions. All passaged fibroblasts cultured were used between passages 5 and 15 and after culturing for 7 days following splitting 1:2. Mϕ were isolated and cultured as described previously [14].

2.3. Immunofluorescent microscopy and antibodies used

Cells were stained and analyzed by a confocal laser microscopy (Zeiss LSM 510, Carl Zeiss Co., Ltd.) as described previously [14]. In order to see the actin cytoskeletons, the cells were stained by Rhodamine-Phalloidin (Molecular Probe).

2.4. Subtraction cDNA library

cDNA subtraction was performed with PCR-select Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's recommendation. Briefly, two cDNA pools were synthesized from total RNA isolated from TD Mϕ and pooled total RNA from control Mϕ. The cDNA pools were digested by *Rsa*I before cDNA subtractions. cDNA from TD cells were subtracted using cDNA from control cells and suppression subtractive hybridization was performed according to the manufacturer's protocol. The remaining cDNAs were randomly subcloned into pGEM-T Easy vector (Promega) and sequenced.

2.5. Plasmid preparation and establishment of stable cell lines expressing dominant active or negative form of Cdc42Hs

Expression vectors, pEF-BOS-myc-V12Cdc42Hs (dominant active form of Cdc42Hs, Cdc42-DA) and pEF-BOS-myc-N17Cdc42Hs (dominant negative form of Cdc42Hs, Cdc42-DN) were constructed and stably expressing MDCK cells were isolated, as described [15,16].

2.6. Cholesterol efflux assay

Subconfluent passaged fibroblasts and MDCK cells were labeled with 2 mCi/well ^{3}H-cholesterol (NEN Life Science Products, Inc., Boston, MA, USA) for 24 h with 2 mg/ml of an ACAT inhibitor, F-1394 (Fujirebio, Tokyo, Japan) in serum-free medium supplemented with 0.1% BSA. After equilibration, cholesterol efflux from the cells was carried out for the indicated hours to a 0.1% BSA serum-free medium with and without Apo AI (Sigma) to measure Apo AI-specific efflux. Cell supernatants were collected and monolayers were lysed in 0.1 M NaOH. Radioactivity was determined by liquid scintillation counting. The percentage efflux is the ratio of counts in the supernatant to the sum of radioactivities in the media plus supernatant. Each experiment was carried out in quadruplicate.

3. Results and discussion

3.1. The cDNA subtraction technique reveals the decreased expression of Cdc42Hs in cells from patients with Tangier disease

We have performed cDNA subtraction with using total RNA from Mϕ from TD1, showing that the expression of cDNA encoding Cdc42Hs (GenBank Accession Number M57298) [8,9] was decreased in Mϕ of TD1, compared with pooled RNA from healthy volunteers (Fig. 1A). Reverse transcription-based polymerase chain reaction (RT-PCR) analyses confirmed that mRNA expression of Cdc42Hs was decreased in macrophages (Mϕ) obtained from the other patient, TD2,

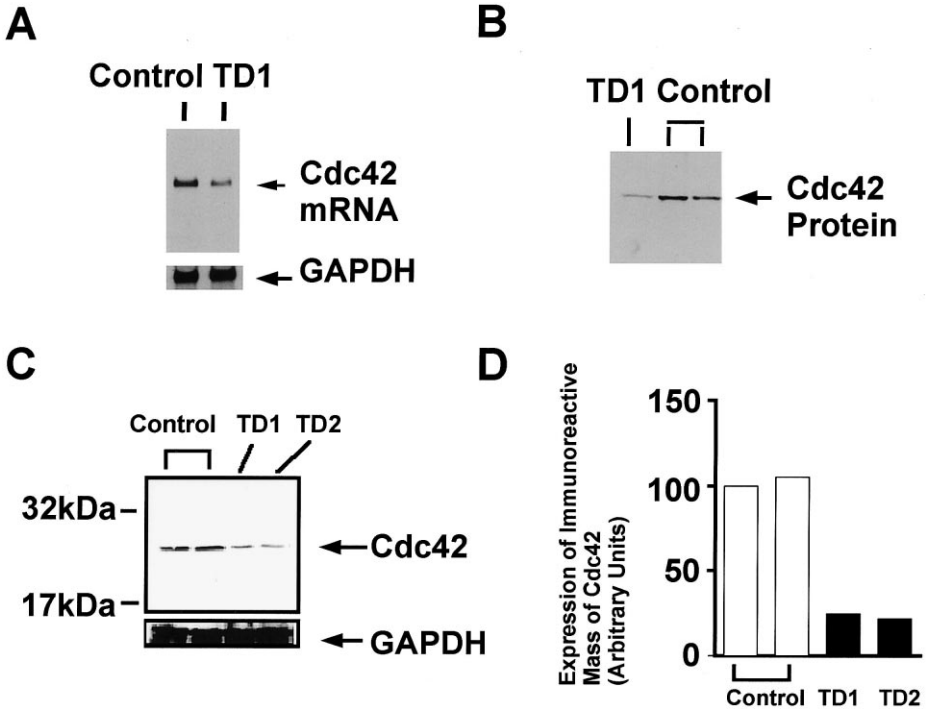

Fig. 1. Decreased expression of Cdc42Hs in cells from patients with Tangier disease. A: 10 μg of total RNA from macrophages (Mϕ) from TD1 and pooled total RNA from control Mϕ were subjected to Northern blot analysis. Biotinylated 576 nt human Cdc42Hs cRNA probes corresponding to nt 70–645 human Cdc42Hs cDNA (GenBank Accession Number M57298) and GAPDH 316 nt cRNA probes transcribed from pTRI-GAPDH Human (Ambion) were used as probes. B: Whole cell lysates were extracted from human Mϕ cultured in 10% human AB serum/RPMI. 20 μg of whole cell lysates were separated by 5–15% SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with anti-Cdc42 antibody (Cat. # sc-87, Santa Cruz) and IgG bound was visualized by ECL plus kit (Amersham). C and D: Whole cell lysates were extracted from fibroblasts of TD1 and TD2 and two control fibroblasts and subjected to Western blot analysis. The immunoreactive mass of GAPDH was detected with anti-GAPDH antibody (Cat. # A9521, Sigma) and shown as an internal standard. The exposed films were subjected to NIH image programs. Bar graph (D) shows the abundance of immunoreactive mass of Cdc42Hs in TD, comparing with that in control.

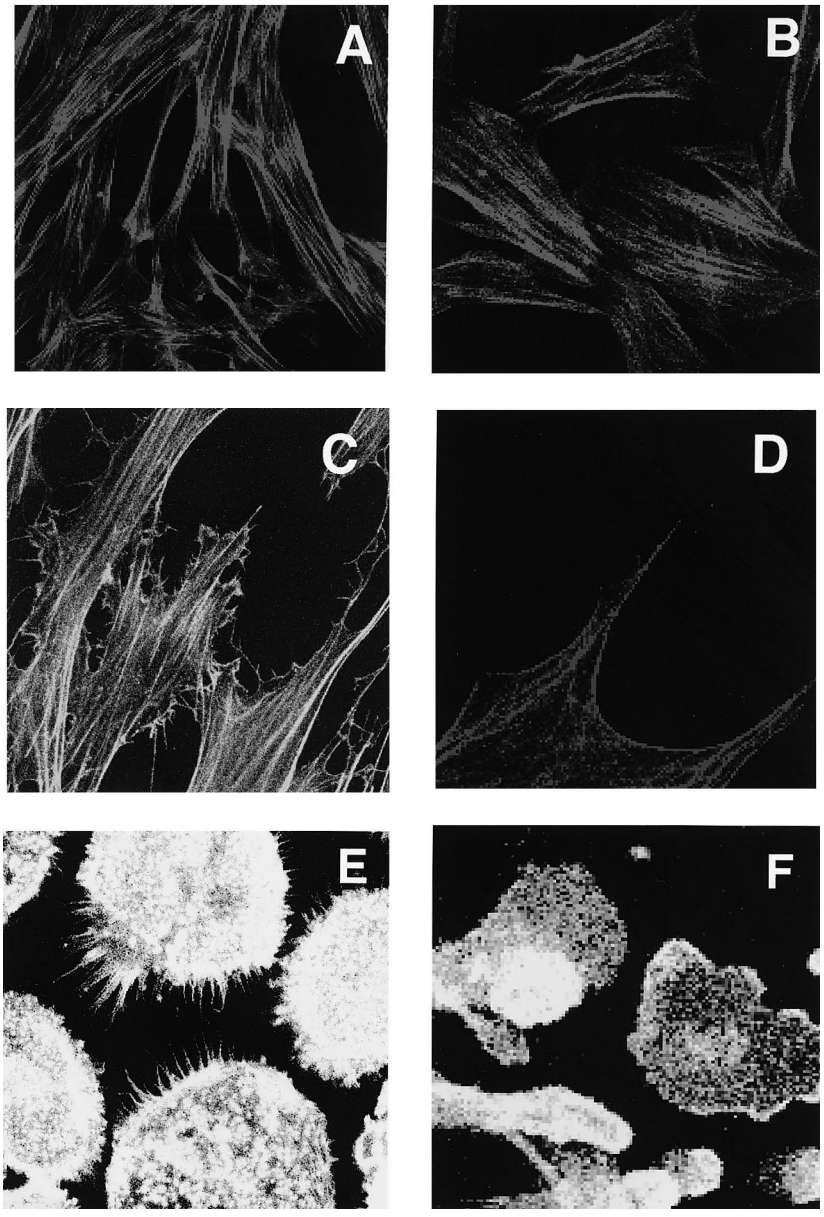

Fig. 2. Abnormal cell shape of fibroblasts (B and D) and macrophages (F) from patients with Tangier disease. Fibroblasts (panels A, B, C, and D) and macrophages (panels E and F) were incubated in a 2-well glass chamber slide (FALCON culture slide; Becton Dickinson Labware, Franklin Lakes, NJ, USA). Normal cells were demonstrated in panels A, C, and E. Cells from TD1 were shown in panels B, D, and F. The cells were stained by Rhodamine–Phalloidin (Molecular probes) to visualize F-actin. Images were acquired by confocal laser microscopy as a single 1 μm thick optical section using a Zeiss LSM 410 confocal microscope. The magnitude for each image was as follows: A and B, 10× objective. C, 100× objective. D, 40× objective. E and F, 100× objective.

as well (data not shown). Western blot analyses showed that the immunoreactive masses of Cdc42Hs were also decreased in both Mϕ (Fig. 1B) and fibroblasts (Fig. 1C and D) obtained from both TD1 and TD2. These results indicated that the expression of this type of small G protein was markedly decreased in both Mϕ and passaged fibroblasts from two unrelated TD patients. Cdc42 belongs to a small G protein, Rho GTPase family. There are two isoforms of this molecule; Cdc42Hs is ubiquitous and Cdc42G25K is in neuron-specific form. Many experimental studies revealed that this G protein has a variety of cell biological functions, however relevance and significance of this molecule in human diseases and pathological conditions is not known yet. To our knowledge, this is the first demonstration that the expression of this molecule is altered in cells from human disease.

3.2. *Alteration of actin cytoskeletons in cells from Tangier disease*

Many experimental studies using dominant active or negative form of Cdc42 have demonstrated that Cdc42 functions to regulate rearrangement of actin cytoskeleton as well as formation of filopodia in cell lines including some fibroblasts and Mϕ [8,9]. Therefore, we have stained F-actin by Rhodamine–Phalloidin in both passaged fibroblasts and monocyte-derived Mϕ obtained from Tangier patients and controls, in order to know whether or not the TD cells have the alteration

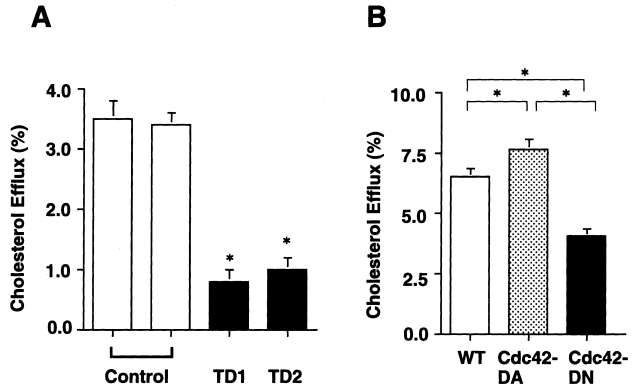

Fig. 3. Apo AI-mediated cholesterol efflux from passaged fibroblasts from Tangier and control subjects (A) and that from MDCK cells stably expressing dominant active or negative mutant of Cdc42Hs (B). Cellular cholesterol efflux was assayed, as shown in Section 2. A: Fibroblasts from Tangier and control subjects were used for the efflux study. The data are expressed as mean ± S.D. Briefly, the release of radioactive cholesterol to a given acceptor was measured by scintillation counting of filtered aliquots of acceptor-containing medium (5 μg/ml of human Apo AI). Cells were pre-labeled by incubation for 24 h at 37°C in a CO_2 incubator with DMEM supplemented with 5% FCS radiolabeled with ^{3}H-cholesterol and an acyl CoA:cholesterol acyltransferase (ACAT) inhibitor (F-1394, Fuji Rebio, Japan). After equilibration, cholesterol efflux was performed with 4 h incubation with Apo AI. Asterisks denote statistical significance compared with controls ($P < 0.05$). B: MDCK cells stably expressing mutants of Cdc42Hs were used for the efflux study. Cholesterol efflux was measured after 18 h incubation with human Apo AI (5 μg/ml of human Apo AI). The data are expressed as mean ± S.D. Asterisks denote statistical significance ($P < 0.05$).

of actin cytoskeletons. As shown in Fig. 2, lower power view of confocal laser microscopy showed that fibroblasts from TD1 (Fig. 2) were larger in size, as reported previously [17]. TD fibroblasts appeared to have coarse actin fibers (Fig. 2B), compared to those from normal controls (Fig. 2A). Higher power view showed that the edge of cells are sharp in TD cells (Fig. 2D), whereas control cells had some spike formations (Fig. 2C). Mϕ from TD1 (Fig. 2F) also had apparent abnormal cell shape with less filopodia formation than those from controls (Fig. 2E). Both fibroblasts and Mϕ from TD2 had similar morphological changes to those of TD1 (data not shown). These observations showed that the TD cells with decreased expression of endogenous Cdc42Hs had abnormal actin cytoskeletons with less filopodia formation, which was similar to those observed in cells expressing the dominant negative form of Cdc42 [8,9]. These data suggested that the altered expression of Cdc42Hs might in some parts contribute to the abnormal actin cytoskeletons in TD cells.

3.3. *Cholesterol efflux was altered in MDCK cells expressing the dominant negative or active form of Cdc42Hs*

In order to search for the possibility that the decreased expression of Cdc42Hs may be related to impaired CE, we have tested CE from the MDCK cells stably expressing dominant active or negative form of Cdc42Hs, which have been developed by us [15,16]. As shown in previous reports, Apo AI-mediated CE was markedly decreased in TD cells (Fig. 3A) [5,6]. It was very interesting that Apo AI-mediated CE is increased from cells expressing Cdc42-DA. Inversely, the CE was significantly decreased in cells expressing Cdc42-DN (Fig. 3B).

3.4. *A novel hypothesis that Cdc42Hs may play a role in some process of cholesterol efflux*

CE from the cells is thought to be a very complicated process. However, it can be divided at least into the following two steps: movement of cholesterol from some intracellular lesions to plasma membrane and release of cholesterol to the acceptors such as small HDL particles or free Apo AI [18]. It is obvious that the ABCA1 gene product is one of the major gate keeper molecules for CE, however other molecules should be involved in the process of CE [11]. We have found that scavenger receptor class B type I (SR-BI) [19], which has an ability to mediate CE, was expressed in the foam cells in the human atherosclerotic lesions [14]. Furthermore, we could purify a GPI-anchored protein on the cell surface of human monocyte-derived Mϕ, which might be involved in CE [20].

Cdc42 was originally identified as a factor necessary for the budding of yeast and reported to be the brefeldin A-sensitive component located in the Golgi apparatus as well as plasma membrane in some mammalian cells [21]. Recently it has been suggested that Cdc42 may control vesicular transport and secretory transport in some cells [22]. We had speculated that vesicular transport might be involved in intracellular movement of cholesterol in association with rearrangement of actin cytoskeletons. Considering the present results with MDCK cells expressing mutants of Cdc42Hs (Fig. 3), we have raised a novel hypothesis that this type of small G protein may be one of the important components for intracellular lipid transport and subsequent CE from the cells. Further studies are going on in our laboratory to elucidate significance of Cdc42Hs in these processes of CE as well as the development of atherosclerosis.

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