The Establishment of Telomerase-immortalized Tangier Disease Cell Lines Indicates the Existence of an Apolipoprotein A-I-inducible but ABCA1-independent Cholesterol Efflux Pathway*

Michael Walter‡§, Nicholas R. Forsyth¶, Woodring E. Wright†, Jerry W. Shay¶, and Michael G. Roth‡

From the ‡Department of Biochemistry and the ¶Department of Cell Biology, the University of Texas, Southwestern Medical Center, Dallas, Texas 75235-9038.

Tangier disease (TD) is a human genetic disorder associated with defective apolipoprotein-I-induced lipid efflux and increased atherosclerotic susceptibility. It has been linked to mutations in the ATP-binding cassette protein A1 (ABCA1). Here we describe the establishment of permanent Tangier cell lines using telomerase. Ectopic expression of the catalytic subunit of human telomerase extended the life span of control and TD skin fibroblasts, and (in contrast to immortalization procedures using viral oncogenes) did not impair apolipoprotein A-I-induced lipid efflux. The key characteristics of TD fibroblasts (reduced cholesterol and phospholipid efflux) were observed both in primary and telomerase-immortalized fibroblasts from two unrelated homozygous patients. Surprisingly, the apolipoprotein-inducible cholesterol efflux in TD cells was significantly improved after immortalization (up to 40% of normal values). In contrast to ABCA1-dependent cholesterol efflux, this efflux was not inhibited by brefeldin A, glybenclamide, or intracellular ATP depletion but was inhibited in the presence of cytochalasin D. Apolipoprotein A-I-dependent cholesterol efflux was inversely correlated with the population doubling number in cell culture and was inhibited up to 40% in near-senescent normal diploid fibroblasts. This inhibition was completely reversed by telomerase. Thus ectopic expression of telomerase is a way to circumvent the lack of critical experimental material and represents a major improvement for studying cholesterol efflux pathways in lipid disorders. Our findings indicate the existence of an ABCA1-independent but cytoskeleton-dependent cholesterol removal pathway that may help to prevent early atherosclerosis in Tangier disease but may also be sensitive to aging phenomena ex vivo and possibly in vivo.

A low HDL1 plasma level is one of the most predictive coronary risk factors (1). It is widely assumed that HDL protects against atherosclerosis by mobilizing excess cholesterol from arterial cells and transporting the cholesterol to the liver, a process called “reverse cholesterol transport” (2). However, the mechanism by which HDL mobilizes cellular cholesterol is still obscure, and the relative contribution of “nonspecific” diffusion-like or more active apolipoprotein-inducible transport components has been hotly debated during the past 2 decades (3, 4).

The discovery of ATP-binding cassette transporter A1 (ABCA1) as the defective protein in the autosomal co-dominant genetic HDL deficiency syndrome Tangier disease (TD) (5–8) initiated a tremendous number of new studies in this field. These studies indicated that ABCA1 is essential in the initial steps of HDL formation and that impaired function of ABCA1 in TD is associated with higher coronary risk (9–12). Based on homology with other ATP-binding cassette transporters, it was suggested that ABCA1 transports lipids across the plasma membrane by an energy-dependent process that helps to protect cells from cholesterol overload (9, 10). However, ABCA1 was also localized to intracellular organelles (13), suggesting a more general role in cellular trafficking; and the exact physiological role of ABCA1 is still unclear. Moreover, it is not understood why the manifestation of atherosclerosis in many TD patients occurs at a relatively advanced age (14) and is apparently completely lacking in some patients (15, 16) despite near-complete HDL deficiency in all homozygous patients. This has been explained by the multifactorial origin of atherosclerosis, the existence of undefined anti-atherosclerotic properties of ABCA1 (that may vary among mutations), long disease process before clinical signs become manifest, low low density lipoprotein cholesterol levels in TD patients, and unknown compensatory mechanisms that may operate more efficiently at younger age.

For further studies on cholesterol efflux in TD and on potentially anti-atherogenic compensatory mechanisms, permanent cell lines from TD patients would be very helpful. The limit on cell proliferation in primary cells represents an obstacle to the study of cultivated fibroblasts, especially since many rounds of cell division are required to produce large quantities of cells for biochemical analysis. Moreover, the cellular phenotype might be influenced by aging in cell culture. Age seems to be an important modulator of cholesterol efflux and a critical factor in TD patients because HDL cholesterol levels in heterozygote TD patients significantly decrease with increasing age (12). Moreover, cultivated TD cells have a reduced in vitro growth.

*This work was supported in part by grants from the National Institutes of Health and the Diane and Hal Brierley Chair in Biomedical Research (to M. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by a research award from the Deutsche Forschungsgemeinschaft.
‡To whom correspondence should be addressed: Dept. of Biochemistry, Southwestern Medical Center, 5523 Harry Hines Blvd., Dallas, TX 75235-9038. Tel.: 214-648-4542; Fax: 214-648-8856; E-mail: michael.roth@utsouthwestern.edu.
§Supported by a research award from the National Institutes of Health and the Diane and Hal Brierley Chair in Biomedical Research (to M. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶Supported by a research award from the National Institutes of Health and the Diane and Hal Brierley Chair in Biomedical Research (to M. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; ABCA1, ATP-binding cassette transporter A1; FAFA, fatty acid-free albumin; hTERT, human telomerase catalytic subunit; PD, passage doubling; TD, Tangier disease; 8-Br-cAMP, 8-bromo-cAMP; DMEM, Dulbecco's modified Eagle's medium.
rate (17) and may have a reduced proliferative life span, which further complicates biochemical studies.

Most experiments on TD have been performed with human skin fibroblasts because this cell type can easily be obtained from patients. The key characteristics of the disease are detectable in these cells, including cholesterol accumulation and reduced cholesterol efflux in response to apolipoproteins (18–21). Therefore, the immortalization of skin fibroblasts by using viral oncogenes dramatically decreases apolipoprotein-inducible cholesterol efflux (22). In the present study, we used the human telomerase catalytic subunit hTERT to immortalize skin fibroblasts from healthy controls and from two homozygous TD patients. Telomerase is a specialized cellular reverse transcriptase that can compensate for the erosion of telomeres by synthesizing new telomeric DNA. It has been shown previously that the forced expression of exogenous hTERT in normal human cells is sufficient to produce telomerase activity in these cells, prevent the erosion of telomeres, and circumvent the induction of senescence and crises (23–25). Our results show that the expression of hTERT efficiently extends the life span of Tangier fibroblasts without changing the key characteristics of this disease. Moreover, we show that hTERT expression circumvents a marked reduction of an ABCA1-independent cholesterol efflux pathway observed at higher cell numbers of normal fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cholesterol, essentially fatty acid-free bovine serum albumin, apolipoprotein A-I (apoA-I), apolipoprotein A-II (apoA-II), oligomycin (dissolved in MeSO as 0.1 μg/ml stock solution), cytochalasin D, glyburide, 8-bromo-cAMP (8-Br-cAMP), brefeldin A, and 2-deoxyglucose were purchased from Sigma. Thin layer chromatography plates (LKB62 Silica Gel 60 A) were purchased from Whatman. [1,2-3H]Cholesterol (48 Ci/mmol) and [methyl-3H]choline chloride (75 Ci/mmol) from PerkinElmer Life Sciences.

**Cell Culture**—Human skin fibroblasts cultured from biopsies of adult human skin were isolated as described previously (19). The Tangier cells were initially maintained using 1.3 split ratios, and based upon this we estimated that they had undergone 22 and 25 population doublings since the start of these experiments. Subsequent doublings were determined by cell counts. For immortalization, cells were infected with retroviral supernatants obtained from packaging cells stably expressing hTERT cloned into the pBabePuro vector (27). Cells were then selected for 2 weeks using puromycin at 750 ng/ml. For lipid efflux measurements, normal cells between passage levels 20 and 25 and immortalized cells at different passage levels, as indicated, were plated at 25,000 cells/15-mm well or 50,000 cells/35-mm well and grown to confluence in DMEM containing 10% fetal bovine serum.

**Telomerase Activity and Telomere Length Analysis**—Telomerase activity was determined using the TRAP assay as described previously (26). Telomere length was measured after DNA extraction from cell samples with different population doublings by digestion with the restriction enzymes AluI, CfoI, HaeIII, HinfI, MspI, and RsaI and electrophoresis on 0.7% agarose gels as detailed elsewhere (29). The gels were stained with ethidium bromide and visualized using a UV transilluminator (28). Telomere length was measured after DNA extraction from cell samples at different passage levels, as indicated, were plated at 0.5 ml of DMEM with 1% fetal bovine serum for 24 h. After labeling, cells were first washed with equilibration medium (DMEM containing 0.2% FAFa) and then equilibrated in the same medium for 1 h. The medium was then replaced with 0.5 ml of DMEM in the presence or absence of apoA-I, apoA-II, or HDL and incubated at 37 °C for 6 h. Medium was collected, and the cells were harvested by centrifuging at 14,000 × g for 5 min, and then 0.4 ml of the supernatant was transferred into a glass test tube. Cells remaining in the dish were lysed in 250 μl of 0.2% SDS by rocking for 10 min at room temperature and transferred to a second glass test tube. Remaining cellular debris was collected by washing the wells with 0.25 ml of water and pooling this with the 0.2% SDS lysates. Lipids were extracted by a 30-s vortex with a 1.5-ml chloroform/methanol mixture (1:2, v/v) followed by incubation for 1 h at room temperature. After adding 0.5 ml of water and 0.5 ml of chloroform, the tubes were vortexed for 30 s and centrifuged for 15 min at 3,000 rpm at room temperature. The organic (lower) phase was transferred to a microcentrifuge tube, dried under vacuum, and resuspended in 0.2 ml of methanol by vortexing. Samples were then mixed with 500 μl of chloroform/methanol/water mixture (65:25:4, v/v/v) and scintillation counting. Percent efflux was calculated by determining the ratio of counts released into the medium over the total counts. Student’s t test was used to detect significant differences among samples.

**For fractionation of phospholipids by TLC, the chloroform/methanol extracts prepared as described above were dried under vacuum and suspended in 35 μl of chloroform/methanol mixture (1:2, v/v) followed by incubation for 1 h at room temperature. After adding 0.5 ml of water and 0.5 ml of chloroform, the tubes were vortexed for 30 s and centrifuged for 15 min at 3,000 rpm at room temperature. The organic (lower) phase was transferred to a microcentrifuge tube, dried under vacuum, and resuspended in 0.2 ml of methanol by vortexing. Samples were then mixed with 500 μl of chloroform/methanol/water mixture (65:25:4, v/v/v) and scintillation counting. Percent efflux was calculated by determining the ratio of counts released into the medium over the total counts. Student’s t test was used to detect significant differences among samples.**

**RESULTS**

**Immortalization of Control and Tangier Cell Lines**—The hTERT cDNA was transferred into cells derived from two normal individuals and two homozygous TD patients (T1 and T2). The patient T1 (63-year-old, female) is homozygous for an asparagine to serine amino acid substitution in exon 19 (AAT-AGT; amino acid position 935 of the primary translation product). The molecular defect (30) and clinical and biochemical manifestations including HDL deficiency, splenomegaly, lipid storage in reticuloendothelial tissues, and lack of severe atherosclerosis have been described in detail in previous reports (17–19, 21). The patient T2 (60-year-old, male) is characterized by a homozygous 1-bp deletion in exon 13 (dG1764:Leu548: Leu575End). This deletion introduces a stop codon at position 575, resulting in deletion of the majority of the ABCA1 protein sequence (8). The virtually complete absence of HDL in three homozygous patients with this mutation is associated with atherosclerosis and coronary heart disease.

Primary fibroblasts from the patients and two healthy controls were infected with a defective retrovirus encoding hTERT (pBabePuro-hTERT). All fibroblasts were derived from skin. Neonatal B-d foreskin fibroblasts have been described elsewhere (31) and will be referred to as control 1 (C1) here. The second control cell line was derived from hip skin of an appar-
Telomerase-immortalized Tangier Disease Fibroblasts

**Fig. 1.** Telomerase activity and telomere length in two control and two Tangier hTERT-infected cell lines. *A,* telomerase activities for a representative set of two uninfected (−) control (C1 and C2) and two TD cell lines (T1 and T2), and the respective cells infected with the hTERT vector (+). Telomerase produces a ladder of 6-bp addition products. *B,* the telomere lengths for the same representative set of samples are shown. The results show the typical smear characteristic of human telomeres and also show that the telomeres are longer in cells that have been infected with the hTERT vector. Positions of molecular weight markers are indicated on the left side (in kb). *PD,* population doublings.

**Fig. 2.** Life span of primary and hTERT-infected Tangier cells with the results expressed in population doublings. T1 cells were infected at population doubling (PD) 25, and T2 cells were infected at PD22. All cells infected with the hTERT vector (TT1 and TT2) grew well past the PD number at which senescence was induced in the corresponding control strain (T1 and T2).

Sufficient phospholipid has complexed with lipid-poor apoA-I to create a cholesterol sink (35). We therefore measured apoA-I-induced phospholipid efflux after labeling cholesterol-loaded and growth-arrested fibroblasts with radioactive [3H]choline. ApoA-I-dependent phospholipid efflux in the normal fibroblast strains was found to increase up to 4–5-fold of basal values during a 6-h incubation period. There was no significant difference in the phospholipid efflux of primary and the respective immortalized control cell lines (Fig. 4).

A possible reason for the marked reduction of apolipoprotein-inducible cholesterol efflux in cells immortalized with oncogenes is the depletion of cAMP that is observed in these cells (22). As shown in Fig. 3C, the apoA-I-inducible lipid efflux in hTERT-immortalized cells was only slightly increased by the cAMP analog 8-Br-cAMP to a similar extent as in the corresponding primary cells. This is in sharp contrast to fibroblasts immortalized with oncogenes (22) and excludes depletion of cAMP as a problem for studying cholesterol efflux in hTERT-immortalized fibroblasts.

**Cholesterol and Phospholipid Efflux from TERT-immortalized Tangier Cell Lines**—Fibroblasts from the TD patient T1 have been found previously to have reduced cholesterol and phospholipid efflux (19, 21). Cholesterol and phospholipid efflux in T1 and T2 fibroblasts was reduced ~85–95% compared...
with fibroblasts from healthy control donors (Figs. 4 and 5). The apoA-I-inducible cholesterol efflux from the immortalized cells TT1 (telomerase-immortalized T1) and TT2 (telomerase-immortalized T2) was reduced by ~65 and 70%, respectively, compared with fibroblasts from healthy control donors (Fig. 5). The apoA-I-inducible phospholipid efflux was reduced by 60–90%, depending on the concentration of apolipoprotein used (Fig. 4). This variability was due to the fact that apoA-I-inducible lipid efflux required higher concentrations of apoA1 in immortalized Tangier fibroblasts (Fig. 4C), results similar to those described previously (19) for non-immortalized Tangier fibroblasts. Thus, the typical characteristics of Tangier cells (reduced cholesterol and phospholipid efflux) were still visible after hTERT infection. However, the lipid efflux capacity was significantly increased in TERT-infected Tangier fibroblasts (TT1 and TT2 in Fig. 5) compared with the parental, non-immortalized Tangier cells. This difference between the immortalized and the parental TD cells was also seen using lower or higher cell densities (50 or 100% confluence) or different labeling conditions (labeling with 0.5 or 5 μCi/ml \[^3H]cholesterol\). Improved cholesterol efflux in immortalized Tangier fibroblasts was also observed with HDL or apoA-II as agonists (Table I). In fibroblasts from the healthy control donors cholesterol and phospholipid efflux was not significantly different in the normal and the respective TERT-immortalized cells (Figs. 4 and 5 and Table I).

Cholesterol and Phospholipid Efflux from Fibroblasts at Different Population Doublings—Because of their limited proliferative capacity, both Tangier strains had been analyzed within a few population doublings before senescence. We therefore hypothesized that lipid efflux data obtained with non-immortalized Tangier cells might not present the true \textit{in vivo}\ pheno-type but might overestimate the degree of impairment due to secondary aging processes in cell culture. To examine this possibility, we measured cholesterol efflux from normal control fibroblasts from the same donor (C1) at low and higher cell passages. As shown in Fig. 6A, we found an inverse relationship between passage doubling (PD) number and apoA-I-inducible cholesterol efflux capacity, resulting in a 17% reduction of lipid efflux at PD45 and a 38% reduction at PD67 compared with cells at PD26. HDL-inducible cholesterol efflux was inhibited by 12% at PD45 and 30% at PD67 (Fig. 6B). PD67 is ~20 doublings prior to senescence in these cells. This reduction in apoA-I-inducible cholesterol efflux dependent on the PD number was similarly observed in fibroblasts treated with retroviral supernatants with empty vector (32% reduction, PD60 versus PD23). High passage TERT-infected cells (PD187) of the same donor, however, showed a cholesterol efflux capacity similar to that observed with young primary cells. Thus, the apoA-I-inducible cholesterol efflux pathway and (to a lesser extent) the HDL-inducible cholesterol efflux continuously decreased with time in cell culture, and this process seems to be counteracted by TERT expression. In contrast, apoA-I-induced phospholipid efflux in \[^3H\]choline-labeled fibroblasts was not significantly different in cells at different cell passages (Fig. 6C).

Characterization of ApoA-I-inducible Cholesterol Efflux in hTERT-TD Fibroblasts—Brefeldin A, an inhibitor of intracellular vesicular trafficking, partially inhibits HDL- and apoA-I-inducible cholesterol and phospholipid efflux from normal fibroblasts (36, 37). It was hypothesized that two cholesterol efflux pathways may exist: a brefeldin-sensitive, apoA-I-inducible pathway that is mediated by ABCA1, and another still undefined brefeldin-insensitive efflux pathway (37).

We were therefore interested to determine whether the increased apoA-I-inducible cholesterol efflux seen in hTERT-TD cells was sensitive to brefeldin A. As shown in Fig. 7, 10 μg/ml brefeldin A inhibited apoA-I-inducible cholesterol efflux in normal control cells during a 4-h incubation phase by 67% but had no inhibitory effect on cholesterol efflux in TT1 or TT2 cells, suggesting that the lipid removal pathway in both TERT-immortalized Tangier cells is not mediated by the ABCA-1 pathway and does not involve the Golgi apparatus. Although a 24-h pre-incubation with brefeldin A completely blocked apoA-I-inducible cholesterol efflux in normal cells, these results are not interpretable since these conditions markedly reduced cell viability.

Next we examined the influence of the sulfonylurea deriva-
tive glybenclamide on apoA-I-inducible cholesterol efflux. Glybenclamide is an effective inhibitor of several ABC transporters including ABCA1 (38). A treatment that was reported to inhibit ABCA1 in cultivated fibroblasts (250 μM, including a 60-min preincubation phase) (38) significantly inhibited apoA-I-inducible cholesterol efflux in normal but not in TT1 or TT2 fibroblasts, and even appeared to enhance cholesterol efflux induced by apoA-I in these cells. To exclude the possibility that a glybenclamide-insensitive ATP-binding cassette transporter is involved in lipid efflux in immortalized Tangier fibroblasts, cells were subjected to ATP depletion by treatment with 30 μM oligomycin and 6 mM 2-deoxyglucose. This treatment almost completely abolished apoA-I-dependent cholesterol efflux in immortalized control cells but not in immortalized Tangier cells (Table II).

It has been suggested previously (30) that Rho family proteins and the cytoskeleton might be involved in ABCA1-dependent and/or alternative cholesterol removal pathways. We therefore examined the possible involvement of the cytoskeleton in lipid efflux using cytochalasin D. Disassembly of actin fibers by 1 μM cytochalasin D markedly inhibited cholesterol efflux both in immortalized control cells and in immortalized Tangier fibroblasts. It is known that cAMP enhances apoA-I-inducible cholesterol efflux (39) and that a cAMP-dependent kinase is involved in this process (26). As shown in Figs. 3 and 7, treatment of normal fibroblasts with 8-Br-cAMP increased apoA-I-mediated cholesterol efflux in immortalized control cells but not in immortalized Tangier cells.

Together, these data suggest that the apoA-I-inducible cholesterol efflux still seen in hTERT-immortalized TD fibroblasts is mediated by an ABCA1-independent pathway. In contrast to ABCA1-dependent cholesterol efflux, this pathway seems to be brefeldin-resistant, is not inhibited by glybenclamide or cellular ATP depletion, but is dependent on a regular cytoskeleton structure.
FIG. 5. Comparison of apoA-I-inducible cholesterol efflux from primary and hTERT-infected control and Tangier fibroblasts. A, apoA-I (10 μg/ml)-inducible cholesterol efflux was compared in primary (C1, C2, T1, and T2) and hTERT-immortalized (CT1, CT2, TT1, and TT2) fibroblasts from two healthy controls (C1 and C2) and the two Tangier patients (T1 and T2) after a 2- and 8-h incubation period in the presence of 1 mg/ml FAFA. The [3H]cholesterol in the medium was calculated as % of total [3H]cholesterol (cells plus medium) and then expressed as “fold increase over baseline.” The base-line value represents the cholesterol released into the medium in the presence of FAFA alone. Cell [3H]cholesterol was compared in primary (C1, C2, T1, and T2) and hTERT-infected control and Tangier cell lines T1 (●) and T2 (▲) before (filled symbols) and after (open symbols) immortalization. Similar results were obtained in four independent experiments.

DISCUSSION

Previous studies (34) have shown that the activity of the apolipoprotein A-I lipid removal pathway is sensitive to the proliferative state of the cells, with quiescent cells exhibiting higher activities than proliferating cells. The apoA-I-dependent cholesterol efflux is relatively inactive in rapidly proliferating cells, and the immortalization of fibroblasts with papillomavirus E6/E7 oncogenes reduces lipid efflux promoted by lipid-free apoA-I or HDL by ~70% (22). In contrast, immortalization of human fibroblasts with hTERT, which leaves normal growth controls intact (40, 41), can circumvent these problems. ApoA-I-inducible as well as HDL-inducible cholesterol efflux was comparable in the respective normal primary and hTERT-immortalized fibroblasts. Moreover, the ability of growth arrest and cholesterol loading to increase apoA-I-inducible cholesterol efflux was present both in primary and hTERT-immortalized cells. Thus ectopic expression of hTERT is a way to circumvent the lack of critical experimental material for studying apoA-I-inducible cholesterol efflux.

Cells immortalized with oncogenes exhibit significant alterations in physiological and biological properties. These cells are associated with aneuploidy, spontaneous hypermutability, loss of contact inhibition, and alterations in biochemical functions related to cell cycle checkpoints. The reduced cholesterol efflux in fibroblasts immortalized with oncogenes can be normalized by preincubating the cells with 8-Br-cAMP (22). It was therefore proposed that decreased cellular cAMP levels in rapidly proliferating cells may be responsible for the marked reduction in apoA-I-inducible cholesterol efflux in tumor cells or cells transformed with oncogenes or carcinogens. In accordance with this concept, ABCA1 was shown recently to be constitutively phosphorylated by a cAMP-dependent protein kinase A (26), suggesting that maximum apoA-I-inducible cholesterol efflux capacity is only possible as long as the cellular cAMP levels do not decline below a certain threshold. In this context, we were interested that 8-Br-cAMP only slightly increased apoA-I-inducible cholesterol efflux in primary as well as hTERT-immortalized fibroblasts. Thus, cAMP depletion can apparently be prevented by immortalization with hTERT.

The key characteristics of the TD phenotype were visible
both in normal and hTERTi-immortalized TD fibroblasts. These include markedly reduced cholesterol and phospholipid efflux in response to apoA-I and a less severely reduced cholesterol and phospholipid efflux in response to HDL. We were surprised, however, that the immortalized cells from both TD patients showed a significant improvement in apoA-I-, apoA-II-, and HDL-inducible cholesterol efflux capacity (Figs. 4 and 5 and Table 1). The increase in apoA-I-inducible efflux capacity of TT2 to 30–40% of normal values was remarkable. This mutant had almost no detectable apoA-I-inducible cholesterol efflux before immortalization. It could not entirely be excluded that a small N-terminal fragment of ABCA1 is synthesized in these cells. However, this fragment, which lacks both ATP-binding sites, is unlikely to be functional. We could not detect any ABCA1 protein fragment using Western blotting or by mass spectrometry analysis. Moreover, the apoA-I-inducible cholesterol efflux in hTERTi-immortalized TD cells differs in several aspects from the ABCA1-dependent cholesterol efflux in normal fibroblasts. In contrast to ABCA1-dependent cholesterol efflux, this pathway is brefeldin-resistant, not inhibited by glybenclamide or cellular ATP depletion, and not activated by a cAMP analog. Remaley and co-workers (37) have previously proposed the existence of a brefeldin-sensitive (ABCA1-dependent) and a brefeldin-resistant cholesterol removal pathway. Data in this report support this model and suggest that the cholesterol efflux remaining after brefeldin inhibition in normal cells is quantitatively the same as the efflux seen with or without brefeldin in the immortalized Tangier cells.

Fig. 6. Promotion of lipid efflux from primary and hTERTi-infected human skin fibroblasts by apoA-I and HDL, using cells from the same donor at different population doublings. A and B, the control cell line C1 (●, ▲, and ▼) or the immortalized cell line CT1 (○) was treated with 10 μg/ml apoA-I (A) or 100 μg/ml HDL (B) at different PDs, as described in Figs. 3 and 5 and under the “Experimental Procedures.” C, phospholipid efflux from primary and hTERTi-infected C1 fibroblasts by apoA-I at different PDs, as described in Fig. 4. The [3H]lipid in the medium (cholesterol) or the organic extract of the medium (phospholipids) was calculated as % of total [3H]radioactivity (cells plus medium) and then expressed as “fold increase over base line” (efflux in the presence of FAFA alone).

Fig. 7. Effect of brefeldin A, 8-bromo-cAMP, glybenclamide, oligomycin, and cytochalasin D on apoA-I-mediated cellular cholesterol efflux from immortalized fibroblasts. hTERTi control fibroblasts (CT1 at PD36) and TT1 and TT2 fibroblasts (both at PD36) were loaded with cholesterol and labeled with [3H]cholesterol as described under “Experimental Procedures.” Cells were incubated with 10 μg/ml apoA-I plus 1 mg/ml FAFA in the presence of 10 μg/ml brefeldin A, 0.5 mM 8-Br-cAMP, or 250 μM glybenclamide. After 4-h incubations, radioactivity in the medium and in the cells was determined. Cells treated with 8-Br-cAMP were preincubated for 16 h with the same concentrations as used during the efflux phase. Cells treated with brefeldin A or glybenclamide were preincubated for 1 h with the same inhibitor concentrations as used during the efflux phase. The [3H]cholesterol levels in the presence of FAFA alone were defined as base-line values. Data represent averages ± S.D. of two experiments performed in triplicate. * indicates p < 0.01 relative to controls (untreated), and # indicates p < 0.05 relative to controls (untreated), determined by Student’s t test.

Table II

| Cholesterol efflux a | CT1 | TT1 | TT2 |
|---------------------|-----|-----|-----|
| No inhibitor        | 2.9 ± 0.4 | 1.8 ± 0.2 | 1.7 ± 0.1 |
| Cytochalasin D      | 1.3 ± 0.3 b | 1.2 ± 0.3 b | 1.1 ± 0.2 b |
| Oligomycin          | 0.8 ± 0.3 b | 1.7 ± 0.4 | 1.6 ± 0.3 |

a Cholesterol-loaded immortalized control (CT1) and Tangier (TT1 and TT2) fibroblasts were radiolabeled with [3H]cholesterol and loaded with non-lipoprotein cholesterol (20 μg/ml) for 24 h, as described under “Experimental Procedures.” Cells were then incubated for 4 h in the presence of 1 mg/ml FAFA and 10 μg/ml apoA-I plus 30 μM oligomycin or 1 μM cytochalasin D. After 4 h incubations, radioactivity in the medium and in the cells was determined. Cells treated with oligomycin were preincubated for 16 h in glucose-free medium containing 6 mM 2-deoxyglucose. Cells treated with cytochalasin D were preincubated for 1 h. Values are expressed as % of total [3H]cholesterol (cells plus medium) appearing in the medium and represent the mean of triplicate wells. Cholesterol efflux in the presence of FAFA alone was 0.9, 1.1, and 1.2% for CT1, TT1, and TT2 respectively. Similar results were obtained in two independent experiments. b p < 0.01 for a comparison of treatment versus no treatment by Student’s t test.

Downloaded from http://www.jbc.org/ by guest on July 18, 2018
What could be the mechanism for ABCA1-independent, apoA-I-inducible cholesterol efflux subsequent to immortalization with hTERT? It was shown recently (42) that amphipathic helical peptides can promote lipid efflux in an ABCA1-independent fashion by a detergent-like action called microsolubilization. Another mechanism of cholesterol removal was described as membrane shedding, a process releasing plasma membrane vesicles that may be enriched in raft lipids (43). There are close structural and functional relationships between lipid rafts and the cytoskeleton (44). In this context, we were interested in the fact that an intact cytoskeleton proved to be necessary for induction of both ABCA1-dependent and ABCA1-independent apolipoprotein-inducible lipid efflux in immortalized Tangier cells, as shown by cytochalasin D treatment.

Cells from TD patients have growth abnormalities (17) and a limited proliferative life span in culture. Studies with premature aging and genomic instability syndromes indicate that it is difficult to distinguish effects intrinsic to a genetic defect from secondary changes due to replicative aging. We therefore hypothesize that an ABCA1-independent cholesterol efflux pathway is sensitive to replicative aging and/or damage phenomena and is therefore hard to detect in near-senescent primary TD cells. This interpretation is further supported by the observation that apoA-I-inducible cholesterol efflux in normal control (but not in hTERT-infected control fibroblasts) is also inhibited at high culture passages.

In summary, immortalization of skin fibroblasts by ectopic expression of telomerase is a way to study cholesterol removal pathways in normal cells and in cells from patients with Tangier disease. This method represents a major improvement over the use of primary cell lines especially at higher cell passages or near senescence. Our data suggest the existence of an ABCA1-independent cholesterol efflux mechanism that may help to compensate for ABCA1 deficiency in young Tangier cells, which declines with replicative aging in culture, and which may thus constitute to decreased cholesterol efflux during aging in vivo.

Acknowledgments—We are grateful to Dr. G. Assmann for providing the primary cell lines. HDL, was kindly provided by Dr. J. L. Goldstein and Dr. M. S. Brown. We thank Maria Kofiszer for expert assistance and Dr. Susanne Steinfurt for helpful comments and advice.

Note Added in Proof—It has recently been shown that lipidation of apoA-I is not completely abolished in ABCA1-deficient hepatocytes (Kiss, R. S., McManus, D. C., Franklin, V., Tan, W. L., McKenzie, A., Chimini, G., and Marcel, Y. L. (2003) Biochemistry 32, 10110–10127; Sahoo, D., Hoh, S., Chimini, G., Agillon, L. B., Agnihotri, R., Francis, G. A., and Lehner, R. (2004) J. Lipid Res. 45, 5027–5035).
The Establishment of Telomerase-immortalized Tangier Disease Cell Lines Indicates the Existence of an Apolipoprotein A-I-inducible but ABCA1-independent Cholesterol Efflux Pathway
Michael Walter, Nicholas R. Forsyth, Woodring E. Wright, Jerry W. Shay and Michael G. Roth

J. Biol. Chem. 2004, 279:20866-20873.
doi: 10.1074/jbc.M401714200 originally published online March 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401714200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 19 of which can be accessed free at http://www.jbc.org/content/279/20/20866.full.html#ref-list-1