Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 850-856

THE HIGH DENSITY LIPOPROTEIN- AND APOLIPOPROTEIN A-I-INDUCED MOBILIZATION OF CELLULAR CHOLESTEROL IS IMPAIRED IN FIBROBLASTS FROM TANGIER DISEASE SUBJECTS

Michael Walter, Ute Gerdes, Udo Seedorf, and Gerd Assmann

Institut für Klinische Chemie und Laboratoriumsmedizin and Institut für Arteriosklerosеforschung, Westfälische Wilhelms-Universität Münster, Domagkstr. 3, 48149 Münster, Germany

Received October 18, 1994

SUMMARY: Tangier disease (also known as familial HDL-deficiency) is characterized by very low high density lipoprotein (HDL) plasma levels, splenomegaly, and massive cholesteryl ester accumulation in the cytoplasm of various cell types. Since this phenotype may in part be caused by a defect in the pathway mediating cholesterol efflux from peripheral cells, we investigated the HDL_3-mediated mobilization of cholesterol synthesized *de novo* from [^{14}C]-mevalonolactone in cultivated fibroblasts from two patients with Tangier disease. Our results indicate that the HDL_3-induced translocation of [^{14}C]-cholesterol from intracellular pools to the plasma membrane and its subsequent secretion into the extracellular medium was approximately 50 % less in the cells from the patients than in controls. The same result was also obtained with artificial apolipoprotein A-I-containing phospholipid vesicles. By contrast, no significant difference in HDL_3-induced cholesterol efflux was observed when plasma membrane was labeled with exogenous [^{14}C]-cholesterol. We conclude that inefficient cholesterol efflux in Tangier disease is primarily caused by impaired HDL_3-induced activation of cholesterol translocation from intracellular pools to the plasma membrane.

Tangier disease is a rare HDL deficiency syndrome with autosomal codominant inheritance. It is characterized by cholesteryl ester deposition in various tissues and markedly reduced HDL cholesterol in the plasma (1). Heterozygotes show half-normal levels of structurally abnormal alpha-lipoproteins in the serum (2). The disorder was first described among inhabitants of Tangier Island in the Chesapeake Bay, most of whom are descendants of the original settlers of 1686 (1). Since then, more than 30 other families have been discovered. The kindred studied in this report was identified in Germany by Assmann et al. in 1977 (3).

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; FC, free (unesterified) cholesterol; apo A-I, apolipoprotein A-I; FAFA, fatty acid-free albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DPPC, dipalmitoylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACAT, acyl-CoA:cholesterol acyltransferase.

0006-291X/94 $5.00

HDL-particles are hypercatabolized in homo- and heterozygous Tangier patients. However, a structural defect in the apolipoprotein A-I, the major protein constituent of HDL, was excluded as the cause of this hypercatabolism (1). Although the molecular defect is unknown, several abnormalities in cellular phospholipid, triglyceride, and cholesteryl ester metabolism point to a defect of cellular lipid metabolism or trafficking (4). Since the early steps in the maturation of HDL particles are dependent on specific interactions between HDL precursors and cells (5), we hypothesized that the HDL-induced cholesterol efflux might be distorted in Tangier disease.

MATERIALS AND METHODS

Cell culture: Human skin fibroblasts cultured from biopsies of adult human hip skin were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution (Sigma). For experiments, cells between passage levels 3 and 6 were seeded in 60-mm culture dishes at a density of approximately 220,000 cells/dish. At the state of 70-80% confluence, the cultures were loaded with free cholesterol (FC). Cell layers were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated for 48 h at 37°C in DMEM supplemented with FC (50 μg/ml), 2 mg/ml fatty acid-free albumin (FAFA) and 1% antibiotic/antimycotic solution.

Probands: The experiments were performed with fibroblasts from five normolipidemic healthy probands and two Tangier patients of the J.S. family (patient J.S.: 60 y, male; patient E.G.: 57 y, female, sister of J.S.) whose clinical, biochemical and histopathological manifestations including severe HDL deficiency, mild hypertriglyceridemia, hypocholesterolemia, splenomegaly and lipid storage in reticulo-endothelial tissues have been described in detail in previous reports (2-4,6,7).

Preparation of lipoproteins and proteoliposomes: HDL_3 (d=1.125-1.210 g/mL) was isolated by standard differential ultracentrifugal flotation from fresh normal human plasma and dialyzed against 0.3 mM Tris-HCl buffer (pH 6.8) containing 0.15 M NaCl (8). Apo A-I proteoliposomes containing normal apo A-I and dipalmitoyl-phosphatidylcholine (DPPC) were obtained by the cholate dialysis method according to Chen and Albers (9). Apo A-I was prepared from HDL_3 by a procedure described previously (10), lyophilized, and stored as 500-μg aliquots at -70°C. The molar ratio of apo A-I to DPPC was 0.8:250.

Selective labeling of sterol pools: Intracellular sterols were labeled by pulse incubations with [^{14}C]mevalonolactone (from Du Pont-New England Nuclear) as described (11). After cholesterol-loading, cell layers were rinsed three times with PBS-FAFA 1 mg/ml and incubated for 3 h at 37°C in bicarbonate-free DMEM-Hepes, 1 mg/ml FAFA, 8 μCi/ml [^{14}C]mevalonolactone, 0.5 mM mevalonolactone, and 2μg/ml of the ACAT inhibitor octimibate (from Rhône-Poulenc). After incubation, cell layers were rinsed three times with PBS-FAFA and then incubated in the same media without label for 1 h at 37°C. To enrich the plasma membrane cholesterol pool with [^{14}C]FC from an exogenous source, cholesterol-loaded cells were chilled on ice, washed five times with ice-cold PBS, and incubated for 3 h at 15°C in DMEM containing 20 mM Hepes, 1 mg/ml FAFA, 0.4 mM mevalonolactone, and 0.3 μCi/ml [^{14}C]FC (50-60 mCi/mmol, Du Pont-New England Nuclear). Labeled cells were rinsed three times with PBS-FAFA and then incubated for 4 h at 37°C in bicarbonate-free DMEM-Hepes, 1 mg/ml FAFA in the presence or absence of HDL_3 or apo A-I/DPPC-complexes.

Cholesterol efflux and translocation: After the 4 h incubation period, the efflux media were collected, one ml of PBS-FAFA was used to rinse the cell layers and the wash was added to the efflux media. Efflux media were extracted three times by the method of Folch et al. (12). Mem-

brane cholesterol was determined by the cholesterol oxidase method described by Lange and Ramos (13). Conversion of [^{14}C]sterols to [^{14}C]cholestenone represented the plasma membrane associated sterol pool. Cell layers were extracted in hexane:isopropanol (3:2, v/v) as described by Hara and Radin (14). Sterol species were separated by thin layer chromatography on silica gel G plates (from Merck) developed in heptane:ether:methanol:acetic acid (80:30:10:1.2, v/v/v/v). Lipids were identified by staining with I_2 vapor and by comigration with standards. Appropriate spots were taken for scintillation counting. The cellular protein content was determined by the method of Bradford (15), the FC mass was determined by a fluoroenzymatic method (16).

RESULTS

The HDL_3- and apo A-I/DPPC-complex-mediated mobilization of *de novo* synthesized free cholesterol (FC) was determined in fibroblasts from normolipidemic control persons and from Tangier patients as described by Oram et al. (11). Cholesterol-loaded cells were treated with 2 μg/ml ACAT inhibitor (octimibate) to prevent esterification of *de novo* synthesized cholesterol. The cells were then pulse-labeled with [^{14}C]mevalonolactone to enrich intracellular pools with labeled sterol. With this treatment less than 2% of the total [^{14}C]-labeled sterol was found in the ester pool. In order to discriminate between HDL_3-specific and non-specific cholesterol mobilization we designed an experimental protocol in which all cells underwent an identical 4 h experimental incubation period in the presence of 1 mg/ml FAFA. At different times before stopping the 4 h incubation period, 20 μg/ml HDL_3 was added and the [^{14}C]FC in the extracellular medium and plasma membrane was quantified as described in the Methods section. The values obtained with FAFA but without HDL_3 were set as 100% (basal value). Under basal conditions neither the total amount of radioactivity incorporated into cellular FC (control: 51,524 ± 6,210 d.p.m./mg cell protein; Tangier: 50,721 ± 4,747 d.p.m./mg cell protein) nor the specific activities of cellular FC (control: 386 ± 52 d.p.m./μg FC; Tangier: 436 ± 38 d.p.m./μg FC) were significantly different in the two cell types.

In control fibroblasts, the addition of 20 μg/ml HDL_3 to the cell cultures caused a time-dependent biphasic increase in both extracellular [^{14}C]FC and membrane [^{14}C]FC (Figs. 1a,b). The first phase was associated with an increase of plasma membrane [^{14}C]FC up to 125% of the basal value (Fig. 1b) and with an increase of extracellular [^{14}C]FC up to 230% of the basal value (Fig. 1a), reaching a maximum after 25-30 min. The second phase resulted in an increase of plasma membrane [^{14}C]FC up to 150% of basal levels and of extracellular [^{14}C]FC up to 410-460% of the basal value after approximately 120 min. The accumulation of [^{14}C]FC in the plasma membrane and in the medium occurred without a lag-phase.

The two efflux phases observed with control cells were also detectable in Tangier fibroblasts. However, the increase of plasma membrane and extracellular [^{14}C]FC occurred with a lag phase of approximately 10 min and the maxima were markedly reduced. The maximal increase of the

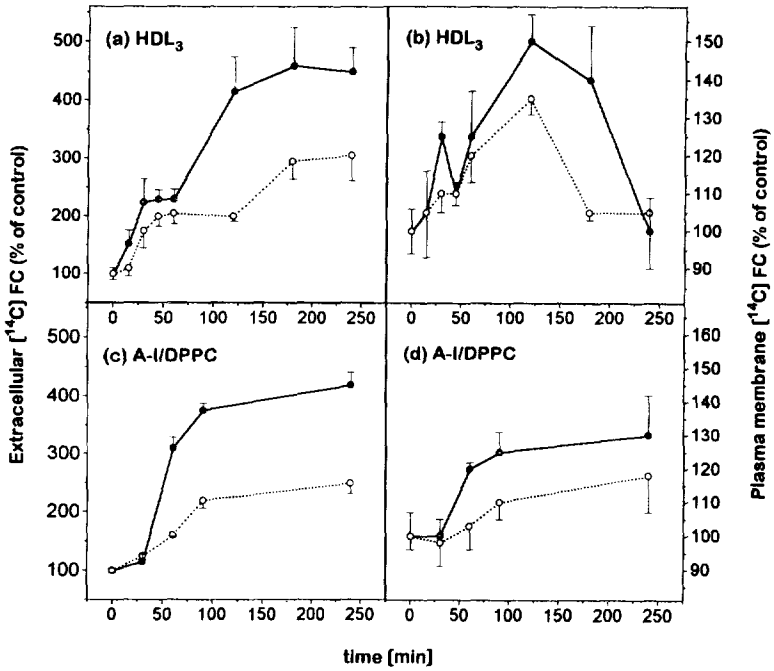

Figure 1. HDL_3- and apo A-I/DPPC-complex-induced mobilization of de novo synthesized cholesterol. Cholesterol-loaded normal (●) and Tangier fibroblasts (○) were pulse-labeled with [^{14}C]mevalonolactone (8 μCi/ml, 0.5 mM) as described in Material and Methods. [^{14}C]FC in the plasma membrane (b,d) and efflux medium (a,c) was measured after a 4 h incubation in the presence of 1 mg/ml FAFA and 50 μg/ml HDL_3 (a,b) or 10 μg/ml apo A-I/DPPC (c,d). The HDL_3 or apo A-I/DPPC was added at the indicated times before stopping the 4 h incubation. The basal levels for [^{14}C]FC in the plasma membrane (in the presence of FAFA alone) were 3,310 ± 868 d.p.m./mg cell protein (control cells) and 4,210 ± 613 d.p.m./mg cell protein (Tangier cells). The basal levels for [^{14}C]FC in the extracellular medium (in the presence of FAFA alone) were 2,480 ± 130 d.p.m./mg cell protein (control cells) and 2,710 ± 667 d.p.m./mg cell protein (Tangier cells). The basal levels were defined as 100%. Values are mean ± S.D. from five incubations on one control proband and one Tangier proband (J.S.). Comparable results were obtained on four other controls and one other Tangier patient (E.G.).

first efflux phase was reduced by 25-55%, the maximal increase of the second phase was reduced by 20-70% (Figs. 1a,b).

To test whether one of the two cholesterol efflux phases is induced by the main protein constituent of HDL_3, apolipoprotein A-I, apo A-I/DPPC-complexes were tested for their ability to enhance the [^{14}C]FC translocation in pulse-labeled fibroblasts. As shown in Figs. 1c and 1d, 10 μg/ml apo A-I/DPPC-complexes enhanced the basal [^{14}C]FC efflux approximately 4-fold and slightly enhanced the amount of [^{14}C]FC in the plasma membrane up to 130% of the basal value. In contrast to HDL_3, the time kinetics of the apo A-I/DPPC-complex-inducible FC efflux was monophasic. In Tangier fibroblasts, the relative increase of plasma membrane and extracellular [^{14}C]FC was delayed and reduced by 40-60% (240 min) in a similar manner to that observed with HDL_3. These data suggest that the molecular defect in Tangier disease is associated with a defect in HDL_3- and apo A-I-mediated cholesterol mobilization.

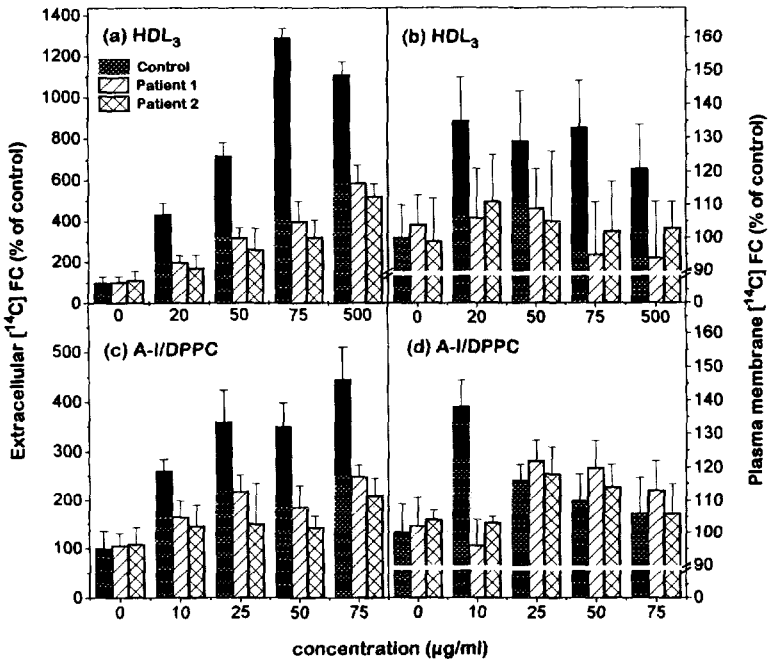

Figure 2. Concentration dependence of the HDL$_3$- and apo A-I/DPPC-complex-induced mobilization of de novo synthesized cholesterol. Cholesterol-loaded normal and Tangier fibroblasts were treated as described in Fig. 1, except that increasing concentrations of HDL_3 (a,b) or apo A-I/DPPC-complexes (c,d) were added for the 4 h incubation period. Values are mean ± S.D. from five incubations on one control proband and two Tangier patients.

To further test this hypothesis, the concentration dependence of the HDL_3- and apo A-I/DPPC-complex-inducible FC mobilization was determined in Tangier fibroblasts and compared with control cells. As shown in Fig. 2, incubation of the cells with HDL_3 or apo A-I/DPPC-complexes resulted in a concentration-dependent increase in the level of [^{14}C]FC in the plasma membrane and extracellular medium. The maximal increase in the level of [^{14}C]FC in the plasma membrane was observed at 10-20 µg/ml HDL_3 or apo A-I/DPPC-complexes. The maximal increase in the level of [^{14}C]FC in the extracellular medium was observed at approximately 75 µg/ml HDL_3 or apo A-I/DPPC-complexes. In cells from the Tangier patients, cholesterol translocation and efflux were significantly reduced over a wide concentration range, as indicated in Fig. 2. Moreover, the concentration maxima were shifted slightly to the right. These data demonstrate that the translocation of *de novo* synthesized FC to the cell membrane and extracellular medium is impaired, but not totally absent in Tangier fibroblasts.

Labeling the plasma membrane with [^{14}C]FC we examined whether the nonspecific efflux of plasma membrane FC to the extracellular medium is impaired in Tangier fibroblasts. As shown in Fig. 3, the [^{14}C]FC efflux from the plasma membrane to HDL_3 was not significantly different in normal and Tangier cells.

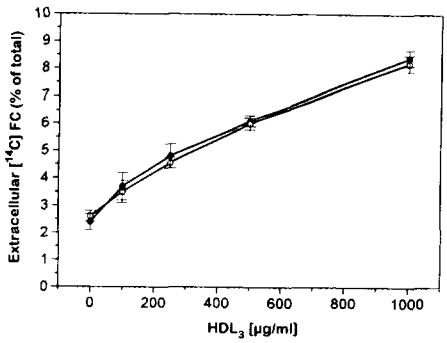

Figure 3. Concentration dependence of the HDL_3-induced efflux of plasma membrane cholesterol. Cholesterol-loaded normal (●) and Tangier fibroblasts (○) were pulse-labeled with 0.3 µCi/ml [^{14}C]FC for 2 h at 15°C. Efflux of [^{14}C]FC was measured at 37°C in the presence of 1 mg/ml FAFA and the indicated protein concentrations of HDL_3 during a 4 h incubation, as described in Material and Methods. Results represent the mean ± S.D. of values for medium [^{14}C]FC from three or four incubations, expressed as percentage of the total cellular [^{14}C]FC.

DISCUSSION

We demonstrate here that the efflux of [^{14}C]FC synthesized from [^{14}C]-mevalonolactone in response to exogenously added HDL_3 or apolipoprotein AI proteoliposomes is markedly lower in fibroblasts from two patients with Tangier disease than in controls. A possible explanation for this result is that the metabolic incorporation of [^{14}C]-mevalonolactone was lower in the cells from the patient than in the normal cells. However, since neither the total amount of radioactivity incorporated into FC nor the specific activities measured in the cells and in the extracellular medium were significantly different in the two types of cells, this explanation can be excluded. Another possible explanation is that the desorption of FC from the plasma membrane and its subsequent transfer to extracellular lipid acceptors is disturbed. This explanation is also unlikely because no significant difference of HDL_3-induced FC efflux was observed when the plasma membrane was labeled with exogenously added [^{14}C]FC. In addition, we did not observe an accumulation of radiolabeled FC in the plasma membrane of the Tangier fibroblasts when [^{14}C]-mevalonolactone was used as the radiolabeled cholesterol precursor. Thus, the most likely explanation for our results is that the increased flux of *de novo* synthesized FC from intracellular pools to the plasma membrane observed in normal cells in response to HDL_3 or apolipoprotein AI proteoliposomes is disturbed in Tangier disease.

The abnormal structure and the hypercatabolism of HDL particles observed in Tangier patients may be secondary to the impaired HDL_3-mediated mobilization of intracellular cholesterol. Since normal HDL particles are virtually absent in the Tangier patients described here (3,6), our findings suggest that the specific HDL-mediated mobilization of intracellular FC may be an important step in the maturation of HDL particles. This hypothesis is further supported by other reports which indicate that early steps in the maturation of HDL particles are dependent upon specific interactions between HDL precursors and competent peripheral cells (5,17). The exact

cellular defect in Tangier disease, however, remains unclear. A disturbance at the level of the plasma membrane, a defect in the cellular signal transduction pathway or in the intracellular cholesterol transport machinery itself are possible causes of this disorder.

We performed our experiments in the presence of an ACAT-inhibitor to ensure sufficient incorporation of radiolabel into the FC available for transfer to the plasma membrane and HDL_3-induced efflux. Therefore, we cannot determine if the reduced HDL_3-induced flux of *de novo* synthesized FC to the plasma membrane might explain the increased rate of cholesterol esterification observed in cells from patients with Tangier disease (4).

In normal and in Tangier cells, the HDL_3-induced mobilization of cellular FC was biphasic, whereas the apo A-I/DPPC-complex-induced FC efflux was monophasic. This indicates that the specific HDL_3-mediated cholesterol efflux is a complex phenomenon. The existence of different HDL receptors, as suggested by Morrison et al. (18), the involvement of different agonists interacting with the same receptor, as suggested by Barkia et al. (19), or the contribution of different intracellular cholesterol pools, as suggested by Rothblat (20) could explain the observed kinetics.

Acknowledgment: We thank Dr. Paul Cullen for critical reading of the manuscript.

REFERENCES

1. Assmann, G., von Eckardstein, A., and Brewer, B. (1994) In: The Metabolic Basis of Inherited Disease (C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, eds.), *in press*.
2. Assmann, G., Simantke, O., Schaefer, H. E. and Smootz, E. (1977) *J. Clin. Invest.* **60**, 1025-1035.
3. Assmann, G., Smootz, E., Adler, K., Capurso, A. and Oette, K. J. (1977) *J. Clin. Invest.* **59**, 565-575.
4. Schmitz, G., Fischer, H., Beuck, M., Hoecker, K.-P. and Robenek, K. (1990) *Arteriosclerosis* **10**, 1010-1019.
5. Castro, G. R., and Fielding, C. J. (1988) *Biochemistry* **27**, 25-29.
6. Assmann, G., Herbert, P. N., Fredrickson, D. S. and Forte, T. (1977) *J. Clin. Invest.* **60** 242-252.
7. Walter, M., Kerber, S., Fechtrup, C., Seedorf, U., Breithardt, G., and Assmann, G. (1994) *Atherosclerosis*, in press.
8. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345-1353.
9. Chen, C., and Albers, J. J. (1982) *J. Lipid Res.* **23**, 680-691.
10. von Eckardstein, A., Funke, H., Walter, M., Altland, K., Benninghoven, A., and Assmann, G. (1990) *J. Biol. Chem.* **265**, 8610-8617.
11. Mendez, A. J., Oram, J. F., and Bierman, E. L. (1991) *J. Biol. Chem.* **266**, 10104-10111.
12. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497-509.
13. Lange, Y., and Ramos, B. V. (1983) *J. Biol. Chem.* **258**, 15130-15134.
14. Hara, A., and Radin, N. S. (1978) *Anal. Biochem.* **90**, 420-426.
15. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254.
16. Heider, J. G., and Boyett, R. L. (1978) *J. Lipid Res.* **19**, 514-518.
17. Huang, Y., von Eckardstein, A., and Assmann, G. (1993) *Arterioscler. Thromb.* **13**, 445-458.
18. Morrison, J. R., McPherson, G. A.., and Fidge, N. H. (1992) *J. Biol. Chem.* **267**, 13205-13209.
19. Barkia, A., Puchois, P., Ghalim, N., Torpier, G., Barbaras, R., Ailhaud, G., and Fruchart, J.-C. (1991) *Atherosclerosis* **87**, 135-146.
20. Mahlberg, F. H., and Rothblat, G. H. (1992) *J. Biol. Chem.* **267**, 4541-4550.