# Reduction in apolipoprotein-mediated removal of cellular lipids by immortalization of human fibroblasts and its reversion by cAMP: lack of effect with Tangier disease cells

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**Abstract** **High density lipoprotein (HDL) phospholipids and apolipoproteins remove cellular lipids by two distinct mechanisms, but their relative contribution to reverse cholesterol transport is unknown. Whereas phospholipid-mediated cholesterol efflux from cultured cells reflects the activity of the HDL receptor SR-BI, apolipoprotein-mediated lipid removal is regulated in response to changes in cellular cholesterol content (positive) and cell proliferation rates (negative). Here we show that immortalization of human skin fibroblast lines with the papillomavirus E6/E7 oncogenes increased their proliferation rates and selectively reduced the activity of the apolipoprotein-mediated lipid removal pathway. This reduction was accompanied by a decrease in cellular cAMP levels and was reversed by treatment with a cAMP analog. The stimulatory effect of cAMP was independent of changes in cellular phenotype or activities of cholesteryl ester cycle enzymes. The severely impaired apolipoprotein-mediated lipid removal pathway in Tangier disease fibroblasts, which persisted after immortalization, was not improved by treatment with a cAMP analog, implying that the cellular defect in Tangier disease is upstream from this cAMP-dependent signaling pathway. These results indicate that papillomavirus-induced immortalization of fibroblasts selectively reduces the activity of the apolipoprotein-mediated lipid removal pathway by a cAMP-dependent process, perhaps to prevent loss of cellular lipids needed for continual membrane synthesis.**—Oram, J. F., A. J. Mendez, J. Lymp, T. J. Kavanagh, and C. L. Halbert. **Reduction in apolipoprotein-mediated removal of cellular lipids by immortalization of human fibroblasts and its reversion by cAMP: lack of effect with Tangier disease cells.** ***J. Lipid Res.*** **1999.** 40: **1769–1781.**

**Supplementary key words** cholesterol efflux • phospholipid efflux • high density lipoprotein • apolipoproteins • immortalized cells • Tangier disease

It is widely believed that HDL protects against cardiovascular disease by removing excess cholesterol from cells, but the proposed mechanisms involved have been controversial. It now appears that HDL components remove cellular lipids by at least two distinct mechanisms (1). First, HDL phospholipids pick up cholesterol that desorbs from the plasma membrane (2). This process is facilitated by binding of HDL to scavenger receptor BI (SR-BI) (3) and may involve caveolae (4). Second, HDL apolipoproteins remove both cholesterol and phospholipids from cells (5–9) by an active excretory pathway (10–12). This mechanism may be mediated by lipid-poor apolipoproteins that transfer from HDL particles to cell-surface binding sites (1, 8, 13, 14). Although the molecular properties of the apolipoprotein-mediated pathway are unknown, it does not appear to involve SR-BI (3) or caveolae (15).

It is unclear to what extent these lipid transport pathways contribute to overall mobilization of cholesterol from tissues in vivo. Studies with mice showed that plasma HDL cholesterol levels correlate inversely with SR-BI expression (16, 17), indicating that SR-BI-mediated selective uptake of HDL cholesteryl esters by tissues overshadows any possible role of this receptor in promoting transport of cholesterol from cells. Support for the physiological relevance of the apolipoprotein-mediated lipid removal pathway comes from studies of a rare HDL deficiency syndrome called Tangier disease (TD). Fibroblasts from TD homozygotes have a molecular defect that almost completely abolishes the ability of apolipoproteins to remove cellular cholesterol and phospholipids (18–21). These patients are characterized by a rapid turnover of apoA-I and deposition of cholesteryl esters in tissue macrophages (22), implying that an inability of newly synthesized apoA-I

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein; SR-BI, scavenger receptor BI; cAMP, cyclic AMP.

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to acquire cellular lipids leads to accumulation of sterols in tissues and degradation of apoA-I. Thus the apolipoprotein-mediated lipid removal pathway clearly plays an important role in mobilizing tissue cholesterol and generating HDL particles.

These recent findings illustrate the importance of developing cell culture systems to characterize the different HDL-mediated lipid removal pathways and to identify the participating cellular proteins. Many laboratories have relied on the use of immortalized cell lines to study HDL-mediated cholesterol efflux. Previous studies have shown, however, that the activity of the lipid removal pathway mediated by lipid-free apolipoproteins is sensitive to the proliferative state of cells, with quiescent cells exhibiting higher activities than proliferating cells (12, 13). This implies that the apolipoprotein-dependent component of HDL-mediated cholesterol efflux would be relatively inactive in rapidly proliferating cells. Because immortalized cell lines proliferate rapidly even in the absence of serum growth factors, they may be poor models for studying apolipoprotein-mediated lipid removal.

In the current study, we examined the effects of immortalization of human skin fibroblasts on the relative activities of the different lipid efflux pathways. Results show that immortalization of fibroblasts with papillomavirus E6/E7 oncogenes selectively reduced lipid efflux promoted by lipid-free apoA-I, and that treatment of immortalized fibroblasts with a cAMP analog largely reversed this reduction in lipid efflux. The severely impaired apolipoprotein-mediated lipid efflux in TD fibroblasts, which persisted after immortalization, was not improved by treatment with a cAMP analog. These findings indicate that papillomavirus-induced immortalization of cells selectively inactivates the apolipoprotein-mediated lipid removal pathway by a cAMP-dependent process downstream from the cellular defect in TD.

## MATERIALS AND METHODS

### Lipoproteins and apoA-I

LDL and $HDL_3$ (hereafter referred to as HDL) were prepared by sequential ultracentrifugation in the density intervals 1.019–1.063 and 1.125–1.21g/ml, respectively, and HDL was depleted of apoE and apoB as previously described (18, 19). ApoA-I was purified from isolated HDL as described previously (8) and was iodinated by the iodine monochloride method (23). Trypsinized HDL was prepared as previously described (13, 14) by treating HDL with trypsin for 30 min at 37°C at an HDL:trypsin protein ratio of 40:1. This procedure digests approximately 25% of the total HDL protein content of HDL particles without disturbing its lipid composition (14). For experiments comparing HDL and trypsinized HDL, molar concentrations were based on particle phospholipid composition measured by the method of Bartlett (24). Molarities of HDL particles and apoA-I were calculated based on protein molecular weights of 100,000 for HDL and 28,000 for apoA-I. LDL was acetylated by the method of Goldstein et al. (25).

### Cell culture

Human fibroblasts were obtained from skin explants from two normal subjects (NL4, NL7) and two patients with homozygous Tangier disease (IM, previously TG1; JW, previously TG2) as described (19). BHK cells and PD388 and RAW247 macrophages were obtained from ATCC, and Fu5AH cells were a gift from Dr. G. Rothblat (Allegheny University of Health Sciences, Philadelphia). Cultured cells were grown and maintained in either DMEM (fibroblasts, Fu5AH cells, and BHK cells) or Ham's F-12 (PD388 and RAW247 macrophages) supplemented with 5–10% fetal bovine serum (growth medium). The same medium supplemented with 1 mg/ml bovine serum albumin (BSA) instead of serum (serum-free medium) was used for all cholesterol loading, equilibration, and efflux incubations. Primary human skin fibroblasts were immortalized as described previously (26, 27) by infection with amphotrophic retroviruses containing vectors with inserts of human papillomavirus 16 oncogenes E6 and E7 and a neomycin selectable marker. Pooled cell populations were selected in the presence of G418 for two passages, after which G418 was excluded from the medium. In some cases, cells were infected with vector alone as controls (mock-infected). Fibroblasts were used between the fifth and thirteenth passage (primary) or sixth and fourteenth passage (immortalized). Cell number per dish was quantified using a Coulter Counter (Hialeah, FA) after cells were removed from the dishes by trypsinization. Cell protein was quantified by the method of Lowry et al. (28) after digestion of cells with 0.1 N NaOH.

For most experiments, cells were seeded into 16-mm wells and grown to confluence. Cells were loaded with cholesterol by incubation for 24 h to 48 h in serum-free medium supplemented with either nonlipoprotein cholesterol (30 μg/ml), LDL (100 μg/ml), or acetylated LDL (50 μg/ml), followed by an 18–20 h incubation with the same medium lacking cholesterol to allow equilibration of cholesterol pools. In some experiments, 8-bromo-cAMP (8-Br-cAMP) was added to the medium during the equilibration incubations. Unless indicated otherwise, cellular cholesterol pools were radiolabeled by addition of 0.2–0.5 $\mu C_i$/ml [1,2-$^3$H]cholesterol (40–60 $C_i$/mmol, Amersham Corp., Arlington Heights, IL) to the growth medium 3 days prior to cholesterol loading. To radiolabel cellular phospholipids in cholesterol-loaded cells, 10 $\mu C_i$/ml [$^3$H]choline chloride (75–85 $C_i$/mmol; Amersham Corp.) was added to the equilibration medium. Cells were washed with phosphate-buffered saline (PBS) containing BSA twice or four times prior to measurements of cholesterol and phospholipid efflux, respectively.

### Cholesterol and phospholipid efflux

Cells were incubated at 37°C with serum-free medium and the indicated additions. After the indicated times, the efflux media were collected and centrifuged to remove cell debris, and cell layers were rinsed twice with ice-cold PBS/BSA and twice with PBS. Media and cells were stored frozen at −20°C until extraction of lipid and protein. Efflux media was either counted directly (for cells labeled with [$^3$H]cholesterol) or extracted by the method of Folch et al. (29) for [$^3$H]phospholipid or cholesterol mass measurements. Cell layers were extracted with hexane–isopropanol 3:2 (vol/vol) as described (19). Sterol species were separated by thin-layer chromatography on silica gel G plates developed in hexane–diethyl ether–methanol–acetic acid 120:30:10:1.5 (vol/vol/vol/vol). Choline-containing phospholipids were separated by thin-layer chromatography on silica gel plates developed in chloroform–methanol–water 65:35:4 (vol/vol/vol). Lipid spots were identified by staining with $I_2$ vapor and by co-migration with standards. Appropriate spots were taken for determination of sterol and phospholipid radioactivity or sterol mass (8, 9). Unless indicated otherwise, efflux of radiolabeled lipids represents the fraction of total radiolabeled lipid (cells plus medium) appearing in the medium.

### Plasma membrane lipid domains

Cholesterol oxidase sensitivity of plasma membrane [$^3$H]cholesterol was determined by treating cells at 37°C with DMEM containing 1 U/ml cholesterol oxidase (30) and measuring fractional conversion of free [$^3$H]cholesterol to [$^3$H]cholestenone as described (12). Accessibility of cholesterol to cyclodextrin was assessed by measuring the fraction of cellular free [$^3$H]cholesterol released into DMEM containing 25 mm hydroxypropyl-β-cyclodextrin (Research Plus, Bayonne, NJ) during 30-min incubations at 37°C. The fraction of cellular [$^3$H]sphingomyelin accessible to sphingomyelinase was determined by treating [$^3$H]choline-labeled cells with DMEM plus or minus 0.1 U/ml sphingomyelinase for 30 min at 37°C and measuring cellular [$^3$H]sphingomyelin content. The percent sphingomyelin-sensitive phospholipid was calculated as 100× the difference in [$^3$H]sphingomyelin content between enzyme-treated and untreated cells divided by values for untreated cells.

To determine the radiolabeled lipid composition of caveolae-rich membranes, washed fibroblasts were dislodged from the wells and pelleted in a microfuge, cells were solubilized in 1% Triton X-100 (in MES buffer, pH 6.5) at 0°C using a 25-g needle to disperse cells, detergent insoluble membranes were sedimented at 4°C by microfugation at 20,000 *g* for 10 min, and lipids in the pellets were Folch-extracted (29) for separation by thin-layer chromatography and scintillation counting. Detergent-soluble radiolabeled lipids were measured in aliquots representing 10% of the total volume of the microfuge supertants to minimize the interference of Triton X-100 with the lipid isolation procedures. The detergent-insoluble pellet contained over 60% of the total cellular sphingomyelin and immunoblot-detectable caveolin-1 and less than 10% of the total phosphatidylcholine and membrane protein.

### Cellular binding of apoA-I

Cholesterol-loaded fibroblasts were incubated at 37°C for 6 h with serum-free medium containing $^{125}$I-labeled apoA-I and unlabeled apoA-I. Cells were washed twice with ice-cold PBS/BSA and twice with PBS. Cell layers were dissolved in 0.1 N NaOH and aliquots were taken for quantitation of radioactivity and protein (8).

### Fluorescent microscopy and cell morphology

Cells were seeded into covered glass chambers and grown to approximately 50% confluence. Cells were washed and treated with PBS containing 4 μg/ml acetoxymethyl ester of calcein (calcein-AM, molecular probes, Eugene, OR) for 10 min at 37°C. After washing with PBS and adding growth medium, the coverslip chambers were mounted on the stage of an ACAS Ultima confocal scanning microscope. Fields of 540 × 540 microns were scanned at a resolution of 2 microns/pixel using a 100× oil immersion objective with 488 nm excitation from an argon–ion laser (300 mwatts). Calcein fluorescence emission was detected with a 530/30 nm band pass filter using a pinhole setting of 100 microns.

Area and perimeter values were determined for individual cells on a DASY 9,000 Workstation (Meridian Instruments, Okemos, MI) using the Single Cell Analysis software package provided by the manufacturer. Shape factor was calculated as perimeter$^2$/4πarea. The shape factor is one for a circle and increases with shape complexity. For each cell line, mean area and shape factor were calculated as the grand mean over all cells. Statistical analysis was done using S-PLUS, Version 3.1 Release 1 for Sun SPARC, Sun OS 4.x (Statistical Sciences, Inc., Seattle, WA), and the Statistical Analysis System, Proprietary Software Release 6.09 (SAS Institute Inc., Carey, NC). Standard errors were weighted by the number of cells per image, and the associated degree of freedom was the number of image fields minus one. Each *P* value was obtained by ANOVA. Log transformations were taken on both area and shape factor to satisfy ANOVA assumptions and adjustment was made for image field. The *P* values are 2-sided and based on the standard ANOVA F-test. For the current study, 3 to 5 images containing at least five isolated cells per image were analyzed for each cell line.

### Other methods

To compare cellular expression of SR-BI and caveolin-1, fibroblasts grown in 60-mm dishes were washed with PBS and dislodged from the dishes in ice-cold 50 mm Tris saline (pH 7.4) containing protease inhibitors and 0.5 mm EDTA (30). After centrifugation, cell pellets were solubilized in buffer containing 2% SDS, aliquots were assayed for protein content, and equal amounts of cellular protein (200 μg) were applied to adjacent lanes of a 15% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose, and SR-BI and caveolin-1 were identified by immunoblot analysis as described previously (31) using 1:5,000 dilutions of rabbit antisera to human SR-BI (a gift from Dr. Monty Krieger, MIT, Boston) and a 1:5,000 dilution of rabbit antiserum to human caveolin-1 (Transduction Laboratories, Lexington, KY). Antibody-positive bands were visualized by enhanced chemiluminescence (ECL, Amersham).

To assess cholesterol esterification rates, cells were washed once with PBS and incubated for 1 h at 37°C with DMEM containing 9 μm [$^{14}$C]oleate (50–60 mCimmol, Amersham Corp.) bound to 3 μm BSA (8, 13, 19). Cell lipids were separated by thin-layer chromatography as described above to determine cholesteryl ester radioactivity. To measure cellular cAMP content, fibroblasts plated in 35-mm wells were treated as described in the text, cell layers were extracted with ice-cold 65% (v/v) ethanol, and cAMP was quantified using the Biotrak Enzyme Immunoassay (non-acetylated) system (Amersham Corp.) according to the manufacturer's directions. Student's *t* test was performed where indicated for determining significances between sets of data.

## RESULTS

### Apolipoprotein-mediated cholesterol efflux from immortalized cell lines

Lipid-free apolipoproteins remove cellular cholesterol by an active process that is up-regulated by both loading cells with cholesterol and inhibiting cell proliferation (1, 12). We found that this lipid removal pathway was absent or had very low activity in many immortalized cell lines, even when previously incubated with cholesterol-rich media. When Fu5AH hepatoma cells were radiolabeled with [$^3$H]cholesterol and then loaded with non-lipoprotein cholesterol, 6-h incubations with HDL led to a dose-dependent increase in [$^3$H]cholesterol efflux (**Fig. 1A**). In contrast, purified apoA-I had virtually no ability to stimulate cholesterol efflux from these cells. We obtained the same results when Fu5AH cells were loaded with LDL-derived cholesterol (data not shown). Cholesterol-loaded BHK cells showed a similar pattern of cholesterol efflux (Fig. 1B). ApoA-I also lacked the ability to remove [$^3$H]cholesterol from RAW247 and PD388 macrophages, even after these cells were loaded with acetylated LDL-derived cholesterol through the scavenger receptor (data not shown). These results indicate that the cholesterol efflux pathway mediated by lipid-free apoA-I is inactive in many immortalized cell lines.

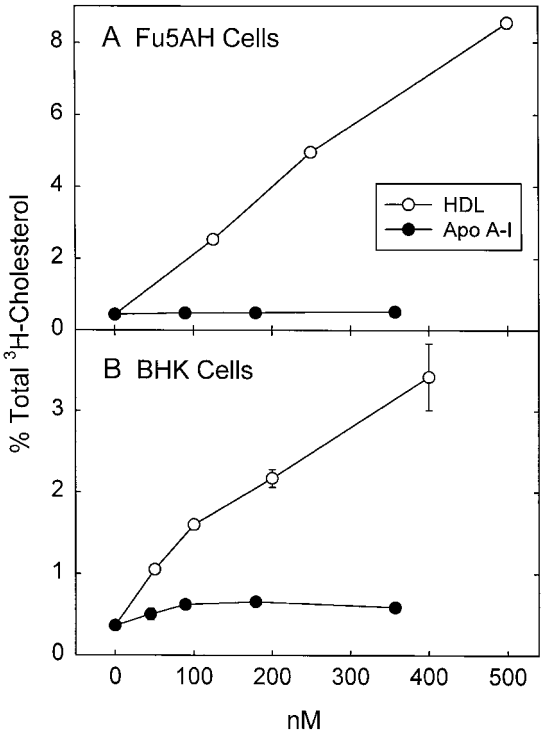


**Fig. 1.** Promotion of cholesterol efflux from Fu5AH and BHK cells by HDL and apoA-I. Cells were radiolabeled with [$^3$H]cholesterol and loaded with non-lipoprotein cholesterol (30 μg/ml) for 24 h as described in Methods. Cells were then incubated for 6 h with medium containing the indicated concentrations of HDL or apoA-I. Values are expressed as % of total [$^3$H]cholesterol (cells plus medium) appearing in the medium and represent the mean of duplicate (A) or the mean ±SD or triplicate (B) wells. Total [$^3$H]cholesterol cpm/μg cell protein were (A)1174 ± 176 (n = 14) and (B) 1629 ± 138 (n = 24).

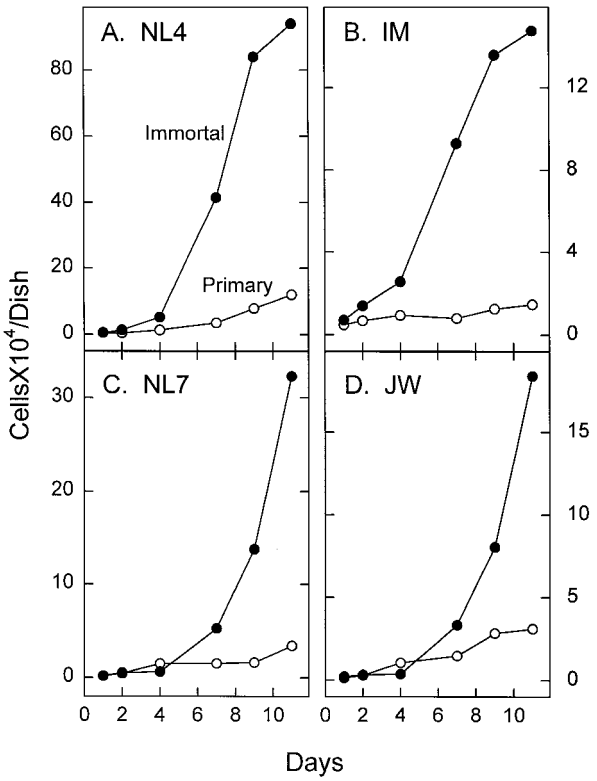


**Fig. 2.** Proliferation rates of primary and immortalized fibroblast lines. Either 2.5 × 10$^4$ (A and C) or 1.0 × 10$^4$ (B and D) cells were seeded in 35-mm dishes, and cell number per dish was measured after the indicated days as described in Experimental Procedures. The growth medium (10% serum) was changed every 2–3 days. Results are the mean of duplicate dishes.

### Proliferation rates and morphology of primary and immortalized fibroblasts

To more directly examine the effects of immortalization on apolipoprotein-mediated cholesterol efflux, we immortalized skin fibroblast lines from two normal subjects (NL4, NL7) and two patients with TD (IM, JW), and we measured changes in proliferation rates, cell morphology, and parameters of lipid efflux. TD cells were chosen as controls because they have a genetic defect that severely impairs apolipoprotein-mediated lipid removal without affecting phospholipid-mediated lipid efflux. Immortalization of these fibroblasts with papillomavirus E6/E7 oncogenes markedly increased the proliferation rates of all four cell lines. During the 10-day growth period shown in **Fig. 2**, the number of cells per dish increased an average of 18-fold for the four primary cell lines and 120-fold for the immortalized cell lines.

We calculated the mean cell sizes and shape complexities of the different primary and immortalized cell lines from fluorescent microscopic images (see Materials and Methods). The four primary fibroblast lines varied nearly 3-fold in the average area of a single cell (2380 ± 200, 1020 ± 220, 1780 ± 160, 2840 ± 320 μm$^2$ for NL4, NL7, IM, JW, respectively), while the immortalized cell lines were more uniform in size (1730 ± 160, 1220 ± 190, 1440 ± 110, 1790 ± 150 μm$^2$, respectively). As with area, shape complexity varied significantly among the primary cell lines. With the exception of TD cell line IM, which initially had the most regular cell shape (value closer to 1.0), the immortalized cells had more regular cell shapes (2.62 ± 0.13, 2.06 ± .30, 2.42 ± 0.14, 2.30 ± 0.03, respectively) than their parental lines (3.54 ± 0.44, 4.09 ± 0.63, 2.47 ± 0.20, 3.98 ± 0.30, respectively). Thus, the papillomavirus-induced immortalizations produced cells that were more uniform in both size and shape among the different normal and TD cell lines.

### Immortalization-induced changes in cholesterol and phospholipid efflux

To examine immortalization-induced changes in the different mechanisms of cholesterol efflux, we compared the abilities of HDL, trypsinized HDL (TrHDL), and purified apoA-I to promote cholesterol efflux from the primary and immortalized cell lines. These particles remove cholesterol from cells by either phospholipid-mediated processes (TrHDL), apolipoprotein-mediated processes (apoA-I), or both processes (HDL) (12–14, 19).

Initially we compared cholesterol efflux from primary and immortalized cells that were not overloaded with cho-

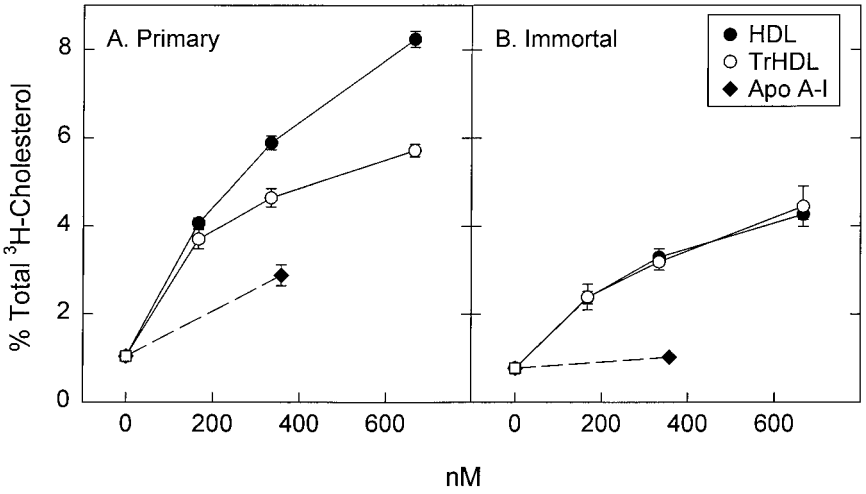


**Fig. 3.** Cholesterol efflux from primary (A) and immortalized (B) normal fibroblasts. Fibroblasts (NL4) were radiolabeled with [$^3$H]cholesterol by addition of cholesterol tracer to the growth medium as described in Methods. After 48 h cells were incubated for 18 h with serum-free medium (equilibration incubation) and then for 6 h with the indicated concentration of HDL, trypsinized HDL (TrHDL), or apoA-I. Cholesterol efflux is expressed as the % total [$^3$H]cholesterol (cell plus medium) released into the medium. Results are the mean ±SD of triplicate wells. Total [$^3$H]cholesterol cpm/μg cell protein were (A) 2169 ± 204 (n = 24) and (B) 1358 ± 66 (n = 24).

lesterol. Cells were radiolabeled by incubation with growth medium containing serum and [$^3$H]cholesterol. Subsequent 6-h incubations with increasing concentrations of HDL or TrHDL revealed that HDL promoted fractional [$^3$H]cholesterol efflux from primary normal fibroblasts to a greater extent than did TrHDL (**Fig. 3A**). The differences in efflux mediated by these particles at their highest concentrations was comparable to that pro-

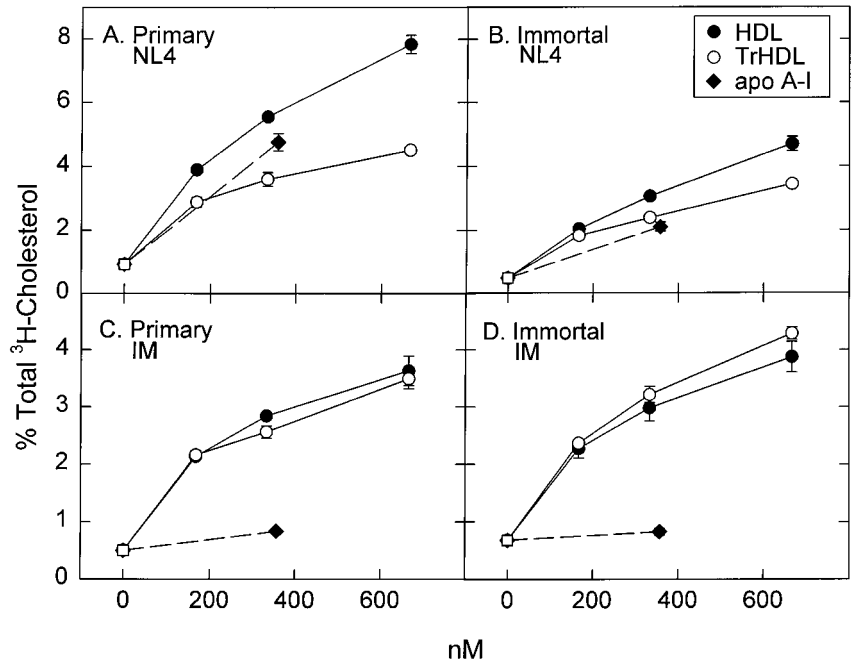


**Fig. 4.** Cholesterol efflux from serum-deprived, cholesterol-loaded primary and immortalized normal and Tangier disease fibroblasts. Fibroblasts (NL4, JW) were radiolabeled with [$^3$H]cholesterol and cholesterol loaded by 48-h incubations with serum-free medium containing 30 μg/ml cholesterol followed by an 18-h equilibration incubation with cholesterol-free medium as described in Methods. Cells were then incubated for 6 h with serum-free medium containing the indicated concentrations of HDL, TrHDL, or apoA-I, and fractional [$^3$H]cholesterol efflux (as percent total) was measured as described in Fig. 3. Total [$^3$H]cholesterol cpm/μg cell protein were (A) 2114 ± 100 (n = 24) (B) 1926 ± 55 (n = 24) (C) 1825 ± 224 (n = 24) (D) 1443 ± 79 (n = 24).

moted by purified apoA-I at a concentration 4-fold above saturation of efflux (8, 12, 19), consistent with this difference reflecting apolipoprotein-mediated cholesterol efflux. In contrast, HDL and TrHDL stimulated cholesterol efflux to the same extent from immortalized normal fibroblasts, and apoA-I had an effect only slightly above background (Fig. 3B). These results indicate that immortalization of normal fibroblasts almost completely abolished the apolipoprotein-mediated component of cholesterol efflux while only partially reducing the phospholipid-mediated component.

As cholesterol loading of primary fibroblasts increases apolipoprotein-mediated lipid efflux, particularly when cell proliferation is also arrested (12), we tested the effects of cholesterol loading of primary and immortalized cells on the different cholesterol efflux processes. Cells were treated as described above, except that they were incubated with serum-free medium supplemented with non-lipoprotein cholesterol for 48 h. In the absence of serum, primary fibroblasts become quiescent whereas immortalized cells continue to proliferate, although at a slower rate. With serum-deprived, cholesterol-loaded primary normal fibroblasts, TrHDL was approximately half as effective as untreated HDL in stimulating [$^3$H]cholesterol efflux, and purified apoA-I stimulated cholesterol efflux to the same extent as TrHDL at the highest concentration tested (**Fig. 4A**). These results suggest that the phospholipid- and apolipoprotein-mediated mechanisms of cholesterol efflux contributed equally to cholesterol efflux from these growth-arrested, cholesterol-loaded cells. When immortalized normal fibroblasts were treated the same way, HDL was more effective than TrHDL in promoting cholesterol efflux and apoA-I stimulated efflux several fold above background (Fig. 4B). Thus, in contrast to cells maintained on serum (Fig. 3B), serum-deprived and cholesterol-loaded immortalized fibroblasts were able to release cholesterol by the apolipoprotein-mediated mechanism, although to a significantly lesser extent than the parental primary line.

With both primary and immortalized TD fibroblasts that were cholesterol-loaded, HDL and TrHDL were equally effective in promoting cholesterol efflux, and apoA-I stimulated efflux only slightly above background (Fig. 4C and D). These results are consistent with previous reports showing that apolipoprotein-mediated lipid efflux is almost completely absent in TD cells, whereas phospholipid-mediated cholesterol efflux is normal. These findings also indicate that immortalization of TD fibroblasts did not correct this lipid transport defect.

Because the immortalized cells continued to proliferate during the incubation with serum-free medium, these cells tended to accumulate less total cholesterol mass than the growth-arrested primary cells when exposed to the same cholesterol-rich medium. To adjust for this, we incubated a normal primary cell line (NL4) and its immortalized transformant with medium containing 10 μg/ml and 30 μg/ml cholesterol, respectively. Under these conditions, the immortalized cells accumulated 60% and 400% more free and esterified cholesterol mass per mg cell protein, respectively, than the parental cells (**Table 1**). Despite a higher cholesterol content, the immortalized cells released less cholesterol mass into medium containing apoA-I than did the primary cells. The fractional choles-

TABLE 1. ApoA-I-mediated cholesterol mass efflux from cholesterol-loaded primary and immortalized normal fibroblasts

| Cells | Medium Free | Cellular Free | Cellular Esterified |
|---|---|---|---|
| | *μg/mg cell protein (% of total cholesterol)* | *μg/mg cell protein* | |
| Primary | 6.20 ± 0.24 (5.81 ± 0.54) | 105 ± 8 | 3.7 ± 0.1 |
| Immortal | 4.97 ± 0.44 (2.34 ± 0.02) | 169 ± 6 | 18.8 ± 1.0 |

Primary fibroblast line NL4 and its immortalized line were grown in 35-mm dishes and incubated for 48 h with serum-free medium containing albumin plus either 10 μg/ml (primary) or 30 μg/ml (immortal) cholesterol. After an overnight incubation in cholesterol-free medium, cells were incubated for 6 h with serum-free medium containing albumin alone or albumin plus 5 μg/ml apoA-I. Medium values represent the difference in cholesterol mass between incubation with and without apoA-I. Cellular values are those for cells incubated with apoA-I. Each value is the mean ± SD of four incubations. Unincubated apoA-I had undetectable amounts of cholesterol.

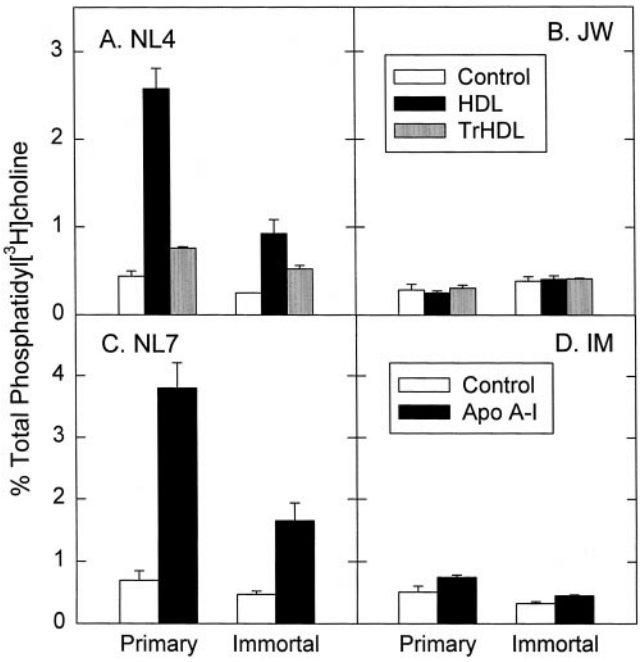


**Fig. 5.** Phosphatidylcholine efflux from cholesterol-loaded primary and immortalized normal and Tangier disease fibroblasts. The indicated primary and immortalized cell lines were loaded with cholesterol as described for Fig. 4, and cellular phospholipids were radiolabeled with [$^3$H]choline during the equilibration incubation as described in Methods. Cells were incubated for 6 h with serum-free medium alone (control) or with the addition of 600 nm HDL, 600 nm TrHDL (A, B), or 360 nm apo A-I (C, D). Each value represents the mean ±SD of triplicate wells expressed as percent total phosphatidyl[$^3$H]choline (cells plus medium) appearing in the medium. Total phosphatidyl[$^3$H]choline cpm/μg cell protein were (A-Pr) 1887 ± 189 (n = 9) (A-Im) 3056 ± 154 (n = 9) (B-Pr) 1600 ± 140 (n = 9) (B-Im) 3607 ± 319 (n = 9) (C-Pr) 1005 ± 50 (n = 6) (C-Im) 3267 ± 258 (n = 6) (D-Pr) 1144 ± 91 (n = 6) (D-Im) 3004 ± 132 (n = 6).

terol mass efflux from the immortalized cells was 42% that of the primary cells. The mean percent cholesterol mass efflux in three separate experiments (total cholesterol ranging from 80 to 189 μg/mg cell protein) was 4.39 ± 1.41 (SD) for primary cell line NL4 and 1.92 ± 0.39 for its immortalized line. Thus immortalization of fibroblasts reduced the apolipoprotein-mediated cholesterol removal pathway by approximately 60% under culture conditions that maximally enhance this pathway.

We also compared the abilities of HDL, TrHDL, and apoA-I to stimulate phospholipid efflux from serum-deprived, cholesterol-loaded primary and immortalized fibroblasts. Addition of HDL or apoA-I to albumin-containing medium increased radiolabeled phosphatidylcholine efflux from normal primary cells approximately 6-fold (**Fig. 5**). HDL and apoA-I were 60–70% less effective in stimulating phospholipid efflux from the immortalized cells than from the parental cells. TrHDL was significantly less effective than HDL at stimulating phosphatidylcholine efflux from both primary and immortalized cells. Neither HDL, TrHDL, nor apoA-I stimulated phosphatidylcholine efflux from primary and immortalized TD fibroblasts (**Fig. 6**). These findings indicate that HDL apolipoproteins, and not HDL lipids, mediate most of the phospholipid efflux stimulated by HDL. As with cholesterol efflux, immortalization of TD fibroblasts did not correct the cellular defect in apolipoprotein-mediated phospholipid efflux.

To ensure that the processes of retroviral infection and selection alone were not responsible for the decreased activity of the apolipoprotein-mediated lipid efflux pathway, we infected normal fibroblasts (NL4) with vector lacking the papilloma virus E6/E7 insert, selected cells for neomycin resistance for two passages (mock-infected), and compared apoA-I-mediated [$^3$H]cholesterol and [$^3$H]phospholipid efflux with uninfected/non-selected cells grown in parallel. Results showed that 6 h incubation of cholesterol-loaded cells with 5 μg/ml apoA-I promoted fractional [$^3$H]cholesterol efflux of 6.10 ± 0.54% and 6.81 ± 0.32% (mean ± SD, n = 4) from mock-infected and uninfected fibroblasts, respectively. Under the same conditions, fractional phosphatidyl[$^3$H]choline efflux was 5.15 ± 0.56% and 5.54 ± 0.59%, respectively. Mock-infected cells also had similar proliferation rates as uninfected cells (not shown). Thus, the reduction in apolipoprotein-mediated lipid efflux could not be attributed to the infection and selection conditions used in this study.

### Caveolae-rich plasma membrane domains

To test the possibility that transformation of fibroblasts modifies accessibility of plasma membrane cholesterol to extracellular molecules, we treated cholesterol-loaded pri-

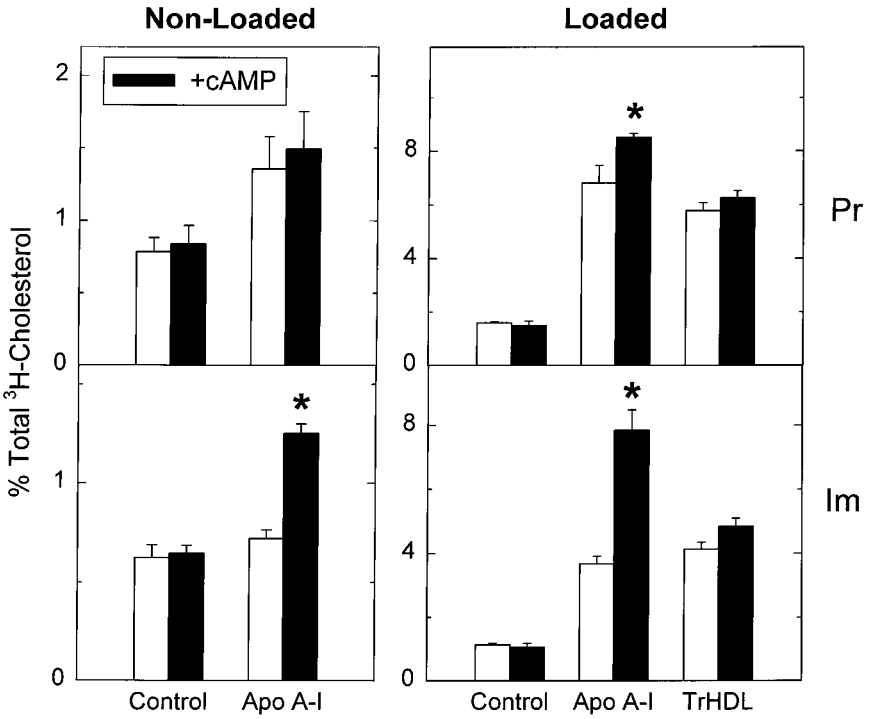


**Fig. 6.** Stimulation of apoA-I-mediated cholesterol efflux from immortalized normal fibroblasts by 8-Br-cAMP. Fibroblasts (NL4) not loaded with cholesterol (non-loaded) were radiolabeled and treated as in Fig. 3, and loaded fibroblasts were radiolabeled and loaded with cholesterol as described for Fig. 4. Cells were then incubated with serum-free medium alone (controls), with 180 nm apoA-I, or with 600 nm TrHDL. Half the wells (filled bars) received 1 mm 8-Br-cAMP (cAMP) during the 18-h equilibration and 6-h efflux incubations. Values are the mean ±SD [$^3$H]cholesterol efflux of triplicate wells expressed as percent total [$^3$H]cholesterol. Significant ($P < 0.03$) effects of apo8-Br-cAMP are indicated by asterisks. Total [$^3$H]cholesterol cpm/μg cell protein were (non-loaded, Pr) 1202 ± 252 (n = 12) (loaded, Pr) 852 ± 76 (n = 18) (non-loaded, Im) 1713 ± 164 (n = 12) (loaded, Im) 700 ± 63 (n = 18).

mary and immortalized normal and TD fibroblasts with cholesterol oxidase and cyclodextrin (see Experimental Procedures). Cholesterol oxidase converts exofacial plasma membrane cholesterol to cholestenone and has been used as a probe to assay cholesterol associated with caveolae (4, 30). Oxidase treatment converted approximately 20% of the total radiolabeled free cholesterol to cholestenone for all primary and immortalized cell lines (data not shown). Cyclodextrin is an efficient acceptor of cholesterol and removes a large fraction of plasma membrane cholesterol after short incubations (32). Treatment with this compound for 30 min removed 40–50% of the free [$^3$H]cholesterol from all cell lines (data not shown). Thus accessibility of [$^3$H]cholesterol to these extracellular molecules was similar for primary and immortalized normal and TD cells despite marked differences in apoA-I-mediated [$^3$H]cholesterol efflux.

To examine directly the lipid composition of caveolae-rich membranes, we treated the four different fibroblast lines with either Triton X-100 or sphingomyelinase (see Experimental Procedures). Caveolae are components of cholesterol- and sphingomyelin-rich membranes that are resistant to solublization in Triton X-100 at 4°C (33–35). Cholesterol loading of fibroblasts increased the fraction of radiolabeled cholesterol resistant to Triton X-100 from approximately 10% to 40%. The fractions of detergent-insoluble cholesterol in all the cholesterol-loaded immortalized cells were the same as that in the parental lines (data not shown). Treatment of cells with sphingomyelinase digested nearly 80% of the radiolabeled sphingomyelin in all four cell lines (data not shown), indicating similar fractions of radiolabeled sphingomyelin in the plasma membrane. These results indicate that the differences in apoA-I-mediated cholesterol and phospholipid efflux among these four primary and immortalized cell lines were not associated with marked differences in the properties of plasma membrane lipid domains.

### Reversion of immortalization-induced reductions in lipid efflux by cAMP

Smith et al. (36) and Sakr et al. (37) reported that treatment of macrophages with cAMP induces apolipoprotein-mediated cholesterol efflux, raising the possibility that changes in the cellular content of cAMP could influence the activity of this lipid transport pathway. Measurements of cellular cAMP levels revealed that papillomavirus-induced immortalization of fibroblasts significantly reduced their cAMP content. For cell line NL4 maintained on serum, mean cAMP levels ($\pm$SD, n = 3) were 8.21 $\pm$ 1.41 and 5.30 $\pm$ 0.66 pmol/mg cell protein in the primary and immortalized cells, respectively. For the same cells deprived of serum and loaded with cholesterol, cAMP levels were 8.07 $\pm$ 1.36 and 4.73 $\pm$ 0.29 pmol/mg cell protein, respectively. Although changes in cAMP levels could not account for the increased apolipoprotein-mediated lipid efflux caused by serum deprivation and cholesterol loading, decreased cAMP levels may have contributed to the inactivation of this pathway after immortalization.

To confirm that cAMP plays a role in modulating apolipoprotein-mediated lipid efflux from fibroblasts, we treated primary and immortalized fibroblasts with 8-Br-cAMP prior to measuring apoA-I-mediated cholesterol efflux. Treatment of primary normal fibroblasts with 1 mm 8-Br-cAMP had a slight stimulatory effect on cholesterol efflux mediated by apoA-I, which was significant for cholesterol-loaded cells (Fig. 6, upper panels). Similar results were observed with mock-infected fibroblasts (data not shown). In contrast, treatment of both non-loaded and cholesterol-loaded immortalized fibroblasts with 8-Br-cAMP enhanced apoA-I-mediated cholesterol efflux over 2-fold (Fig. 6, bottom panels). Non-loaded immortalized cells showed the largest relative increase because apoA-I had no significant stimulatory effect on cholesterol efflux in the absence of 8-Br-cAMP. The maximum effect of 8-Br-cAMP required pretreatment of cells, as addition of this analog to the apoA-I-containing media caused only a modest (20–30%) increase in cholesterol efflux during the 6 h incubations. As with primary cells, 8-Br-cAMP had no effect on cholesterol efflux from cholesterol-loaded immortalized cells during control incubations or in the presence of TrHDL. These results indicate that 8-Br-cAMP selectively enhances the apolipoprotein-mediated cholesterol removal pathway in immortalized fibroblasts.

In contrast to its effects on normal cells, 8-Br-cAMP had no significant effect on apoA-I-mediated cholesterol efflux from cholesterol-loaded immortalized TD cells (data not shown). Thus, this cAMP analog was unable to correct the lipid transport defect in TD cells.

Previous studies showed that cAMP can stimulate neutral cholesteryl ester hydrolase activity in some cell types (38), suggesting that the enhanced cholesterol efflux may be secondary to an increased cholesteryl ester turnover. To test this possibility, we measured changes in medium and cellular radiolabeled cholesterol after treatment of cholesterol-loaded fibroblasts with increasing concentrations of 8-Br-cAMP. With the immortalized cells, 8-Br-cAMP caused a dose-dependent, saturable increase in apoA-I-mediated cholesterol efflux (**Fig. 7**). During the 6-h efflux incubations, apoA-I-stimulated cholesterol efflux from both primary and immortalized cells was associated with a decrease in the radiolabeled unesterified cholesterol content of the cells. The fraction of cellular cholesterol that was esterified was much lower in immortalized cells than in the parental line, as would be expected for more rapidly proliferating cells (Fig. 7). Addition of apoA-I and 8-Br-cAMP had no effect on the radiolabeled cholesteryl ester content in either cell line, indicating that increased turnover of cholesteryl esters could not account for the enhanced cholesterol efflux. One-hour pulse incubation with [$^{14}$C]oleate revealed that incorporation of oleate into cholesteryl esters was unaffected by 24-h treatments of both primary and transformed cells with 8-Br-cAMP (not shown), implying that this agent had no effect on either the activity of acyl CoA:cholesterol acyltransferase or the availability of cholesterol substrate for this enzyme.

Treatment of cells with 8-Br-cAMP for 24 h had no significant effect on the cellular protein content per well (Fig. 7), excluding the possibility that the enhanced cho-

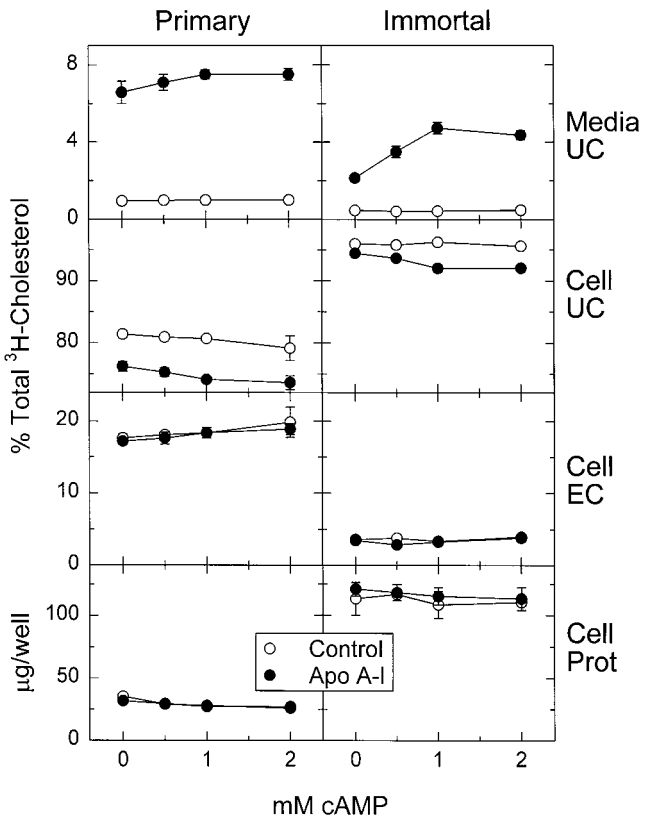


**Fig. 7.** Dose-dependent effects of 8-Br-cAMP on [$^3$H]cholesterol efflux, cellular free and esterified [$^3$H] cholesterol content, and cellular protein content of primary and immortalized normal fibroblasts. Radiolabeled and cholesterol-loaded fibroblasts (NL4) were incubated for 6 h with serum-free medium containing either zero (open symbols) or 180 nm (closed symbols) apoA-I. The indicated concentrations of 8-Br-cAMP were added to the medium during the 18-h equilibration and 6-h efflux incubations. Media and cellular unesterified [$^3$H]cholesterol (UC), cellular esterified [$^3$H]cholesterol (EC), and cellular protein (prot) content of each well were measured after the efflux incubations. Results for media and cellular sterols are expressed as percent of total (medium plus cell) free and esterified [$^3$H]cholesterol. Each value is the mean ± SD of triplicate wells. Total [$^3$H]cholesterol cpm/µg cell protein were (Pr) 1378 ± 104 (n = 24) (Im) 1294 ± 103 (n = 24).

lesterol efflux was secondary to inhibition of cell proliferation per se.

Phospholipid efflux from the different cell lines showed a similar pattern as cholesterol efflux. Treatment with 8-Br-cAMP increased apoA-I-mediated phosphatidyl[$^3$H]choline and [$^3$H]sphingomyelin efflux to a greater extent from immortalized normal cells than from the parental primary cells (**Fig. 8**) or mock-infected cells (not shown), and again it had no effect on phospholipid efflux from either immortalized or primary TD cells (Fig. 8).

### Enhancement of apoA-I binding to immortalized cells by cAMP

Immortalization of fibroblasts led to a reduction in the number of apoA-I binding sites on cells. After loading with cholesterol, immortalized fibroblasts bound 50–60% less $^{125}$I-labeled apoA-I (at 1 µg/ml) per unit cell protein than did the parental primary cells (data not shown). When immortalized normal cells maintained on serum were treated with 8-Br-cAMP, binding of $^{125}$I-labeled apoA-I to cells was increased approximately 60% (**Fig. 9A**). With cholesterol-loaded cells, the percent stimulation was less (~35%) (Fig. 9B), presumably because the cholesterol loading protocol increased basal apoA-I binding. Treatment with 8-Br-cAMP had no effect on apoA-I binding to either non-loaded or cholesterol-loaded immortalized TD cells (Fig. 9), and it increased binding less than 10% to cholesterol-loaded normal primary cells (data not shown). Thus the stimulatory effects of 8-Br-cAMP on lipid efflux was associated with increased binding of apoA-I to cells.

## DISCUSSION

HDL phospholipids and lipid-free apolipoproteins remove cholesterol from cells by two independent mechanisms (1). We found that the apolipoprotein-mediated cholesterol removal pathway has little or no activity in many cultured immortalized cell lines despite attempts to overload these cells with cholesterol. This low activity was evident even with cell lines that exhibit high rates of cholesterol efflux in the presence of HDL phospholipids. As an example, purified apoA-I had virtually no ability to remove cholesterol from Fu5AH cells, a hepatoma cell line exhibiting an exceptionally high rate of phospholipid-mediated cholesterol efflux because of abundant expression of SR-BI (Fig. 1) (3). These findings raised the possibility that immortalizing primary cells selectively inactivates the apolipoprotein-mediated lipid removal pathway.

To test this hypothesis in more detail, we immortalized primary human skin fibroblasts by infection with papillomavirus E6/E7 subgenes. Immortalization of normal fibroblasts markedly reduced the activity of the apolipoprotein-mediated lipid removal pathway. When cells were maintained on serum-containing growth medium, purified apoA-I had a modest ability to promote cholesterol efflux from the primary cells but had essentially no ability to remove cholesterol from the immortalized cells. Exposure of cells to serum-free and cholesterol-rich medium for 2–3 days increased apoA-I-mediated cholesterol efflux from both primary and immortalized cells, but the maximum activity of this pathway was 60% lower after immortalization. The ability of apoA-I to remove phospholipids from cholesterol-loaded cells was also lower in the immortalized cells compared to the parental cells. The lower activity in immortalized cells does not appear to be attributable to selection of unique cell populations, as it was observed with two different fibroblast lines assayed over a wide passage range (6–14) and was not seen with cells infected with vector alone.

In contrast to its effects on the apolipoprotein-mediated pathway, immortalization of fibroblasts had little or no effect on cholesterol efflux promoted by HDL phospholipids. This was evident from results showing that TrHDL, which removes cholesterol from cells by the phospholipid-mediated process only (12–14, 19), had a similar ability to promote

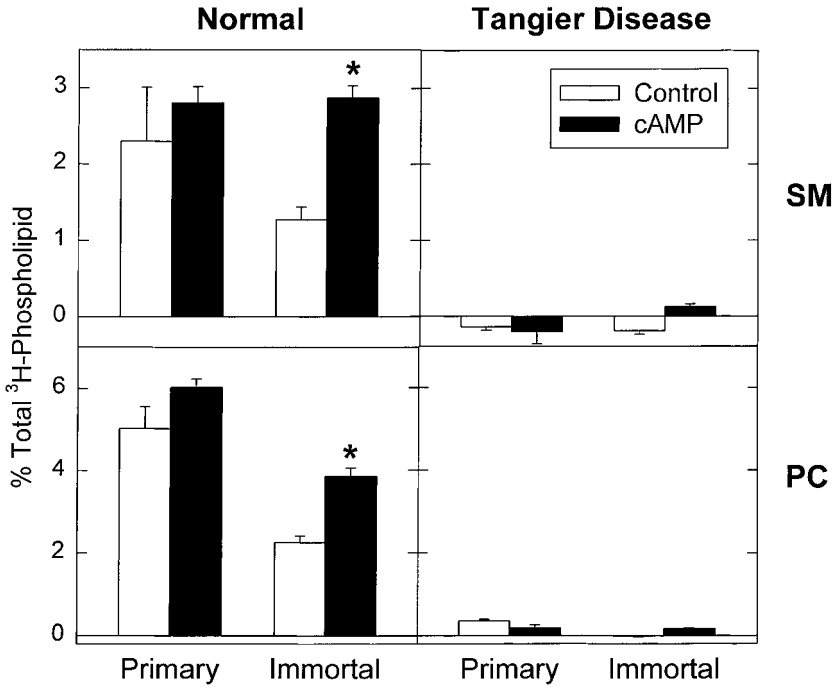


**Fig. 8.** Stimulation of apoA-I-mediated phospholipid efflux from primary and immortalized normal and Tangier disease fibroblasts. Fibroblasts (NL4, JW) were cholesterol-loaded and labeled with [$^3$H]choline as described in Methods and then incubated for 6 h with serum-free medium alone or with 180 nm apoA-I. Half the wells (solid bars) received 1 mm 8-Br-cAMP during the 18-h equilibration/radiolabeling and 6-h efflux incubations. The cAMP analog had no effect on incorporation of [$^3$H]choline into sphingomyelin and phosphatidylcholine. Results are expressed as the percent total [$^3$H]sphingomyelin (SM) and phosphatidyl[$^3$H]choline (PC) appearing in the apoA-I-containing medium minus the mean percent appearing in the control medium, which in some cases yielded negative numbers. Each value is the mean ± SD of triplicate wells. Total [$^3$H]SM and [$^3$H]PC cpm/μg cell protein (n = 12 each) were respectively (Normal, Pr) 227 ± 28, 3283 ± 288; (Normal, Im) 206 ± 33, 2491 ± 190; (Tangier, Pr) 209 ± 17, 3153 ± 193; (Tangier, Im) 272 ± 37, 3876 ± 337.

cholesterol efflux from primary and immortalized cells. The immortalization also reduced cholesterol efflux promoted by native HDL, but to a lesser extent than that seen with purified apoA-I, presumably because HDL promotes cholesterol efflux by both apolipoprotein- and phospholipid-mediated processes (1, 12–14, 19). Thus, as predicted from the studies with different immortalized cell lines, immortalization of fibroblasts selectively reduced the apolipoprotein-mediated lipid removal pathway.

Previous studies have suggested that caveolae-rich plasma membrane domains are the primary source of cholesterol released from fibroblasts to HDL particles (4), raising the possibility that changes in the properties of these domains may account for the reduction in lipid efflux after immortalization. Enzyme and detergent treatment protocols, however, showed similar fractions of radiolabeled cholesterol and sphingomyelin in caveolae-containing domains of the plasma membranes of primary and immortalized fibroblasts exposed to cholesterol-rich medium, despite marked differences among cell lines in the ability of apoA-I to remove these lipids. Based on immunoblot analysis, we also found no detectable differences in expression of caveolin-1 between immortalized cells and their parental cells (data not shown). Thus, in contrast to a previous study showing a decreased caveolin-1 expression after oncogenic transformation of NIH3T3 cells (39), papillomavirus-induced immortalization of fibroblasts had no effect on expression of this protein. These findings further support previous evidence (15) that caveolae-rich membranes do not participate in the lipid efflux pathway mediated by lipid-free apolipoproteins.

We also tested the possibility that the immortalization-induced reduction in this lipid efflux pathway reflected differences in expression of SR-BI, a caveolae-associated HDL receptor reported to bind lipid-free apoA-I as well as HDL phospholipids (40, 41). Immunoblot analysis, however, did not reveal any reproduceable differences in SR-BI levels between primary and immortalized fibroblasts (data not shown), consistent with a previous report that SR-BI does not play a role in cholesterol efflux mediated by lipid-free apoA-I (3). This conclusion is further supported by results showing that apoA-I has virtually no ability to remove cholesterol from Fu5AH cells despite very high levels of SR-BI expression (3).

Although immortalizing cells can alter many cellular processes involved in lipid trafficking, it is likely that increased proliferation rates play a major role in suppressing the apolipoprotein-mediated lipid removal pathway.

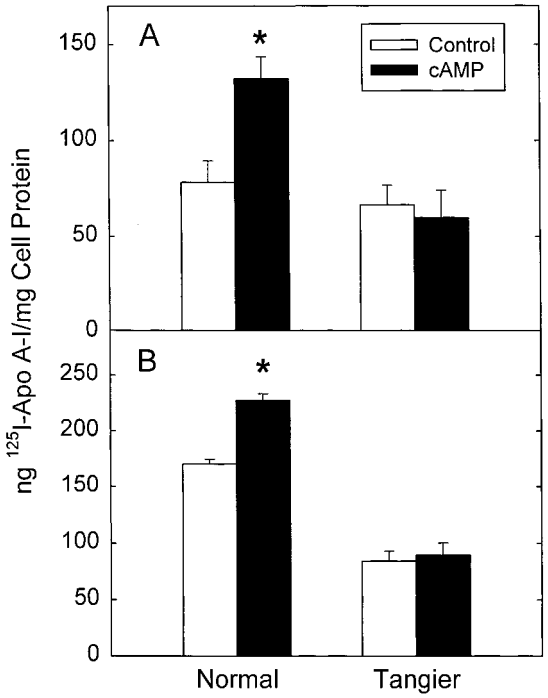


**Fig. 9.** Effect of 8-Br-cAMP on apoA-I binding to immortalized normal and Tangier disease fibroblasts. Immortalized normal (NL4) and Tangier disease fibroblasts (JW) were either maintained on growth medium (A) or loaded with cholesterol (B) as described for Fig. 3 and Fig. 4. Cells were then incubated for 6 h with serum-free medium containing 1 μg/ml $^{125}$I-labeled apoA-I, and cell-associated radioactivity was measured as described in Methods. Half the wells (solid bars) received 1 mm 8-Br-cAMP during the 18-h equilibration and 6-h binding incubations. Results are the means ± SD of quadruplicate wells. Significant ($P < 0.01$) effects of 8-Br-cAMP are indicated by asterisks. Similar results were observed with immortalized cell lines NL7 and IM.

Papillomavirus-immortalized fibroblasts proliferate at much faster rates that their parental primary cells (Fig. 2). In contrast to primary cells, which become quiescent, removal of serum growth factors from the medium only partially inhibits proliferation of immortalized fibroblasts. Previous studies have shown that serum deprivation or treatment with growth-inhibiting cytokines increases the activity of this lipid removal pathway in primary fibroblasts (12, 13, 42). Conversely, exposure of quiescent fibroblasts to growth factors suppresses HDL-mediated cholesterol efflux from these cells (43, 44). Thus the apolipoprotein-mediated lipid removal pathway is most active in cells that do not require cholesterol for continual membrane synthesis during proliferation. Immortalization of fibroblasts also produced morphological changes, including decreased size or less complex shape, which may contribute to the reduced activity of this pathway.

Smith et al. (36) and Sakr et al. (37) reported that the apolipoprotein-mediated lipid removal pathway was induced by treatment of immortalized RAW264 and J774 macrophages with cAMP analogs. This enhanced activity was associated with increased binding of apoA-I to cells and required new protein synthesis. These findings implicate cAMP as an important modulator of the apolipoprotein-mediated lipid pathway, in some cells. The current study provides additional support for this concept. Here we show that immortalization of fibroblasts reduced the cellular cAMP content, and that treatment of these cells with 8-Br-cAMP increased apoA-I-mediated cholesterol and phospholipid efflux to levels near that seen with the parental primary cells. This stimulation was specific for the apolipoprotein-mediated lipid efflux pathway, as evidenced by a lack of effect of 8-Br-cAMP on cholesterol efflux promoted by TrHDL. As also shown previously for macrophages (36, 37), the stimulatory effect of cAMP on lipid efflux from immortalized fibroblasts was associated with an increased binding of apoA-I to cells. In contrast to the immortal cells, primary fibroblasts showed little or no increase in apolipoprotein-mediated lipid efflux when treated with 8-Br-cAMP, perhaps because their cAMP content was already above maximal levels for modulating this pathway. These results support the possibility that a reduction in cAMP levels contributes to the decreased activity of the apolipoprotein-mediated lipid removal pathway after immortalization.

Decreased cellular cAMP levels may contribute to the phenotype of these immortalized fibroblasts, particularly the rapid proliferation. This signaling agent is anti-proliferative for many cultured cell lines, including fibroblasts (45, 46). It is possible that cAMP-dependent protein kinases play a role in coupling mitogenesis to lipid excretion so as to maintain an adequate supply of cholesterol and phospholipids. Marked changes in the immortalized phenotype, however, are not required for the stimulatory effects of cAMP on lipid excretion to apolipoproteins, for this stimulation occurs under conditions where there is no significant inhibition of cell proliferation nor visible changes in cell morphology. Also, immortalization per se is not required, because cAMP analogs stimulate this lipid removal pathway in primary mouse peritoneal macrophages while having no effect in many other immortalized cell lines (37). Because 8-Br-cAMP treatment had no effect on cholesteryl ester turnover or cholesterol esterification, its lipid efflux-enhancing effects are not attributable to changes in the activities of cholesteryl ester cycle enzymes or the size of the cholesterol pool that feeds into this cycle. It is more likely that a cAMP-dependent signaling pathway directly induces or activates key proteins involved in the apolipoprotein-mediated lipid removal pathway.

Fibroblasts from subjects with homozygous TD have an unknown molecular defect that nearly abolishes the ability of apoA-I to remove cellular lipids. Treatment of immortalized TD fibroblasts with 8-Br-cAMP was unable to correct this defect. Also, immortalized TD fibroblasts did not exhibit increased apoA-I binding after 8-Br-cAMP treatment. These results provide additional support for the conclusion that apolipoprotein-mediated lipid removal is closely coupled to the interaction of apoA-I with plasma membrane binding sites. The lack of cAMP effect in immortalized TD cells implies that the defective protein in TD is upstream from the cAMP-dependent signaling pathway involved in modulating removal of lipids by apolipoproteins.

In summary, the current study shows that immortalization of human skin fibroblasts reduces the activity of the apolipoprotein-mediated lipid removal pathway while having no substantial effect on HDL phospholipid-mediated cholesterol efflux. The immortalization-induced reduction in apolipoprotein-mediated lipid removal was largely reversed by treatment of cells with a cAMP analog, further implicating protein kinase A as an important modulator of this pathway. Neither immortalization nor 8-Br-cAMP treatment corrected the impaired apolipoprotein-mediated lipid removal pathway in TD fibroblasts, indicating that the molecular defect expressed in these cells is independent of the immortalized phenotype and cellular cAMP levels.

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