Expression of the ATP-Binding Cassette Transporter Gene ABCG1 (ABC8) in Tangier Disease

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 Several members of the ATP-binding cassette (ABC) transporter family are involved in cholesterol efflux from cells. A defect in one member, ABCA1, results in Tangier disease, a condition characterized by cholesterol accumulation in macrophages and virtual absence of mature circulating high-density lipoproteins. Expression of a second member, ABCG1, is increased by cholesterol-loading in human macrophages. We now show that ABCG1, which we identified by differential display RT-PCR in foamy macrophages, is overexpressed in macrophages from patients with Tangier disease compared to control macrophages. On examination by confocal laser scanning microscopy, ABCG1 was present in perinuclear structures within the cell. In addition, a combination of *in situ* hybridization and indirect immunofluorescence microscopy revealed that ABCG1 is expressed in foamy macrophages within the atherosclerotic plaque. These data indicate that not only ABCA1 but also ABCG1 may play a role in the cholesterol metabolism of macrophages *in vitro* and in the atherosclerotic plaque.   © 2001 Academic Press

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ATP-binding cassette (ABC) transporters form a large gene family that is highly conserved in evolution (1–3). The products of this family use ATP to actively transport many different substrates including nutrients, endogenous toxins, xenobiotic compounds, peptides and organic and inorganic ions across cell membranes in prokaryotes and eukaryotes (2–4). All ABC transporters contain a well-defined nucleotide binding fold, the eponymous ATP-binding cassette (ABC) of about 200 amino acids, first characterized by Walker in 1982 (5). Each transporter is thought to be relatively specific for a single substrate and consists of four domains: two ATP-binding domains and two hydrophobic parts, each of which consists of five or six transmembrane domains (1, 3, 6).

Within the past 2 years, it was shown by our institute (7, 8) and by others (9, 10) that Tangier disease, a rare form of lipid abnormality characterized by low levels of high density lipoprotein cholesterol, cholesterol accumulation in macrophages and, possibly in some cases, premature atherosclerosis, is due to mutations in the ABCA1 gene which codes for the ABCA1 protein (synonym ABC1). The exact function of ABCA1 is unknown; however, its expression was found to be increased upon cholesterol loading in macrophages from healthy controls but not in macrophages from patients with Tangier disease (8). In addition, a recent report showed that ABCA1 might be involved in absorption of cholesterol from the gut, and thus in regulation of whole-body cholesterol balance in humans (11).

In a further recent report, another member of the ABC transporter family, ABCG1 (synonyms: ABC8, human homologue of *Drosophila* white), encoded by the ABCG1 gene, as well as many other ABC genes were shown to be regulated by cholesterol loading in macrophages (12). The half transporter ABCG1, a member of the ABC transporter subfamily G, was first described

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by Chen *et al.* (13) and independently by Croop *et al.* (2) and Savary *et al.* (14) as a homologue of the *Drosophila* white protein. A further ABC transporter that is expressed in the trachea of *Drosophila melanogaster* shows higher homology to the human ABCG1 gene product than the *Drosophila* white protein (15). As an interesting historical point, the *Drosophila white* gene was the first locus mapped by Thomas Hunt Morgan, the founder of modern experimental genetics, in 1910 (16). Recently, Venkateswaran *et al.* showed that ABCG1 mRNA levels in human macrophages are regulated by the liver-specific X receptor/retinoid X receptor (LXR/RXR) pathway (17), which recently has been shown to play an important role in ABCA1-mediated lipid metabolism (11) and in the regulation of mRNA expression of apolipoprotein E (apoE) (18), cholesterol ester transfer protein (CETP) (19), and sterol regulatory element-binding protein-1c (SREBP-1c) (20).

In this report we confirm the cholesterol-dependent regulation of ABCG1 in human macrophages and now show that ABCG1 is upregulated in macrophages isolated from individuals with Tangier disease even without loading of these cells with cholesterol.

MATERIALS AND METHODS

Macrophage culture. Monocytes were isolated from the peripheral blood of six healthy volunteers and of two patients with Tangier disease by cell separation and countercurrent elutriation and cultured as previously described (21). The local Hospital Ethics Committee approved all procedures. The diagnosis of Tangier disease was confirmed in each case by the demonstration of a mutation in the ABCA1 gene. The purity of monocyte fractions was tested by flow cytometry (FACScan, Becton–Dickinson, Heidelberg, FRG) and only fractions containing more than 95% monocytes were pooled for further use.

ApoE genotyping. We have previously shown that the apoE phenotype may influence the cholesterol metabolism of human macrophages (22). We therefore ensured that all donors were homozygous for the apoE3 allele. We performed ApoE genotyping by mutagenically separated PCR as previously described (23).

Isolation and chemical modification of lipoproteins. Low-density lipoprotein (LDL) was obtained from human plasma from healthy volunteer donors by sequential ultracentrifugation (d = 1.019–1.063) for preparation of acetylated (ac-) LDL (24) and by density gradient centrifugation for preparation of oxidized (ox-) LDL (25). LDL protein concentration was determined using a modified method described by Hartree (26). Cholesterol content of LDL and chemically modified LDL was measured by the CHOD-PAP method (Roche Molecular Biochemicals, Mannheim, FRG). Acetylated LDL (acLDL) was obtained by repeated additions of acetic anhydride to LDL as described by Basu *et al.* (24). OxLDL was prepared by oxidation at 37°C for 400 min by the addition of $CuSO_4$ (final concentration 6 μM) to 400 μg LDL per mL (protein concentration) using a modification of the method of Esterbauer *et al.* (25) and used immediately.

Formation of foam cells in vitro and HPLC analysis of lipid content. To generate foam cells, macrophages were loaded with cholesterol by incubation with serum-free RPMI supplemented with 80 μg acLDL (protein concentration) per mL medium or 80 μg oxLDL (protein concentration) per mL medium for 48 h. Control macrophages were incubated in RPMI alone for the same period. Cells were harvested in 2 mL 0.9% (w/v) sodium chloride solution for lipid

analysis or in RNA extraction buffer (Qiagen, Hilden, FRG). Cellular cholesterol and cholesteryl esters were analyzed by high performance liquid chromatography as previously described (27).

mRNA differential display PCR. Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen). Contaminating DNA was removed by digestion with DNase using the MessageClean kit (Gene Hunter, Brookline, MA). Messenger RNA differential display PCR (DD-PCR) was performed according to the instruction manual of the Differential Display PCR kit (MoBiTec, Göttingen, FRG) on a Gene-Amp 9600 (Applied Biosystems, Norwalk, CT) using $T_{12}AA$ as downstream primer and 24 different upstream primers. DD-PCR samples were separated using 6% (w/v) denaturing long-run polyacrylamide sequencing gels (Genomyx, Foster City, CA) that were examined by exposition to BioMax MR-1 films (Eastman Kodak Co., Stuttgart, FRG). To exclude spurious differences in band pattern (false positives, spurious true positives), all experiments were performed and examined using cells from two different donors as previously described (28).

Bands showing differential expression in foam cells versus non-cholesterol-loaded cells were eluted from the gel as previously described (28) and reamplified using appropriate primers extended with a nonamer nucleotide sequence using a modification of the technique described by Wang and Feuerstein (29). The PCR products were cloned into pCRII vectors for sequencing using the TA Dual Promoter Cloning Kit (Invitrogen, Lek, The Netherlands). Plasmid DNA was sequenced on an automatic laser fluorescence sequence analyzer (ABI Prism 377, Applied Biosystems). Sequences were identified by database searching using the BLAST algorithm (30).

Northern blot analysis. Results of DD-PCR analysis were confirmed by Northern blot analysis using standard methods and 15 μg of total RNA per slot were loaded onto the gel. The digoxigenin-labeled probe used for detection consisted of the entire differential display fragment and was prepared by random priming. Lane-loading differences were normalized with a probe specific for the mRNA of the housekeeping gene glycerin aldehyde phosphate dehydrogenase (GAPDH).

Real-time RT-PCR. Five micrograms of total RNA were transcribed to cDNA using SuperScript II reverse transcriptase (Gibco BRL Life Technologies, Karlsruhe, FRG) and an oligo(dT)$_{12–18}$ primer (Gibco). Real-time PCR analysis was performed using the SYBR Green PCR Reagents Kit (Applied Biosystems) with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions. Each PCR amplification sample of cDNA was then performed with the cDNA derived from 50 ng total RNA. For amplification of ABCG1 cDNA, the oligonucleotides used were 5′-ccg acc gac gac aca gag a-3′ and 5′-ctg agc acg aga cac cca ca-3′; amplification of 18S ribosomal RNA cDNA, the internal standard, was performed using the primers 5′-cgg cta cca cat cca gga a-3′ and 5′-gct gga tta ccg cgg ct-3′. Each sample was measured in triplicate and a blank containing no template cDNA was used as a negative control.

Generation of antibodies. Polyclonal antisera against amino acids 611–625 and amino acids 47–61 of the human ABCG1 protein (13) were generated in rabbits using the keyhole limpet hemocyanin-coupled antigen (Eurogentec, Seraing, Belgium). The ABCG1 antisera were purified by affinity-chromatography. Therefore ABCG1 peptides (same as used for immunization of rabbits) were coupled to Sulfolink beads (Pierce, Rockford, IL) according to manufacturer's instructions. The peptide-specific antibodies were obtained from the serum using standard methods as previously described (31). Optimal titers for Western blot analysis, immunocytochemistry and immunohistochemistry were determined by titration assays.

Western blot analysis. Cells were washed, lysed in SDS polyacrylamide gel electrophoresis (PAGE) buffer containing 5% (v/v) 2-mercaptoethanol, and analyzed by SDS–PAGE on 12.5% (w/v) polyacrylamide gels. Proteins were transferred overnight at 4°C onto

nitrocellulose membranes (Bio-Rad Laboratories, München, Germany) and probed with rabbit anti-human ABCG1 antibodies (Eurogentec) after blocking in a solution of 1% (w/v) BSA in TBS with 0.05% (v/v) Tween 20 overnight at 4°C. Horseradish peroxidase donkey anti-rabbit antibody conjugate was used for detection (Amersham Pharmacia Biotech, Freiburg, FRG). Visualization was performed with the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). The chemiluminescence image was recorded on Hyperfilm MP (Amersham Pharmacia Biotech).

Indirect immunofluorescence microscopy. Cells were cultured on glass cover slips, washed, fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), washed again with PBS and 50 mM NH_4Cl in PBS and permeabilized as required using 0.1% (v/v) Triton X-100 in PBS. The cells were washed and blocked with 0.2% (v/v) fish gelatin in PBS for 40 min at room temperature. Thereafter, the cells were incubated for 60 min with the rabbit anti-ABCG1 antibody (Eurogentec) in 0.2% (v/v) gelatin in PBS. The cells were washed several times and incubated for 40 min in a solution containing the fluorescently labeled goat anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands) in 0.2% (v/v) gelatin in PBS. Finally, the slips were washed several times and mounted onto slides using the ProLong Antifade Kit (Molecular Probes). Samples were examined directly using an Olympus Fluoview IX 70 confocal laser scanning microscope (Olympus Optical Co. Europe, Hamburg, FRG).

In situ hybridization studies in human coronary artery. Biotin and digoxigenin (DIG) labeling of a 300 bp ABCG1 antisense RNA probe was carried out according to manufacturer's instructions using the Biotin-DIG-RNA Labeling Kit (Roche Molecular Biochemicals). Human coronary artery was obtained from hearts which were removed during heart transplantation and which were dissected on ice and frozen in liquid nitrogen within 15 min after explantation. The artery explantation procedure was approved by the local Hospital Ethics Committee. *In situ* hybridization was performed as previously described (32) with modifications as described in reference (33).

Indirect immunofluorescence. Sections were fixed in 4% (w/v) paraformaldehyde in PBS, washed copiously in PBS, and incubated in 0.2% (v/v) Tween 20 in PBS for 3 min. The sections were blocked with 1% BSA in PBS and incubated with the primary antibodies diluted in 1% (w/v) BSA in PBS for 45 min at room temperature. ABCG1 was detected using the anti-ABCG1 peptide antibodies described above; macrophages were identified using mouse antibodies to CD68 as previously described (34). After copious washing in PBS, the sections were incubated with fluorescently labeled secondary antibodies (goat anti-rabbit F(ab')2-Cy2 conjugates (Dianova, Hamburg, FRG) for detection of ABCG1 antibodies; goat anti-mouse F(ab')2-Cy3 conjugates (Dianova) for detection of CD68 antibodies) diluted in 1% (w/v) BSA in PBS for 30 min at 37°C. The cell nuclei were stained with Hoechst nuclear dye 3258 and mounted in fluorescence mounting medium (DAKO, Hamburg, FRG).

RESULTS

In Vitro Foam Cell Formation

Incubation of the human monocyte-derived macrophages with 80 μg acLDL per mL medium increased the cellular content of free and esterified cholesterol, while in the control cells incubated in lipid-free medium the cellular cholesterol content decreased during the incubation period (Figs. 1A and 1B).

Identification and Regulation of ABCG1 in Normal Macrophages

A band with clearly increased amplification in acLDL-treated foam cells on DD-PCR (Fig. 1C) con-

tained a 628 bp fragment showing 99% homology to the 3′-UTR of the human ABCG1 mRNA. The time-dependent upregulation of ABCG1 mRNA expression during *in vitro* foam cell formation was confirmed by Northern blot analysis (Fig. 1D) and Western blot analysis (Fig. 1E). Expression of ABCG1 in normal macrophages was also increased by incubation with mildly oxidized LDL as measured by real-time PCR (data not shown).

In Vitro Foam Cell Formation in Tangier Macrophages, Effect on Expression of ABCG1

Macrophages from two well-characterized patients with Tangier disease were examined. Patient TD1 has been shown to express a completely nonfunctional ABCA1 protein which is truncated at amino acid position 935 of the primary translation product after the first ATP-binding cassette because of a base deletion leading to a premature stop codon, while patient TD2 has a missense mutation in the domain coding for the first ATP-binding cassette of his ABCA1 protein (data not shown).

Loading with acLDL increased the cholesterol content of the cells from patient TD1 3.2-fold while increasing the cholesterol content of the control cells 3.5-fold (Figs. 2A and 2B). In the non-cholesterol-loaded macrophages from patient TD1 the level of ABCG1 mRNA was approximately equal to that of the cholesterol-loaded control macrophages (Fig. 2C). While the ABCG1 mRNA level in the control macrophages increased 1.7-fold upon loading of the cells with acLDL, ABCG1 mRNA levels in the cells from patient TD1 did not change under these conditions (Fig. 2C).

In cells from patient TD2, loading with acLDL increased the cholesterol content 3.4-fold, while the cholesterol content of control cells in this experiment were increased 4.9-fold by loading with acLDL (Figs. 2A and 2B). In the non-cholesterol-loaded macrophages from patient TD2 the level of ABCG1 mRNA was approximately 3.5 times greater than that of the cholesterol-loaded control macrophages (Fig. 2C). While the ABCG1 mRNA level in the control macrophages increased 8.0-fold upon loading of the cells with acLDL, ABCG1 mRNA levels in the cells from patient TD2 increased only 5.5-fold under these conditions (Fig. 2C).

Of note in these experiments was the significantly higher cholesteryl ester content of the nonloaded macrophages from both Tangier disease patients (6.9-fold greater than control for patient TD1 and 8.1-fold greater than control for patient TD2; Fig. 2B).

Expression of ABCA1 in Tangier Macrophages

For comparison purposes, the levels of ABCA1 mRNA were also measured in the above experiments. Whereas in the macrophages of healthy donors the
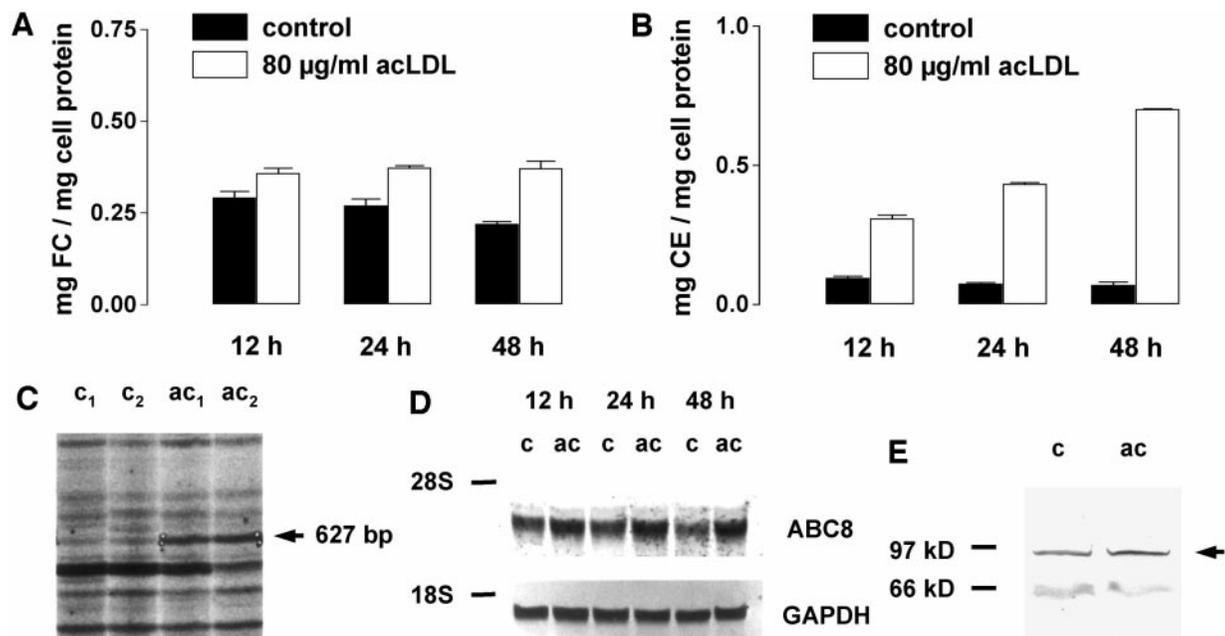
FIG. 1. Identification and expression of ABCG1 mRNA during foam cell formation. HPLC analysis of cellular content of free cholesterol (A) and cholesteryl ester (B) in human monocyte-derived macrophages. Incubation of human macrophages with 80 μg/ml acLDL for the times shown increased the cellular content of free cholesterol and cholesteryl ester, whereas the cholesterol content of the non-cholesterol-loaded cells decreased over time. (C) mRNA differential display PCR identifies ABCG1. Amplification by differential display PCR of cDNA isolated from 14-day-old human monocyte-derived macrophages derived from two donors and incubated for 48 h in the presence of RPMI 1640 cell culture medium alone (lanes C1 and C2) or RPMI 1640 containing 80 μg/mL acLDL (lanes Ac1 and Ac2). The arrow indicates a band that is more strongly expressed in the cells loaded with acLDL than in the control cells. This band was isolated and amplified as described under Materials and Methods section. On sequencing, the band was found to consist of a 628-bp fragment with more than 99% homology to the 3'-untranslated region of the human ABCG1 mRNA. (D) Cholesterol loading increases expression of ABCG1 mRNA in a time-dependent manner. Human macrophages were incubated with 80 μg/ml acLDL for the indicated times and the levels of ABCG1 mRNA were assessed by Northern blot analysis. Over time, ABCG1 expression increases in cholesterol-loaded cells but decreases in the control macrophages as quantified by densitometric scanning of the Northern blot shown. (E) Cholesterol loading increases expression of ABCG1 protein. Human macrophages were incubated for 48 h with 80 μg/ml acLDL and cellular production of ABCG1 protein was measured by Western blot analysis. Expression of ABCG1 protein was increased in the acLDL-treated cells.

levels of ABCA1 mRNA were slightly increased by cholesterol loading (1.2-fold in TD1-control, 1.7-fold in TD2-control), in the Tangier macrophages, expression of ABCA1 was not changed by loading the cells with cholesterol (data not shown).

Expression of ABCG1 in Human Atherosclerosis

Examination of human coronary artery by *in situ* hybridization using an antisense probe specific for ABCG1 mRNA showed that this mRNA was widely expressed throughout atherosclerotic intima. A further striking feature was the strong expression of ABCG1 mRNA within neural bundles in the adventitia of the artery.

Combined indirect immunofluorescence microscopy and *in situ* hybridization revealed that ABCG1 mRNA colocalized with foamy cells expressing the macrophage-specific marker CD68 (Fig. 3) in the atherosclerotic plaque of coronary arteries. This colocalization of ABCG1 and foamy macrophages was confirmed by double labeling studies using an antibody directed toward

amino acids 47–61 of the ABCG1 protein and antibodies to CD68 (Fig. 4). Based on the putative structure of the ABCG1 protein (2, 13), these amino acids are predicted to be located intracellularly between the amino terminus and the Walker A motif (5).

Expression Patterns of ABCG1 in Macrophages

The intracellular localization of ABCG1 in macrophages was examined by indirect confocal laser scanning immunofluorescence microscopy using an antibody directed toward the putative extracellular segment of the loop joining the last two putative transmembrane domains of ABCG1 (amino acids 611–625). Investigation of permeabilized macrophages revealed cytoplasmic staining of structures in the perinuclear region (Fig. 5). This investigation showed further that ABCG1 sometimes seems to be located on the cell surface of non-permeabilized human macrophages (data not shown). Immunocytochemical staining of permeabilized and non-permeabilized human fibroblasts re-
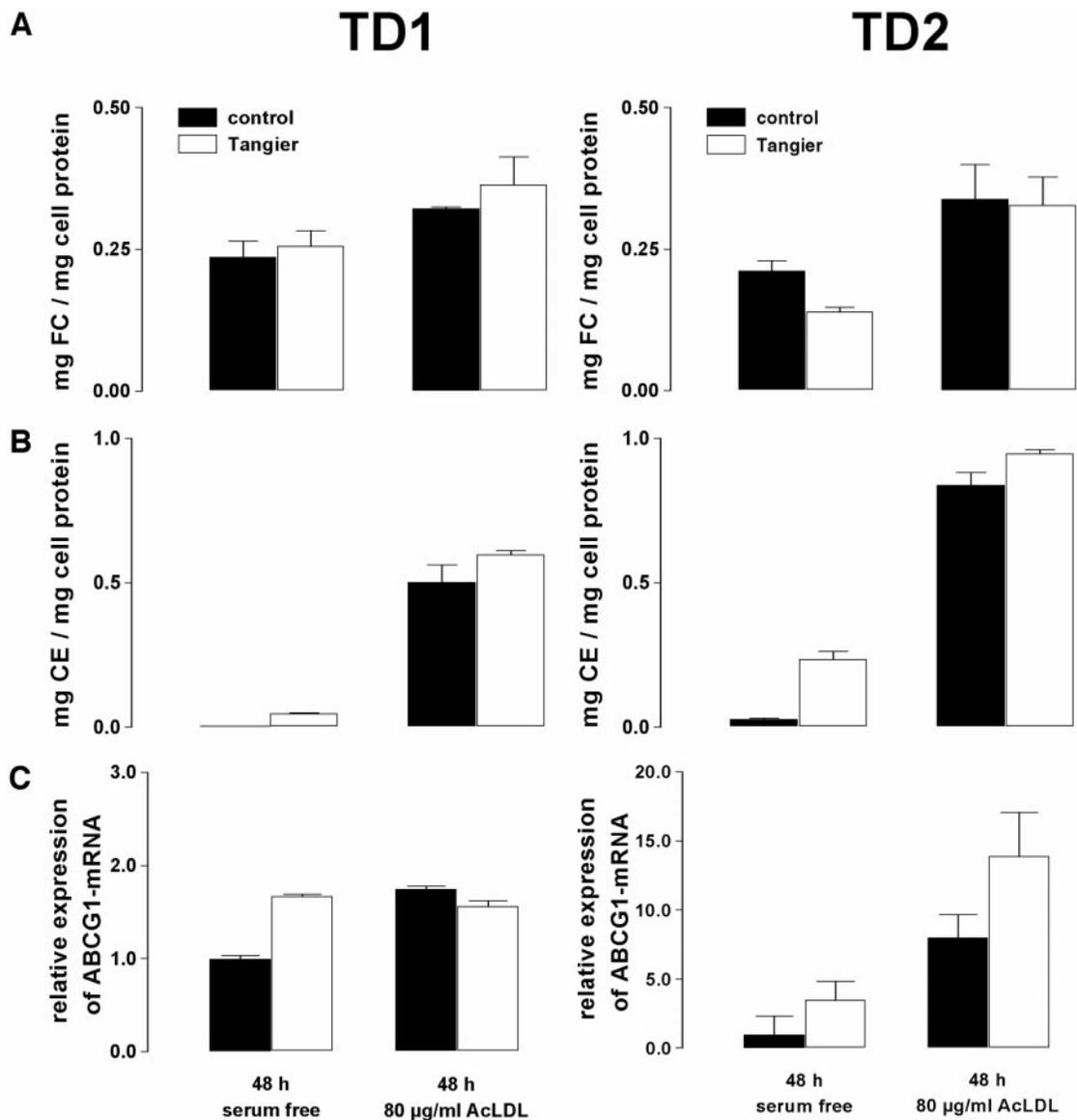
FIG. 2. Levels of ABCG1 mRNA, free cholesterol and cholesteryl ester in human monocyte-derived macrophages from two patients with Tangier disease and two normal controls. Levels of free cholesterol (A) and cholesteryl ester (B) in macrophages from two patients with Tangier disease (TD1 and TD2) and two normal controls as measured by HPLC. The cells from both Tangier patients contained more total cholesterol, free cholesterol and cholesteryl ester both before and after loading by incubation with 80 μg/ml acLDL for 48 h. (C) Levels of ABCG1 mRNA in macrophages from two patients with Tangier disease and two normal controls. Levels of ABCG1 mRNA in monocyte-derived macrophages from two patients with Tangier disease and two normal controls as measured by real-time reverse transcription polymerase chain reaction after loading for 48 h with 80 μg/mL acetylated low density lipoprotein and after incubation for 48 h with serum-free cell culture medium. Levels of ABCG1 mRNA were significantly greater in non-cholesterol-loaded macrophages from both Tangier patients and increased significantly on cholesterol-loading in Tangier patient TD2 (C).

vealed only a cytoplasmic staining of intracellular structures (data not shown).

DISCUSSION

Recently, it was concluded that ABCG1 also participates in cholesterol transport in macrophages (12, 17); we now extend these results to show by confocal laser microscopy that ABCG1 is expressed both on the sur-face and in perinuclear structures within the cytoplasm of human monocyte-derived macrophages. We also show that macrophage-derived foam cells in the atherosclerotic plaque express ABCG1. In normal macrophages we demonstrate that ABCG1 expression is increased by cholesterol loading in accordance with results recently reported by Klucken *et al.* (12). Moreover, we now show that in non-cholesterol-loaded macrophages from individuals with Tangier disease, the
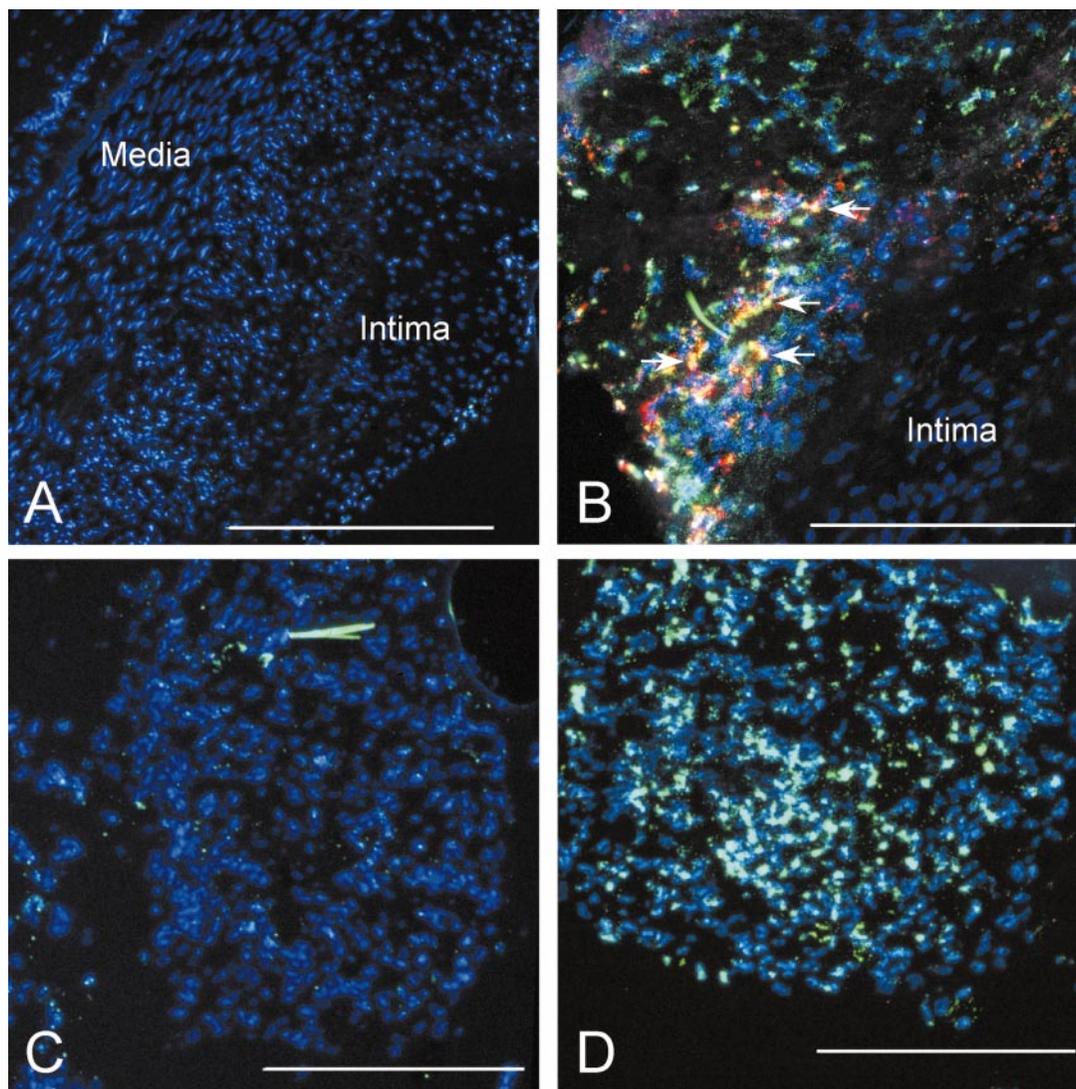
FIG. 3. ABCG1 mRNA is expressed by macrophage-derived foam cells in the human atherosclerotic plaque and by nerve bundles within the adventitia. Sections of human coronary artery were stained by *in situ* hybridization using an antisense RNA probe directed against ABCG1 mRNA. Macrophages were identified using a monoclonal antibody directed toward the macrophage marker protein CD68. Nuclei were visualized using Hoechst nuclear stain 3258 and are shown in blue. (A) Negative control for detection of ABCG1 mRNA (sense RNA probe). (B) Expression of ABCG1 mRNA in the plaque core by foamy macrophages. Macrophage-specific anti-CD68 staining is shown in red. ABCG1 mRNA is shown in bright green. The yellow staining is produced by the overlap of green ABCG1 mRNA stain and red macrophage CD68 stain (arrows). (C) Nerve bundle stained with ABCG1 sense RNA (negative control). (D) ABCG1 mRNA is strongly expressed in the nerve bundles in the adventitia of human coronary arteries (green color). Size bars: 500 μm.

level of ABCG1 expression is increased compared to that in non-cholesterol-loaded macrophages from healthy volunteers.

It has been shown that loading of macrophages with cholesterol also induces upregulation of several other members of the ABC family in addition to ABCA1 and ABCG1, notably the full transporters ABCA7, ABCC5 which encodes the multidrug resistance protein 5 (MRP5) and ABCB11 which encodes the bile salt export protein (BSEP) (12, 35). Inhibition of ABCG1 by antisense oligonucleotides has been shown to reduce HDL-mediated cholesterol efflux (12), while the role of BSEP and MRP5 in cholesterol efflux is not known.

Inactivity of ABCA1 whether in humans in the form of Tangier disease (36) or familial HDL deficiency (37) or in the ABCA1 knockout mouse (38) leads to virtually complete absence of normal HDL, although minor HDL subfractions such as preβ1-HDL are normal (38, 39).

Thus, current results indicate that ABCA1 is necessary for the formation of mature HDL from precursor particles and for efficient cholesterol efflux from cells. However, expression of ABCA1 alone may not be sufficient for this purpose, since other ABC transporters are upregulated by cholesterol loading and since blocking ABCG1 protein expression with antisense oligonucleotides decreases cholesterol efflux to HDL (12). In
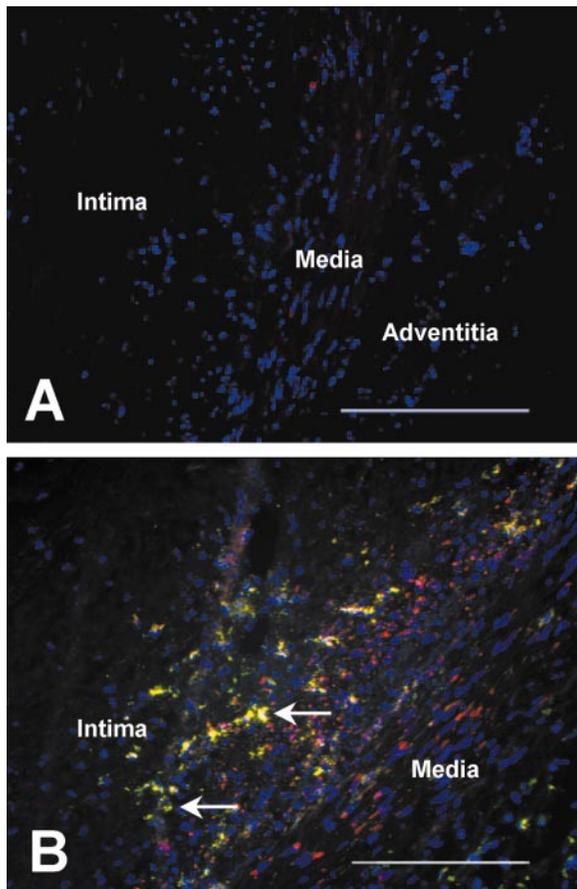
FIG. 4. ABCG1 protein is expressed by macrophage-derived foam cells in the human atherosclerotic plaque. (A) Section of human coronary artery performed without primary antibody as negative control. (B) Section of human coronary artery stained by indirect immunofluorescence using primary antibodies against ABCG1 and the macrophage-specific marker CD68. ABCG1 is shown in dark-green and CD68 in red, the colocalization of these markers appears as bright yellow staining. Size bars: 200 μm.

this context, our finding of an upregulation of ABCG1 in non-cholesterol-loaded Tangier macrophages may indicate a compensatory response in these cells to the absence of normal efflux via the putative ABCA1-mediated pathway.

As described above, non-cholesterol-loaded Tangier macrophages contained more cholesterol than non-cholesterol-loaded control macrophages. Moreover, the intracellular cholesterol content of non-cholesterol-loaded control macrophages decreased over an incubation period of 48 h in the absence of cholesterol acceptors. This decrease was less marked in macrophages derived from patients with Tangier disease, which may indicate that ABCA1 is also involved in cholesterol efflux that is not mediated by HDL. We have previously shown that macrophages export cholesterol in the absence of acceptors such as HDL, possibly by an apoE-linked mechanism (22). In recent studies Venkateswaran and colleagues have shown that expres-

sion of ABCG1 is increased by oxysterols and by expression of the nuclear oxysterol receptor LXRα in RAW264.7 macrophages (17, 40), and that stimulation of the LXRα receptor also increases expression of apolipoprotein E (18). Moreover, in Tangier fibroblasts, residual cholesterol efflux could be induced by incubation of the cells with oxysterols (17).

These data agree with our finding of an upregulation of ABCG1 by mildly oxidized LDL and indicates that ABCG1 may be implicated in apoE-mediated cholesterol efflux from macrophages.

The mechanism by which ABCG1 functions in cholesterol efflux is not known. Although it is present on the surface of cells, we have found that it is also located within the cell in perinuclear structures. Klucken *et al.* also localized ABCG1 to the surface of cells and to perinuclear membranes (12), while other report finding it only in perinuclear membranes and not on the cell surface (40). It is possible that at least a part of the apparent localization of ABCG1 to the surface of macrophages may be related to the binding of anti-ABCG1 antibodies to the FC-γ receptors on the macrophage surface. This hypothesis is supported by the observation that in fibroblasts ABCG1 was located only within the cell and not on the cell surface. Whether or not ABCG1 is present on the cell surface of macrophages, the intracellular location of this protein in macrophages and fibroblasts may indicate that it is also involved in intracellular vesicular trafficking, which may be related to mobilization of intracellular cholesterol stores.

The marked expression of ABCG1 in nerve bundles is intriguing. Peripheral neuropathy is a prominent feature of Tangier disease. During nerve injury and regeneration, cholesterol is stored and redistributed in a coordinated mechanism that is known to involve macrophages, apoE and HDL (41). It is possible, therefore, that the ABC transporters may also have a role in maintaining nervous viability.

Reduction of the cholesterol content of the atherosclerotic plaque in the coronary arteries has been shown to stabilize the lesion and lessen the risk of myocardial infarction (42). In this context, our finding that ABCG1 is expressed by macrophage foam-cells in the plaque is of relevance, since this may be a means of cholesterol removal from the atherosclerotic lesion.

Taken together, our findings and those of the literature indicate that at least two pathways of ABC transporter-associated cholesterol efflux exist, a major pathway mediated via ABCA1 and HDL and a minor pathway that operates even in the absence of either ABCA1 or HDL. ABCG1 has been shown to contribute to HDL-mediated efflux. However, the contribution of ABCG1 or of other ABC transporters such as BSEP or MRP5 to the minor efflux pathway is not known. It has been suggested that pharmacological modulation of ABCA1 thereby increasing HDL concentration may
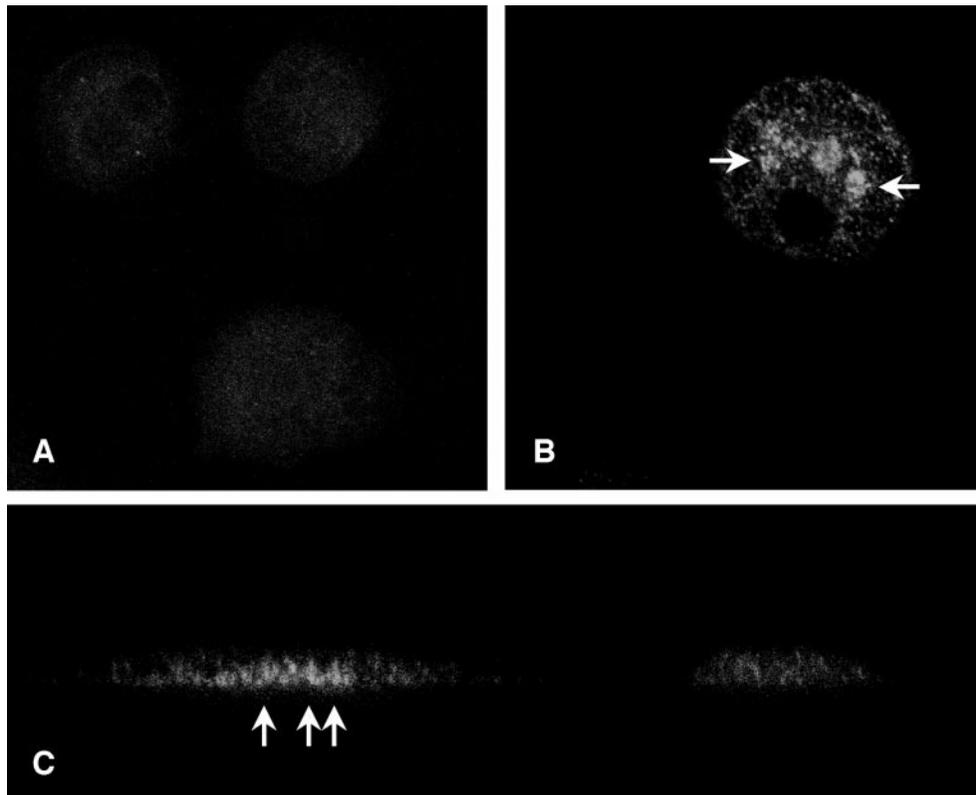
FIG. 5. ABCG1 is expressed in tubular perinuclear structures within macrophages. (A) Human monocyte-derived macrophages stained by immunofluorescence without primary antibody (negative control). (B) Macrophages were permeabilized, stained by immunofluorescence with a primary antibody directed against human ABCG1 (amino acids 611–625), and examined by confocal laser scanning microscopy. ABCG1 is expressed in structures arranged around the cell nucleus (arrows). The cells were scanned in the horizontal plane (cell diameter 10 μm). (C) Human macrophage stained by immunofluorescence as described in B. The arrangement of the ABCG1 protein near the cell nucleus is similar to that shown in B. The cells were scanned in the coronal plane (cell height about 18 μm).

have potential as an anti-atherogenic therapy (37). It is now becoming clear that ABC transporter-mediated cholesterol efflux is more complex than originally thought and that several members of this gene family may be potential therapeutic targets. The expression of ABCG1 by macrophages in the atherosclerotic plaque may be of relevance in this regard.

ACKNOWLEDGMENTS

REFERENCES

1. Luciani, M. F., Denizot, F., Savary, S., Mattei, M. G., and Chimini, G. (1994) Cloning of two novel ABC transporters mapping on human chromosome 9. *Genomics* **21,** 150–159.

2. Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S., and Wu, R. L. (1997) Isolation and characterization of a mammalian homolog of the *Drosophila* white gene. *Gene* **185,** 77–85.

3. Higgins, C. F. (1992) ABC transporters: From microorganism to man. *Annu. Rev. Cell Biol.* **8,** 67–113.

4. Ames, G. F. L., Mimura, C. S., and Shyamala, V. (1990) Bacterial periplasmic permeases belong to a family of transport proteins operating from *Escherichia coli* to human: Traffic ATPases. *FEMS Immunol. Med. Microbiol.* **75,** 429–446.

5. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the a and b subunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1,** 945–951.

6. Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990) Structural model of ATP-binding proteins associated with cystic-fibrosis, multidrug resistance and bacterial transport. *Nature* **346,** 362–365.

7. Rust, S., Walter, M., Funke, H., vonEckardstein, A., Cullen, P., Kroes, H. Y., Hordijk, R., Geisel, J., Kastelein, J., Molhuizen, H. O. F., Schreiner, M., Mischke, A., Hashmann, H. W., and Assmann, G. (1998) Assignment of Tangier disease to chromosome 9q31 by a graphical linkage exclusion strategy. *Nat. Genet.* **20,** 96–98.

8. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J. C., Deleuze, J. F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) Tangier disease is caused by mutations in

the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22,** 352–355.

9. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., vanDam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O. F., Loubser, O., Ouelette, B. F. F., Fichter, K., Ashbourne-Excoffon, K. J. D., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J. P., Genest, J., and Hayden, M. R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22,** 336–345.

10. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W. E., Hahmann, H. W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K. J., and Schmitz, G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22,** 347–351.

11. Repa, J. J., Turley, S. D., Lobaccaro, J. A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R. A., Dietschy, J. M., and Mangelsdorf, D. J. (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289,** 1524–1529.

12. Klucken, J., Büchler, C., Orsó, E., Kaminksi, W. E., Porsch-Özcürümez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R., and Schmitz, G. (2000) ABCG1 (ABC8), the human homolog of the *Drosophila white* gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc. Natl. Acad. Sci. USA* **97,** 817–822.

13. Chen, H. M., Rossier, C., Lalioti, M. D., Lynn, A., Chakravarti, A., Perrin, G., and Antonarakis, S. E. (1996) Cloning of the cDNA for a human homologue of the *Drosophila* white gene and mapping to chromosome 21q22.3. *Am. J. Hum. Genet.* **59,** 66–75.

14. Savary, S., Denizot, F., Luciani, M. F., Mattei, M. G., and Chimini, G. (1996) Molecular cloning of a mammalian ABC transporter homologous to *Drosophila* white gene. *Mamm. Genome* **7,** 673–676.

15. Kuwana, H., Shimizu-Nishikawa, K., Iwahana, H., and Yamamoto, D. (1996) Molecular cloning and characterization of the ABC transporter expressed in Trachea (ATET) gene from *Drosophila melanogaster. Biochim. Biophys. Acta* **1309,** 47–52.

16. Morgan, T. H. (1910) Sex limited inheritance in *Drosophila. Science* **32,** 120–122.

17. Venkateswaran, A., Repa, J. J., Lobaccaro, J. M., Bronson, A., Mangelsdorf, D. J., and Edwards, P. A. (2000) Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J. Biol. Chem.* **275,** 14700–14707.

18. Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J., and Tontonoz, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA,* in press.

19. Luo, Y., and Tall, A. R. (2000) Sterol upregulation of human CETP expression *in vitro* and in transgenic mice by an LXR element. *J. Clin. Invest* **105,** 513–520.

20. Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L., and Mangelsdorf, D. J. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* **14,** 2819–2830.

21. Cullen, P., Mohr, S., Brennhausen, B., Cignarella, A., and Assmann, G. (1997) Downregulation of the selectin ligand-producing fucosyltransferases Fuc-TIV and Fuc-TVII during foam cell formation in monocyte-derived macrophages. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1591–1598.

22. Cullen, P., Cignarella, A., Brennhausen, B., Mohr, S., Assmann, G., and von Eckardstein, A. (1998) Phenotype-dependent differences in apolipoprotein E metabolism and in cholesterol ho-

meostasis in human monocyte-derived macrophages. *J. Clin. Invest.* **101,** 1670–1677.

23. Weisgraber, K. H., Newhouse, Y. M., and Mahley, R. W. (1988) Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide probes. *Biochem. Biophys. Res. Commun.* **157,** 1212–1217.

24. Basu, S. K., Goldstein, J. L., Anderson, R. W. G., and Brown, M. S. (1976) Degradation of cationized low density lipoproteins and regulation of cholesterol metabolism in homozygous familial hypercholesterolemic fibroblasts. *Proc. Natl. Acad. Sci. USA* **73,** 3178–3182.

25. Esterbauer, H., Striegl, G., Puhl, H., and Rotheneder, M. (1989) Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **6,** 67–75.

26. Hartree, E. F. (1972) Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48,** 422–427.

27. Cullen, P., Fobker, M., Tegelkamp, K., Meyer, K., Kannenberg, F., Cignarella, A., Benninghoven, A., and Assmann, G. (1997) An improved method for quantification of cholesterol and cholesteryl esters in human monocyte-derived macrophages by high performance liquid chromatography with identification of unassigned cholesteryl ester species by means of secondary ion mass spectrometry. *J. Lipid Res.* **38,** 401–409.

28. Mohr, S., Cullen, P., Schmidt, R., Cignarella, A., and Assmann, G. (1997) Avoidance of false positives in PCR-Based mRNA differential display during investigation of nonstandardized tissues or cells. *Clin. Chem.* **43,** 182–184.

29. Wang, X. K., and Feuerstein, G. Z. (1995) Direct sequencing of DNA isolated from mRNA differential display. *BioTechniques* **18,** 448.

30. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.

31. Harlow, E., and Lane, D. (1988) Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

32. Plenz, G., Kugler, S., Schnittger, S., Rieder, H., Fonatsch, C., and Muller, P. K. (1994) The human vigilin gene: Identification, chromosomal localization and expression pattern. *Hum. Genet.* **93,** 575–582.

33. Plenz, G., Gan, Y., Raabe, H. M., and Muller, P. K. (1993) Expression of vigilin in chicken cartilage and bone. *Cell Tissue Res.* **273,** 381–389.

34. Weitkamp, B., Cullen, P., Plenz, G., Robenek, H., and Rauterberg, J. (1999) Human macrophages synthesize type VIII collagen *in vitro* and in the atherosclerotic plaque. *FASEB J.* **13,** 1445–1457.

35. Kaminski, W. E., Orso, E., Diederich, W., Klucken, J., Drobnik, W., and Schmitz, G. (2000) Identification of a novel human sterol-sensitive ATP-binding cassette transporter (ABCA7). *Biochem. Biophys. Res. Commun.* **273,** 532–538.

36. Assmann, G., von Eckardstein, A., and Brewer, H. B. (1995) *in* The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.), pp. 1981–2030. McGraw-Hill, New York.

37. Marcil, M., Brooks-Wilson, A., Clee, S. M., Roomp, K., Zhang, L. H., Yu, L., Collins, J. A., van Dam, M., Molhuizen, H. O., Loubster, O., Ouellette, B. F., Sensen, C. W., Fichter, K., Mott, S., Denis, M., Boucher, B., Pimstone, S., Genest, J., Jr., Kastelein, J. J., and Hayden, M. R. (1999) Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* **354,** 1341–1346.

38. Orsó, E., Broccardo, C., Kaminski, W. E., Böttcher, A., Liebisch, G., Drobnik, W., Götz, A., Chambenoit, O., Diederich, W., Lang-

mann, T., Spruss, T., Luciani, M.-F., Rothe, G., Lackner, C., Chimini, G., and Schmitz, G. (2000) Transport of lipids from Golgi to plasma membrane is defective in Tangier disease patients and *ABC1*-deficient mice. *Nature Genet.* **24,** 192–196.

39. Huang, Y. D., von Eckardstein, A., Wu, S. L., Langer, C., and Assmann, G. (1995) Generation of pre-beta-1-HDL and conversion into alpha-HDL - evidence for disturbed HDL conversion in Tangier disease. *Arterioscler. Thromb. Vasc. Biol.* **15,** 1746–1754.

40. Venkateswaran, A., Laffitte, B. A., Joseph, S. B., Mak, P. A., Wilpitz, D. C., Edwards, P. A., and Tontonoz, P. (2000) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXRα. *Proc. Natl. Acad. Sci. USA* **97,** 12097–12102.

41. Mahley, R. W. (1988) Apolipoprotein E. A cholesterol transport protein with expanding role in cell biology. *Science* **240,** 622–630.

42. Archbold, R. A., and Timmis, A. D. (1999) Modification of coronary artery disease progression by cholesterol-lowering therapy: The angiographic studies. *Curr. Opin. Lipidol.* 527–534.