Vimentin-dependent utilization of LDL-cholesterol in human adrenal tumor cells is not associated with the level of expression of apoE, sterol carrier protein-2, or caveolin

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Abstract **SW-13 adrenal tumor cells that lack detectable intermediate filaments (IF-free) exhibit an impaired capacity to esterify lipoprotein-derived cholesterol compared with cells that contain vimentin filaments. IF-free cells were found to synthesize and secrete significant amounts of apoE, while apoE secretion was nearly undetectable in cell lines that spontaneously express vimentin. However, stable transfectants that express a mouse vimentin cDNA exhibited elevated levels of cholesterol esterification and apoE secretion compared with untransfected IF-free cells, indicating that apoE secretion is not directly related to the capacity of these cells to esterify cholesterol. Some of the cell lines that differed in the level of apoE synthesis and secretion had similar levels of apoE mRNA, suggesting that the differences in expression involve a post-transcriptional mechanism. Treatment of these cells with forskolin and IBMX, 8br-cAMP, or TPA had no effect on apoE secretion. The level of sterol carrier protein-2 (SCP_2) synthesis and the distribution of SCP_2 between membrane and soluble cellular fractions was not observably different in cells that contained or lacked vimentin. SW-13 cell lines contained little or no detectable caveolin-1 or caveolin-2. These studies demonstrate that the difference in the capacity of these adrenal tumor cells that contain or lack vimentin filaments to esterify low density lipoprotein-cholesterol is not obviously associated with the level of expression or distribution of apoE, SCP_2, or caveolins.**—Holwell, T. A., S. C. Schweitzer, M. E. Reyland, and R. M. Evans. **Vimentin-dependent utilization of LDL-cholesterol in human adrenal tumor cells is not associated with the level of expression of apoE, sterol carrier protein-2, or caveolin.** ***J. Lipid Res.*** **1999.** 40: **1440–1452.**

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Lipoprotein-derived cholesterol is an important substrate for steroidogenesis (1). Although little is known about the mechanisms responsible for the post-lysosomal transport of cholesterol (2), studies of adrenal tumor cells have indicated that the intermediate filament (IF) component of the cell cytoskeleton may be involved (3). Fluorescence microscopy of lipid droplets and IFs in Y-1 adrenal cells has shown that the cholesteryl ester-rich lipid droplets often co-localize with filaments of the vimentin type (4). When viewed by whole mount electron microscopy, the vimentin IFs appear to form contacts with the surface of the lipid droplets in primary adrenal cells (5). Examination of the lipid metabolism of SW-13 adrenal tumor cells that either contain or lack cytoplasmic IFs has demonstrated that expression of a vimentin-type IF-network had a specific effect on cholesterol metabolism. IF-free SW-13 cell lines were found to have a lower capacity to esterify lipoprotein-derived cholesterol, compared with cells that contained vimentin filaments (6). In these studies the impaired capacity of IF-free adrenal tumor cells to utilize lipoprotein cholesterol was restored in cell lines that expressed a mouse vimentin cDNA. The difference in the capacity of SW-13 cells to utilize lipoprotein cholesterol was not associated with a difference in receptor-mediated endocytosis of lipoprotein or the inherent capacity of the cells to esterify cholesterol, but rather appeared to be associated with post-lysosomal transport (6).

The post-lysosomal events that mediate the intracellular movement of lipoprotein-derived cholesterol have not been well characterized (reviewed in 2, 7, 8). Studies of the human disease Niemann-Pick type C (see ref. 9), mutant cells selected for defective cholesterol transport (see 10, 11), and the effect a variety of inhibitors (12) have indicated that the transport of cholesterol derived from lipoprotein may occur independently from the movement of endogenously produced cholesterol, but very little is known about the mechanism or mechanisms. Although there is strong evidence that lipoprotein-

Abbreviations: SCP_2, sterol carrier protein-2; apoE, apolipoprotein E; TPA, phorbol-12-myristate-13-acetate; IBMX, 3-isobutyl-1-methylxanthine; IF, intermediate filament; PKA, protein kinase A; PKC, protein kinase C.

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derived cholesterol transport involves specific cytoplasmic components, it is not clear whether this involves vesicle-mediated transport or another carrier molecule. There is evidence that proteins such as apolipoprotein E (apoE) (13, 14), sterol carrier protein 2 (SCP_2) (15, 16), and caveolin-1 (8) could be involved, but the precise role of these proteins in intracellular cholesterol transport has not been determined.

The importance of secreted apoE in cholesterol transport between peripheral tissues and the liver is well established (17) and there is now evidence that apoE expression also affects the intracellular utilization of cholesterol that is required for the synthesis of steroid hormones (18, 19). Steroidogenic tissues are a site of significant apoE synthesis (18). In the adrenal gland, apoE mRNA levels are directly related to the level of cholesteryl ester stores and inversely related to the level of steroidogenesis (20). Purified apoE added to primary rat ovarian cell cultures has been reported to inhibit LH-dependent androgen synthesis (21), and expression of a human apoE gene in mouse Y-1 adrenal cells has been shown to produce a significant suppression of steroidogenesis (13). In Y-1 cells, apoE expression was found to be associated with an increased cellular cholesterol content and decreased efflux of free cholesterol, indicating that apoE expression can affect intracellular cholesterol homeostasis (14). Recently, Swarnakar et al. (22) reported that apoE expression in Y-1 cells was associated with an increase in the selective uptake of cholesteryl ester derived from LDL. While these studies have suggested that apoE can affect some aspects of intracellular cholesterol metabolism, the mechanism by which a secreted protein could mediate these effects remains unknown.

Numerous studies have suggested a role for SCP_2 in intracellular sterol transport (7). The SCP_2 gene encodes both a 58 kD and a 15 kD protein (23). The 58 kD protein is localized within peroxisomes, while the 15 kD SCP_2 protein is proteolytically cleaved to a 13.2 kD form that is found in mitochondria, cytosol, the cytosolic surface of peroxisomes (24, 25) and is the form that has been implicated in intracellular cholesterol trafficking. While it may not be required for the intracellular movement of cholesterol (26), the 13.2 SCP_2 protein has been shown to enhance sterol transfer between various membranes in vitro (27, 28), and to stimulate steroidogenesis and esterification of plasma membrane cholesterol when expressed in cells (15, 29). Although there is no known connection between SCP_2 and the cytoskeleton, there is at least a superficial similarity in the phenotype of SCP_2-deficient and IF-deficient cells. CHO cell mutants that lack peroxisomes and are deficient in the 13.2 kD SCP_2 protein have been reported to exhibit an increase in cholesterol synthesis and increased cholesterol efflux, while uptake and hydrolysis of LDL-cholesterol was not affected (30). SW-13 adrenal tumor cells that lack IFs also exhibit an increase in cholesterol synthesis and efflux without apparent effects on the uptake and hydrolysis of LDL-cholesterol (6).

Caveolae are specialized clatherin-free areas of plasma membrane that are rich in cholesterol and glycolipids, and appear to be involved a variety of transport processes (31, 32) including transcytosis of LDL in brain capillary endothelial cells (33). Caveolin-1 is a major caveolae coat protein that has been shown to bind cholesterol (34), and appears to cycle between the cell surface and the Golgi complex (35, 36). This has led to speculation that caveolin may be involved in trafficking and cholesterol homeostasis (36). The subcellular distribution of caveolin has been shown to be affected by agents that disrupt microtubules and IFs (32), however, the possibility of an interaction between IFs and caveolin or caveolae has not been directly examined.

To try to gain some insight as to how vimentin IFs affect the metabolism of cholesterol, studies were carried out to determine whether the differences in the capacity of SW-13 adrenal tumor cells that contain or lack vimentin filaments to utilize lipoprotein-derived cholesterol could be related to the expression or distribution of apoE or SCP_2. We have found that SW-13 cells that contain or lack vimentin IFs have similar levels of SCP_2 and little or no caveolin-1 or caveolin-2. While these cell lines were found to differ substantially in apoE synthesis and secretion, there was no direct relationship between the level of apoE secretion and the capacity of these cells to esterify lipoprotein-derived cholesterol. These observations indicate that the effect of vimentin-type IFs on the capacity of SW-13 cell lines to utilize lipoprotein-derived cholesterol is not obviously associated with the level of expression or subcellular distribution of apoE, SCP_2, or caveolins 1 or 2.

MATERIALS AND METHODS

Cells and cell culture

SW-13 cell lines that spontaneously contain (vim^+) or lack (IF-free) vimentin filaments (37, 38) were grown in monolayer culture in a lipid-free medium consisting of a 1:1 mixture of Ham's F12:Dulbecco's MEM containing either 5% CPSR-1 (Sigma) or 20 ml/L TCM (Celox), 0.5 mg /ml fetuin, and 10 μg/ml gentamicin. Additional cell lines stably transfected with a mouse vimentin cDNA (6) were grown in medium containing 5% CPSR-1 or 20 ml/L TCM, 0.5 mg/ml fetuin, and 200 μg/ml G418. Preliminary experiments indicated that the relative rates of cholesterol esterification in SW-13 cell lines were similar in medium supplemented with either CPSR-1 or TCM (data not shown). Low density lipoprotein (LDL) was purified from human plasma by KBr density gradient ultracentrifugation by the method of Goldstein, Basu, and Brown (39). Forskolin (Sigma), 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma), 8-bromo-cAMP (Boehringer Mannheim), chelerythrine (LC Laboratories), and phorbol-12-myristate-13-acetate (TPA)(Sigma) were added to cultures at the indicated concentrations.

Immunoprecipitation

Approximately 2×10^6 SW-13 cells that spontaneously contain or lack vimentin were plated per 10 cm dish, 48 h prior to radiolabeling. The stable transfectant cell lines grow more slowly and were plated at an initial density of 3×10^6 cells per 10 cm dish. The cells were radiolabeled with 50–100 μCi/ml [^{35}S]methionine in 4 ml of complete medium for the indicated times. In experiments that involved secreted protein, the culture medium was recovered and centrifuged at approximately 500 *g* for 5 min.

The resulting supertnatant was recovered and PMSF and leupeptin was added to final concentrations of 0.2 mm and 5.0 μg/ml, respectively. The medium was centrifuged again at 10,000 *g* for 5 min. Triton X-100 was added to the resulting supernatant to a final concentration of 1% and this solution was centrifuged again at 5000 *g* for 10 min. In experiments involving immunoprecipitation from monolayer cells, the monolayer was rinsed 3 times with PBS. The cells were then lysed in 1 ml of 145 mm NaCl, 20 mm Tris-HCl, pH 7.4, 1 mm EDTA (TBS) containing 0.5% Triton X-100, 0.5 mm PMSF, 0.5 μg/ml leupeptin. The cell lysate was centrifuged at 14,000 *g* for 2–5 min, and the supernatant was recovered for immunoprecipitation. For each experiment, the samples were normalized to contain the same amount of ^{35}S-labeled protein. Fifty μl of goat anti-apoE (Calbiochem), or 4 μg monoclonal anti-caveolin-1 (Transduction Laboratories), or anti-caveolin-2 (Transduction Laboratories), 10 μl of rabbit anti-SCP_2 (gift from J. Billheimer) diluted 1:10 in TBS, was added to 0.5–1.5 ml of sample (either culture medium or monolayer Triton-lysate). For anti-caveolin-1 and caveolin-2 immunoprecipitates, the antibodies were diluted in the lysis buffer. Similarly diluted nonimmune goat or rabbit serum (NGS or NRS) was added to duplicate samples as a negative control. Preliminary experiments determined that increasing the antibody concentration did not result in increased recovery of apoE or SCP_2. The samples were incubated for 1.5 h at 4°C. For anti-caveolin-1 and caveolin-2 immunoprecipitates, 12.5 μl of rabbit anti-mouse serum was then added as a bridge antibody and incubated an additional 1 h. Two hundred μl of blocked rProteinA-agarose (Repligen) slurry (100 μl packed volume) was then added to each sample and mixed for at least 1 h at 4°C. The samples were then centrifuged at 11,000 *g* for 1–2 min. The supernatant was removed and the agarose immunoprecipitate was resuspended in 1 ml TBS. This washing step was repeated 3 or 4 times. The final precipitate was resuspended in 15 μl of 0.25 m Tris, 40% glycerol, 6% SDS, 2 mm DTT, pH 6.8, and stored at −20°C. Precipitated proteins were analyzed by SDS-PAGE (40) in 12.5% (for SCP_2) or 10% (for apoE and caveolin-1) polyacrylamide gels. After electrophoresis, gels were stained with Coomassie blue, destained, and infiltrated with Amplify (Amersham) or 1 m salicylic acid. The gels were dried and ^{35}S-labeled proteins were directly quantitated using an imaging scanner (Bioscan System 200). The gels were scanned for 30 min per lane using the two-dimensional analysis program. The ^{35}S radioactivity for each individual protein band was determined using the manual mode. The gels were then fluorographed on Hyperfilm (Amersham) at −70°C. In experiments where the radiolabeled monolayer was not used for immunoprecipitation, the monolayer was rinsed 2 times with PBS, 1.0 ml of 2% SDS, 0.5% deoxycholate, 1 mm EDTA, 1 mm dithiothreitol was added, and the resulting cell lysate was recovered. These cell lysates were then used to determine ^{35}S incorporated into TCA-precipitable protein.

RNA isolation and Northern analysis

Cells were solubilized in 4 m guanidinium thiocyanate, 5 mm Na citrate, 0.5% sarcosyl, 0.1 m 2-mercaptoethanol, and total RNA was prepared by pelleting the lysate through a 5.7 m CsCl cushion in an SW41 rotor at 150,000 *g* for 16 h. The RNA (20 μg/lane) was separated on agarose formaldehyde gels, transferred to Nytran (Scheicher and Schuell), and cross-linked with ultraviolet radiation. To determine apoE mRNA levels, the blots were hybridized with a ^{32}P-labeled human apoE cDNA probe. The blots were then stripped and re-probed with a rat glyceraldehyde phosphate dehydrogenase cDNA probe as a control for RNA loading (13). Probes were radiolabeled with [α^{32}P]-dCTP using a random primer kit (Stratagene). After hybridization, ^{32}P-labeled bands on the dried Nytran were directly quantitated using a Molecular Dynamics PhosphorImager. The blots were autoradiographed on Hyperfilm (Amersham) at −70°C.

Cell cholesterol content and esterification

In some experiments, the secretion of apoE, cell cholesterol content and esterification were measured. Cells were labeled with [^{35}S]methionine as described above. After 20 h, 0.5 μCi/ml [1-^{14}C]oleate–BSA (39) was added to the medium and the cells were incubated in the presence of both radiolabels for 4 h. The medium was then removed for immunoprecipitation of [^{35}S]apoE. The cell monolayer was rinsed 2 times with TBS, 50 μg of cholesteryl heptadecanoate was added as an internal standard, and then extracted twice with hexane–isopropyl alcohol 3:2. The combined extracts were dried under nitrogen, resuspended in chloroform, and an aliquot was taken for analysis by either TLC or HPLC. Neutral lipids were separated by TLC on activated Silica Gel G plates (Analtech) using a solvent system of hexane–ethyl ether–acetic acid 60:40:1 (v/v). The lipids were visualized by spraying the chromatograms with an aqueous solution of 0.5% 8-anilino-1-naphthalenesulfonic acid and detected with UV light. Lipids were identified by co-chromatography with appropriate purified standards added to each sample (Avanti). Areas of the chromatograms representing resolved individual lipids were scraped into vials and radioactivity was determined in 4 ml of Ecolume (ICN Radiochemicals) by scintillation counting. For HPLC analysis, samples were dried under nitrogen, resuspended in 20 μl of chloroform, and diluted with 190 μl of isopropyl alcohol–acetonitrile 1:1 (v/v). The labeled cholesterol and cholesteryl esters were separated by HPLC as described by Vercaemst, Union, and Rosseneu (41) on a 4.6 × 125 mm Asahipak C18 column using an isopropyl alcohol–acetonitrile mobile phase at 0.6 ml/min. Eluted cholesterol and cholesteryl esters were detected by absorption at 214 nm and identified by co-elution with cholesterol, cholesteryl oleate, and cholesteryl heptadecanoate standards (Sigma). Peak areas were determined using a Gilson 714 analytical program and corrected for recovery using the cholesteryl heptadecanoate internal standard. [^{14}C]oleate incorporated into triglycerides and cholesteryl oleate was detected using an in-line radiodetector (β-Ram, IN/US systems) and peak areas were determined using the Gilson 714 analytical program. The extracted cell monolayer was dissolved in 0.1 N NaOH, neutralized with 1 m HCl, 0.2 m phosphate, and aliquots were taken for protein determination by the Bradford assay (42).

Immunofluorescence microscopy

Cells were plated on sterile glass cover slips. The cells were rinsed with warm PBS and then fixed in freshly prepared 4% paraformaldehyde in PHEM buffer (60 mm PIPES, 25 mm HEPES, 10 mm EGTA, 3 mm $MgCl_2$, pH 6.1) (43) for 5 min at room temperature. The monolayers were then extracted with PHEM buffer containing 0.15% Triton X-110 for 2 min, and then rinsed with PBS. For indirect immunofluorescence, the cells were preincubated in PBS containing 1% ovalbumin and 1% normal goat serum for 10 min, and then incubated with monoclonal anti-apoE for 40 min. The cells on cover slips were then rinsed in PBS and incubated with secondary antibody for 30 min. Monoclonal anti-human apoE (clone 2034, Bioreclamation) was used at 10 μg/ml. Lissamine–rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim), diluted 1:100, was used as the second antibody. All antibodies were diluted in PBS containing 1% ovalbumin and 1% normal goat serum. The cover slips were mounted in Immunomount (Lerner Labs) and viewed on an Olympus microscope equipped with epifluorescence optics. Photographic exposures were made for 40 sec using Kodak TMax

400 film and the film processed with an exposure index of 1200 using Kodak HC-110 developer.

RESULTS

Vimentin expressing and IF-free SW-13 cell lines secrete different amounts of apoE

In previous studies we have found that SW-13/cl.2 cells that lack detectable cytoplasmic intermediate filaments (IF-free) had a substantially lower capacity to esterify LDL-cholesterol than SW-13/cl.1 cells that contain vimentin IFs (vim$^+$). Moreover, expression of a mouse vimentin transgene in T3M cells, derived from the IF-free cl. 2 cell line, increased the capacity of these cells to esterify LDL-cholesterol. To determine whether the difference in intracellular cholesterol metabolism in vim$^+$ and IF-free SW-13 cells could be associated with differences in apoE expression, anti-apoE immunoprecipitation experiments were carried out to determine whether these cells express detectable levels of apoE. Initial experiments were performed with cl.1 vim$^+$, and IF-free cl.2 cells because these cell lines exhibit similar growth characteristics. As a positive control, the hepatoma HepG2 cell line that has been reported to secrete high levels of apoE (44) was also examined. Culture medium from [^{35}S]methionine-labeled cl.2 IF-free cells contained significant levels of [^{35}S]apoE (**Fig. 1**), although the amount of apoE that was produced was clearly less than that obtained from the hepatoma cell line. Little if any detectable [^{35}S]apoE was immunoprecipitated from cl.1 vim$^+$ cell medium under identical conditions. Treatment of cells with LDL stimulated apoE expression in all three cell lines. Quantitation of [^{35}S]apoE immunoprecipitated in triplicate samples indicated that LDL treatment approximately doubled the level of apoE secreted by the IF-free cl.2 cells (data not shown). Because [^{35}S]apoE was barely detectable in the medium from cl.1 vim$^+$ cells in the absence of LDL, it was not possible to accurately determine the actual level of induction of apoE secretion in these cells after LDL treatment. When the level of apoE secretion was compared in cultures treated with LDL, the IF-free cl.2 cell medium contained more [^{35}S]apoE than the medium from the cl.1 vim$^+$ cells. Anti-apoE immunoprecipitation from cell lysates indicated that [^{35}S]apoE was easily detected in HepG2 cell lysates, and could also be detected as a much less abundant band in the IF- free cl.2 cells. However, [^{35}S]apoE was undetectable in cl.1 vim$^+$ cell lysates, indicating that the low level of apoE secretion by these cells was not associated with a detectable accumulation of intracellular apoE. Comparison of the [^{35}S]apoE immunoprecipitated from the culture medium and cell lysates of the cl.2 and HepG2 cells indicated that most of the radiolabeled apoE was present in the culture medium. In addition, the [^{35}S]apoE immunoprecipitated from the monolayers was 2–3 kD smaller than the apoE band detected in the culture medium (see also Fig. 5). This is consistent with the increase in apparent molecular weight associated with glycosylation of the secreted form of apoE (44).

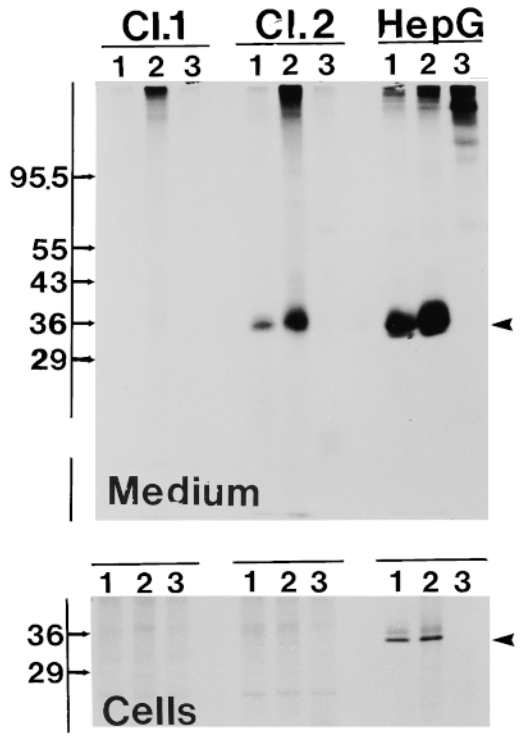


Fig. 1. Immunoprecipitation of apoE from the culture medium and cell lysates of SW-13/cl.1 (vim$^+$), SW-13/cl.2 (IF-free), and HepG2 cells. Anti-apoE immunoprecipitation of culture medium (Medium) and cell lysates (Cells) was performed from cells labeled for 24 h with 100 μCi/ml [^{35}S]methionine. The figure shows the fluorograph of a 10% SDS-polyacrylamide gel of anti-apoE immunoprecipitates from untreated cultures (lane 1), anti-apoE (lane 2), and non-immune serum (lane 3) precipitates from cultures treated with 50 μg/ml LDL. The immunoprecipitations of the cl.1 and cl.2 cell preparations represent material from equivalent numbers of cells. The position of the immunoprecipitated apoE is indicated (◄). The mobilities of MW standards are indicated in kD.

As the observed difference in the capacity of cl.1 vim$^+$ and IF-free cl.2 cells to secrete apoE could simply be a reflection of clonal variation between individual cell lines, apoE secretion was compared in four additional, independently isolated SW-13 cell lines that spontaneously contain or lack vimentin IFs, where the absence of filaments is associated with a lower level of cholesterol esterification (6). As shown in **Fig. 2**, quantitation of the [^{35}S]apoE immunoprecipitated from the culture medium of LDL treated cultures indicated that all three IF-free cell lines exhibited significant levels of apoE secretion, while apoE was barely detectable in the culture medium from the three vim$^+$ cell lines. Examination of total ^{35}S-labeled protein in the culture medium (**Fig. 3**) showed no prominent quantitative or qualitative differences in total protein secretion between the cell lines. This indicated that the observed difference in secretion of apoE in these cells was not associated with an overall difference in secretion of radiolabeled proteins.

The stimulation of apoE secretion observed in the cl.2 cells treated with LDL is consistent with previous studies indicating that cholesterol loading of macrophages stimu-

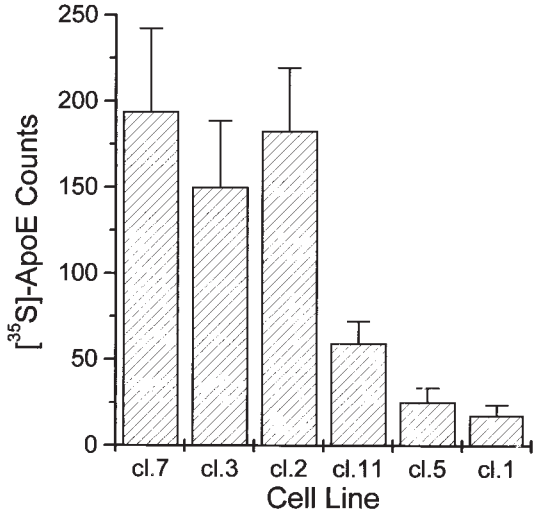


Fig. 2. ApoE secretion in SW-13 cell lines that spontaneously contain or lack vimentin filaments. Cultures of vim$^+$ cl.1, cl.5, and cl.11 cells and IF-free cl.2, cl.3, and cl.7 cells were treated with 50 μg/ml LDL for 24 h. The cells were then labeled with [^{35}S]methionine for 24 h in medium containing 50 μg/ml LDL and anti-apoE immunoprecipitates from equivalent numbers of cells prepared as shown in Fig. 1. The dried gels were directly scanned for ^{35}S and the cpm incorporated into the 36 kD apoE band were determined as described in the Methods section. The values represent the mean of immunoprecipitated [^{35}S]apoE from triplicate cultures ± SE.

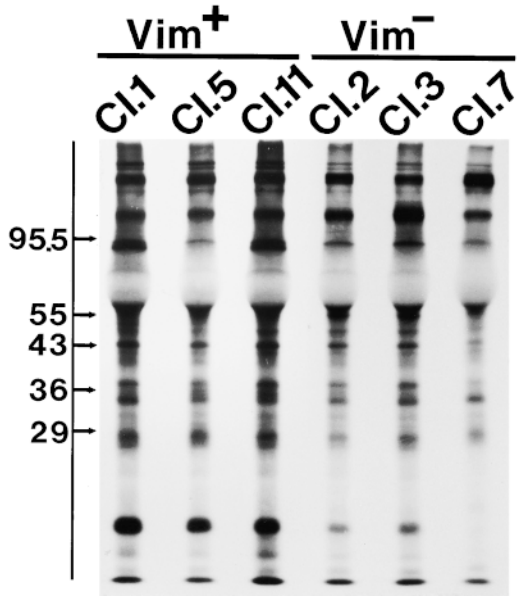


Fig. 3. Total ^{35}S-labeled protein released into the culture medium by SW-13 cell lines. Cultures of vim$^+$ cl.1, cl.5, and cl.11 cells and IF-free cl.2, cl.3, and cl.7 cells were treated with 50 μg/ml LDL for 24 h. The cells were then radiolabeled with [^{35}S]methionine for 24 h in medium containing 50 μg/ml LDL and samples of the culture medium representing equivalent numbers of cells were acetone precipitated. The figure shows the fluorograph of the precipitated ^{35}S-labeled protein separated on a 10% SDS-PAGE.

lates apoE synthesis (45, 46) by increasing apoE gene expression (20, 47). As IF-free SW-13 cells exhibit an impaired capacity to utilize lysosomal cholesterol compared with vim$^+$ cells, it was possible that the elevated level of apoE expression in the IF-free cells could have been a reflection of an accumulation of free cholesterol in a regulatory pool that does not occur in the vim$^+$ cells. The human apoE gene possesses a sterol regulatory element in its promoter region (48). In cultured macrophages, apoE expression can be stimulated with the soluble oxysterol, 25-hydroxycholesterol (47). We have previously shown that cholesterol esterification in cl.1 vim$^+$ and IF-free cl.2 cells is stimulated to the same extent by 25-hydroxycholesterol (6). Experiments were therefore conducted to determine whether treatment of the cl.1 vim$^+$ cells with 25-hydroxycholesterol would stimulate apoE expression to levels similar to that of the IF-free cl.2 cells. However, treatment of cl.1 and cl.2 cells with 25-hydroxycholesterol at concentrations up to 4 μg/ml had little effect on the appearance of [^{35}S]apoE in the culture medium of either cell line (data not shown).

Elevated cholesterol esterification associated with expression of a mouse vimentin cDNA does not inhibit apoE secretion in cells that lack an endogenous vimentin network

It is likely that vimentin is not the only gene that is differentially expressed in the vim$^+$ and IF-free SW-13 cell lines. Therefore, to help determine whether observed differences in apoE expression between the cells that contain or lack vimentin are associated with intermediate filament effects on cholesterol metabolism, the level of apoE secretion was examined in cl.2-derived cell lines that stably express a mouse vimentin cDNA. Previous experiments have demonstrated that the capacity of these stable transfectants to esterify LDL-derived cholesterol is related to the level of the expression of the vimentin transgene (6). Three stable transfected cell lines that express different levels of mouse vimentin and exhibit increasing levels of cholesterol esterification were compared with the parental IF-free cl.2 cells and the cl.1 vim$^+$ cells as positive and negative controls respectively. As shown in **Fig 4**, immunoprecipitation of [^{35}S]apoE from the culture medium of LDL treated cultures revealed that in contrast to the cells that spontaneously express vimentin IFs and have a barely detectable level of apoE secretion, the transfectant cell lines all exhibited levels of [^{35}S]apoE secretion that were equal to or greater than the parental cell line that lacks any detectable IF expression. The level of immunoprecipitated [^{35}S]apoE secreted into the culture medium was quantitated and compared with cholesterol esterification (**Table 1**). These results indicated that unlike the cell lines that spontaneously contain or lack vimentin filaments, where apoE secretion appears to be associated with reduced cholesterol esterification, the increased cholesterol esterification observed in cells that express the vimentin transgene was not associated with a decreased level of apoE secretion. Rather, the vim$^+$ transfectant line (T3M) that showed the highest level of cholesterol esterification exhibited a level of apoE secretion that was significantly higher than that of the parental IF-free cl.2 cell line (Table

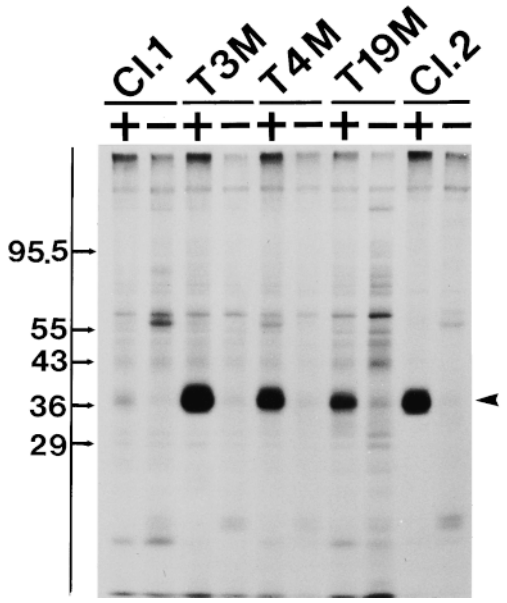


Fig. 4. ApoE secretion in SW-13 cell lines stably transfected with a mouse vimentin cDNA. SW-13/cl.2 cells that lack detectable IFs were transfected with a mouse vimentin cDNA and three stable cell lines that express different amounts of mouse vimentin, T3M, T4M, and T19M were obtained and previously characterized for vimentin content (15). Cultures of SW-13 cells that spontaneously express human vimentin (cl.1), the transfectant cell lines (T3M, T4M, and T19M), and the parental IF-free cl.2 cells were treated with 50 μg/ml LDL for 24 h. The cells were then radiolabeled with 100 μCi/ml [^{35}S]methionine for 24 h in medium containing 50 μg/ml LDL, immunoprecipitated, and analyzed by SDS-PAGE. The figure shows the fluorograph of ^{35}S-labeled proteins from samples of the culture medium representing equivalent numbers of cells that were immunoprecipitated with goat anti-apoE (+lanes) or non-immune goat serum (−lanes). The position of the immunoprecipitated apoE is indicated (◄).

TABLE 1. ApoE secretion and cholesterol esterification in SW-13 cell lines stably transfected with a mouse vimentin cDNA

Cell Line	^{35}S-ApoE Secreted[a]	[^{14}C]Oleate Incorporated into Cholesteryl Oleate	Vimentin Content[b]
	cpm	*nmol/mg protein/h*	*% control*
C1.2 IF-free	349 ± 33	1.67 ± 0.42	0
T19M	147 ± 18	2.40 ± 0.15	5
T4M	234 ± 8	3.12 ± 0.31	9
T3M	493 ± 49	6.16 ± 0.48	21
C1.1 Vim$^+$	37 ± 18	7.99 ± 0.44	100

Values represent the average of triplicate samples ± SE. T19M, T4M, and T3M cells were derived from c1.2 vim cells by stable transfection with a mouse vimentin cDNA (15). In separate experiments, cells were treated with 50 μg/ml LDL and then radiolabeled with 100 μCi [^{35}S]methionine for 20 h, or 0.5 μCi [^{14}C]oleate for 4 h. ApoE was immunoprecipitated from the culture medium, separated by SDS-PAGE, and the gels were directly scanned for ^{35}S incorporated into the 36 kD apoE band. Incorporation of [^{14}C]oleate into cholesteryl oleate was determined by TLC analysis as described in Methods.

[a] The volume of medium used for immunoprecipitation was normalized for total acid-precipitable ^{35}S.

[b] Excerpted from (6).

1). These results indicated that the capacity of SW-13 cell lines to esterify cholesterol that is associated with vimentin expression was not correlated with the level of apoE expression.

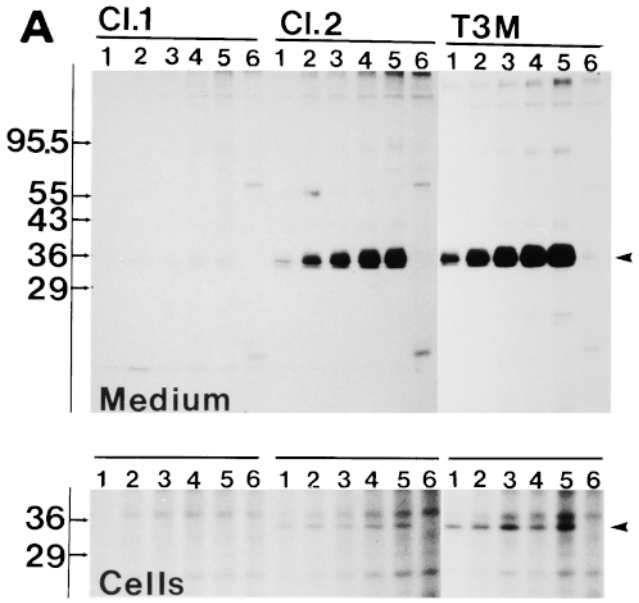
Differences in the level of apoE secretion among SW-13 cell lines are associated with differences in apoE synthesis that are partially regulated at a post-transcriptional level

To begin to differentiate whether the observed differences in apoE recovered from the culture medium of the vim$^+$ and IF-free cells were a reflection of differences in apoE synthesis or possibly due to differences in the activity of the secretory pathway, experiments were carried out to detect apoE in the cell monolayers and the medium with time after addition of [^{35}S]methionine. As shown in **Fig. 5A**, under conditions of continuous radiolabeling, while incorporation of ^{35}S into newly synthesized apoE was not detectable in the cl.1 cells, [^{35}S]apoE could be detected in the cl.2 and T3M cells within 6 h, and the apparent amount of intracellular [^{35}S]apoE did not then appreciably change during the remainder of the labeling period. In contrast, the amount of secreted [^{35}S]apoE that was immunoprecipitated from the culture medium continued to increase throughout the 30 h labeling period. Quantitation of the immunoprecipitated [^{35}S]apoE (Fig. 5B) confirmed the previous observation that T3M vim$^+$ cells secrete significantly higher levels of apoE than the parental IF-free cl.2 cells. Although these results cannot formally exclude the possibility that the protein is synthesized and then rapidly degraded in the cells that secrete little apoE, the differences in the level of apoE secretion in cl.1, cl.2 and T3M cells reflected the differences in the apparent rate of synthesis and amount of intracellular apoE.

To determine whether the differences in apoE synthesis and secretion observed in the SW-13 cell lines were a result of differential regulation of apoE gene transcription, the relative level of apoE mRNA was determined in cl.1, cl.2, and T3M cells. As shown in **Fig. 6**, Northern blot analysis of total cellular RNA indicated that the T3M cells that exhibit the highest level of apoE synthesis contained more apoE mRNA than the cl.1 or cl.2 cells. Phosphorimager analysis of blots of RNA obtained in two separate experiments indicated that the T3M cells contained approximately six times more apoE mRNA than the other two cell lines. However, despite significant differences in the rate of apoE synthesis, cl.1 and cl.2 cells did not detectably differ in apoE mRNA levels. These results indicated that while the difference in the rate of apoE secretion between the parental IF-free cl.2 cells and the T3M vim$^+$ transfectant cell lines was associated with a difference in apoE mRNA, the observed difference in apoE secretion between the cl.1 vim$^+$ and IF-free cl.2 cell lines was largely associated with some post-transcriptional regulatory mechanism.

Effect of agents that alter the activity of protein kinases A and C on apoE secretion and cholesterol content and esterification in SW-13 cell lines

Recent studies with vimentin "knockout" mice have indicated that a lack of vimentin impairs the transduction of chemical signals in the vascular endothelium (49, 50). In



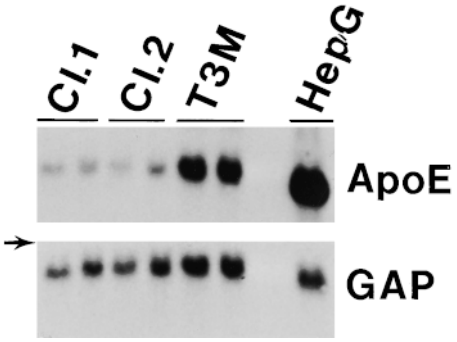


Fig. 6. Northern analysis of apoE mRNA levels. The figure shows the autoradiograph of duplicate samples of total RNA from cl.1 vim$^+$, IF-free cl.2 and T3M cells vim$^+$ (20 μg/lane) hybridized with a human apoE probe (ApoE). The blot was stripped and re-hybridized with a rat glyceraldehyde phosphate dehydrogenase probe (GAP) as a control for RNA loading (7). A sample of RNA from HepG2 cells (10 μg)(HepG) was included as a positive control. The position of the 18S rRNA is indicated (→). The relative amount of apoE and GAP ^{32}P-probe that hybridized was determined by phosphorimaging. The average ratio of apoE/GAP was 1.24, 0.95, and 4.28 for cl.1, cl.2, and T3M cells respectively.

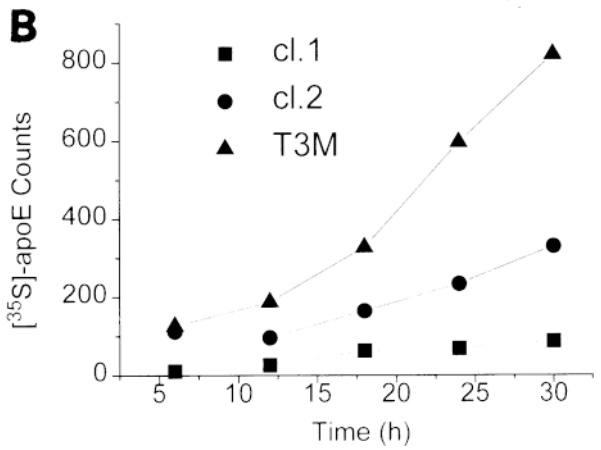


Fig. 5. ApoE synthesis and secretion with time. SW-13 cells that spontaneously express human vimentin (cl.1), IF-free cells (cl.2), and a stable transfectant line that expresses a mouse vimentin cDNA (T3M) were treated with 50 μg/ml LDL and then radiolabeled with 100 μCi/ml [^{35}S]methionine from 6 to 30 h. (A) ^{35}S-fluorograph of a 10% SDS-PAGE of anti-apoE immunoprecipitated proteins from the culture medium (Medium) and cell lysates (Cells) after 6 h (lane 1), 12 h (lane 2), 16 h (lane 3), 24 h (lane 4), and 30 h (lane 5) of continuous labeling, and a non-immune serum precipitate from the 30 h sample (lane 6). The position of the immunoprecipitated apoE is indicated (◄). (B) The dried gels of the culture medium immunoprecipitated apoE were directly scanned for ^{35}S and the cpm incorporated into the 36 kD apoE band were determined as described in the Methods section (cl.1, -■-; cl.2, -●-; T3M, -▲-).

steroidogenic cells, apoE expression and cholesterol homeostasis are affected by agents that alter cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) activities (51, 52). As the cl.1 vim$^+$ and IF- free cl.2 cells do not appear to substantially differ in apoE mRNA levels, we explored the possibility that the observed differences in apoE secretion and cholesterol utilization could reflect differences in PKA- or PKC-dependent regulatory mechanisms. The effects of agents that elevate cAMP or activate PKC on apoE secretion as well as cholesterol and cholesterol esterification were determined in cl.1 and cl.2 cells. Treatment of cells with either forskolin and IBMX or 8-bromo cAMP (8br-cAMP) did not significantly increase the capacity of the cl.1 vim$^+$ cells to secrete apoE (**Table 2**). Although treatment of the IF-free cl.2 cells with forskolin and IBMX did appear to reduce apoE secretion, there was no significant reduction of apoE secretion in cells treated with 8br-cAMP. The free cholesterol and cholesteryl oleate content as well as the overall rate of cholesterol esterification of these cells was consistent with previous studies (6). Untreated cells that lack vimentin filaments exhibited about half the rate of total cholesterol esterification and contained less esterified cholesterol compared with cells that contain vimentin (Table 2). However, these experiments failed to reveal any consistent effect of forskolin and IBMX or 8br-cAMP treatment on the level of cholesterol or cholesterol esterification in either cell line. Similarly, treatment of SW-13 cells with TPA or TPA and the PKC inhibitor chelerythrine did not significantly affect the secretion of apoE or the level of cholesterol esterification in either the cl.1 vim$^+$ or IF-free cl.2 cells (**Table 3**).

Intracellular distribution of apoE in SW-13 cells that differ in the level of apoE secretion

Little is known about the intracellular distribution of apoE in adrenal cortical cells. While apoE is a secreted protein and has been localized in the endoplasmic reticulum, Golgi apparatus, and secretory granules in macrophages (53), apoE in liver cells has also been reported in one study to be a component of peroxisomes (54) while a subsequent study failed to detect peroxisomal apoE (55). To determine whether the observed differ-

TABLE 2. Effect of agents that elevate cAMP on apoE secretion and cholesterol metabolism in vim$^+$ and vim$^-$ SW-13 cells

Cell Line	[^{35}S]ApoE Secreted	Free Cholesterol	Cholesteryl Oleate	[^{14}C]Oleate Incorporated into Cholesteryl Oleate	[^{14}C]Oleate Incorporated into Triglycerides
	cpm/mg protein	*μg/mg protein*	*μg/mg protein*	*nmol/mg protein*	*nmol/mg protein*
C1.1 Vim$^+$					
Untreated	26[a]	24.1 ± 0.5	16.3 ± 1.4	8.1 ± 0.4	15.1 ± 0.5
IBMX/F	14 ± 4	26.4 ± 1.8	17.5 ± 1.4	9.8 ± 0.7	20.8 ± 0.6
8br-cAMP	18 ± 5	33.0 ± 1.7	20.5 ± 1.0	11.0 ± 0.6	18.4 ± 0.6
C1.2 IF-free					
Untreated	96 ± 15	28.3 ± 1.1	8.8 ± 0.1	4.2 ± 0.4	20.8 ± 1.6
IBMX/F	56 ± 8	33.1 ± 6.2	15.9 ± 2.8	7.2 ± 1.2	29.8 ± 5.1
8br-cAMP	99 ± 10	33.8 ± 3.5	10.0 ± 1.1	5.9 ± 1.2	23.5 ± 3.9

Unless otherwise indicated, values represent the average of triplicate samples ± SE. Cells were treated with 1 mm IBMX and 10 μm forskolin or 1 mm 8 br-cAMP in medium containing 50 μg/ml LDL and 100 μCi/ml [^{35}S] methionine for 20 h. [^{14}C]oleate BSA (0.5 μCi/ml) was then added and the cells were incubated an additional 4 h. Radiolabeled apoE was immunoprecipitated from the culture medium and cholesterol and cholesteryl esters were extracted from the monolayers for HPLC analysis as described in Methods.

[a] Duplicate samples.

ences in apoE secretion between SW-13 cell lines might be due to differences in the intracellular distribution of apoE, indirect immunofluorescence experiments were carried out to visualize apoE in cl.1, cl.2, T3M, and HepG2 cells. As shown in **Fig. 7**, monoclonal anti-apoE staining of the cl.1 vim$^+$ cells that secrete little detectable apoE showed no detectable specific staining (Fig. 7A) compared with controls stained with the fluorescent conjugate alone (data not shown). In contrast, the anti-apoE staining pattern of IF-free cl.2 (Fig. 7B) and T3M vim$^+$ cells (Fig. 7C), which secrete detectable amounts of apoE, were characterized by both a faint perinuclear fluorescence, which resembles the staining pattern of the Golgi apparatus in these cells, and a more prominent punctate cytoplasmic fluorescence. As a positive control, anti-apoE immunofluorescence of HepG2 cells showed areas of intense punctate cytoplasmic staining. The intensity of the anti-apoE staining observed in these cells was consistent with the amount of apoE detected by immunoprecipitation from cell lysates (Figs. 1 and 5). The anti-apoE fluorescence shown in Fig. 7 was obtained using a monoclonal anti-human apoE (Bioreclamation clone 2034), and similar results were obtained with two other anti-apoE monoclonal antibodies (Chemicon MAb 1062 and Biodesign clone 3D12, data not shown). These results indicated that the expression of vimentin was not associated with any obvious difference in the intracellular distribution of apoE.

Vimentin expressing and IF-free SW-13 cell lines contain similar levels of SCP_2

Adrenal tissue has been shown to contain a significant level of SCP_2 (56). To determine whether the difference in the capacity of these adrenal tumor cells to utilize LDL-cholesterol might be related to a difference in the level of expression of SCP_2, we carried out immunoprecipitation

TABLE 3. Effect of TPA and chelerythrine on apoE secretion and cholesterol metabolism in vim$^+$ and vim$^-$ SW-13 cells

Cell Line	[^{35}S]ApoE Secreted	Free Cholesterol	Cholesteryl Oleate	[^{14}C]Oleate Incorporated into Cholesteryl Oleate	[^{14}C]Oleate Incorporated into Triglycerides
	cpm/mg protein	*μg/mg protein*	*μg/mg protein*	*nmol/mg protein*	*nmol/mg protein*
C1.1 Vim$^+$					
Untreated	9 ± 3	63.1 ± 12.1	42.6 ± 2.2	19.2 ± 2.5	32.0 ± 4.0
TPA	22 ± 13	67.1 ± 0.8	47.4 ± 5.9	25.2 ± 2.3	44.4 ± 1.5
TPA + Che	23 ± 12	69.9 ± 8.3	38.3 ± 3.0	25.2 ± 1.3	47.8 ± 0.9
C1.2 IF-free					
Untreated	245 ± 18	47.9 ± 5.1	17.1 ± 0.8	12.6 ± 0.5	47.7 ± 7.1
TPA	211 ± 22	38.9 ± 2.3	13.6 ± 0.6	11.5 ± 0.8	50.7 ± 0.5
TPA + Che	207 ± 19	43.7 ± 4.3	15.4 ± 1.6	12.5 ± 1.0	54.3 ± 3.4

Values represent the average of triplicate samples ± SE. Cells were treated with 0.2 μm TPA or 0.2 μm TPA and 10 μm chelerythrine (Che) in medium containing 50 μg/ml LDL and 100 μCi/ml [^{35}S]methionine for 20 h. [^{14}C]oleate BSA (0.5 μCi/ml) was then added and the cells were incubated an additional 4 h. Radiolabeled apoE was immunoprecipitated from the culture medium and cholesterol and cholesteryl esters were extracted from the monolayers for HPLC analysis as described in Methods.

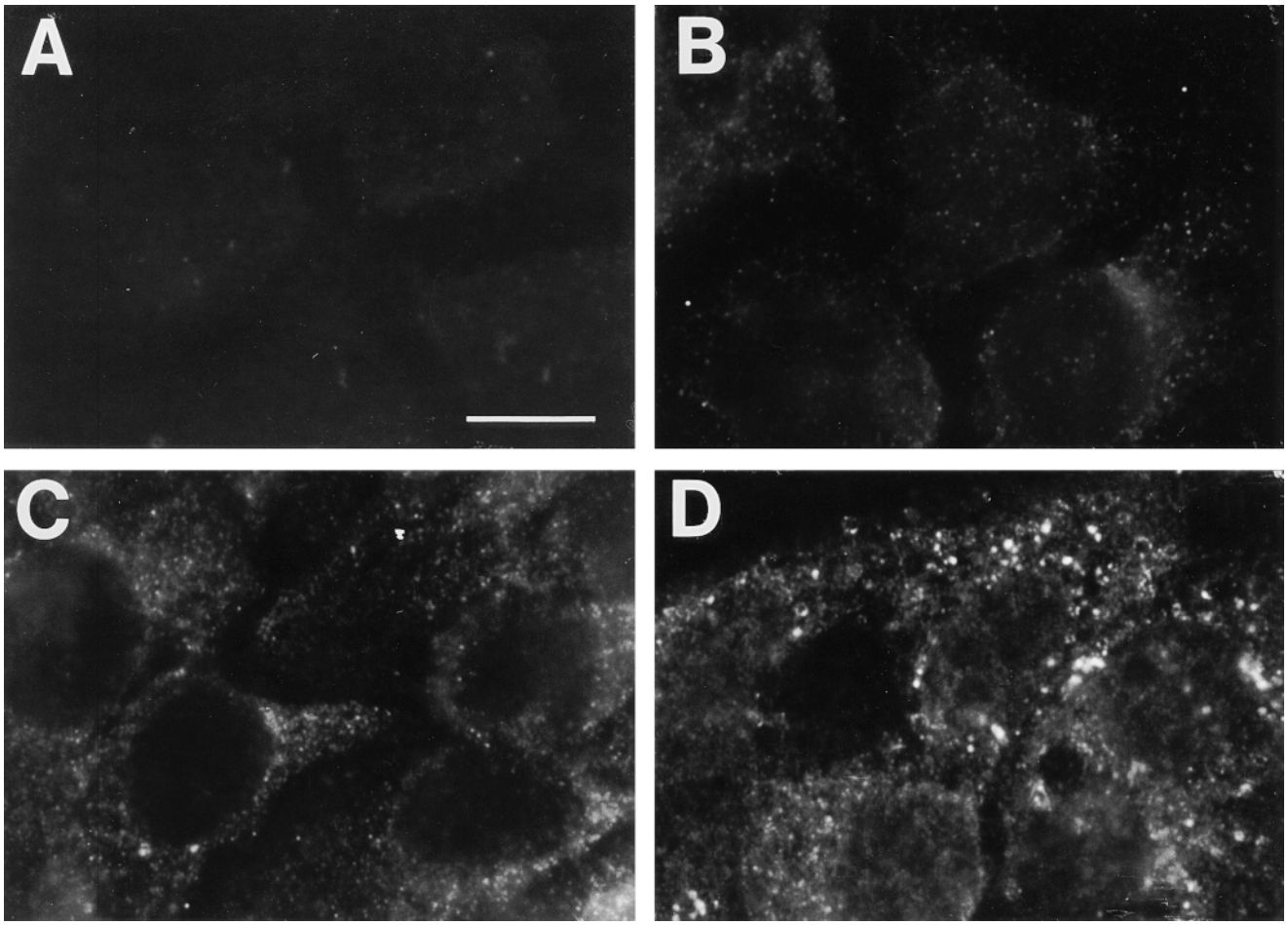


Fig. 7. Anti-apoE indirect immunofluorescence. SW-13 cl.1 vim$^+$ (panel A), IF-free cl.2 (panel B), T3M vim$^+$ (panel C), and HepG2 (panel D) cells were treated with 50 μg/ml LDL for 20 h and stained with a monoclonal anti-apoE antibody. Bar, 10 μm.

experiments with [^{35}S]methionine-labeled cl.1, cl.2, and T3M cells that had been cultured in a lipid-free defined medium in the presence or absence of LDL as the only source of exogenous cholesterol. The anti-SCP_2 antibody specifically precipitated an approximately 13–14 kD ^{35}S-labeled protein from all 3 cell lines (**Fig. 8A**). As a positive control, the antibody precipitated a protein of identical mobility from CHO-K1 cells that have been previously demonstrated to contain the 13.2 kD SCP_2 protein (57). Quantitation of the [^{35}S]SCP_2 that was immunoprecipitated from triplicate cultures after a 24 h labeling period indicated that there was no significant difference in the level of SCP_2 detected in the cells that contain or lack vimentin (Fig. 8 B). Treatment of the cultures with LDL had no apparent effect on the level of SCP_2 in any of the cell lines. Because the 13.2 kD SCP_2 protein is distributed between membranous and cytosolic compartments (56), we also examined the relative amount of SCP_2 in cytosolic and microsomal fractions of cl.1, cl.2, and T3M cells and failed to find any obvious difference in the relative amounts of soluble and membrane-associated SCP_2 in these vim$^+$ and IF-free cell lines (not shown). These results indicate that the difference in the capacity of vim$^+$ and IF-free SW-13 cells to esterify LDL-cholesterol is not associated with the amount or relative distribution of the 13.2 kD SCP_2 protein.

Vimentin expressing and IF-free SW-13 cell lines do not contain detectable levels of caveolins

To determine whether the difference in the capacity of SW-13 cell lines to utilize LDL-cholesterol might be associated with differences in caveolin, the content and distribution of caveolin-1 and caveolin-2 were examined in vimentin expressing and IF-free cells. Under conditions that easily detected the ~22 kD caveolin-1 protein in CHO fibroblasts, neither cl.1 vim$^+$ or IF-free cl.2 cells contained detectable levels of immunoprecipitable caveolin-1 (**Fig. 9**). Similar results were obtained in Western blotting experiments (not shown). In addition, anti-caveolin-1 immunofluorescence studies failed to detect any specific caveolin-1 staining in the SW-13 cell lines. Similarly, under conditions that readily detected the ~20 kD caveolin-2 protein in RSV-3T3 cells, neither cl.1 vim$^+$ nor IF-free cl.2 cells contained detectable levels of caveolin-2 as determined by immunofluorescence, immunoprecipitation, or Western blotting (data not shown). These results demonstrate that SW-13 cells contain little or no caveolin, indicating that the difference in the capacity of these cells to

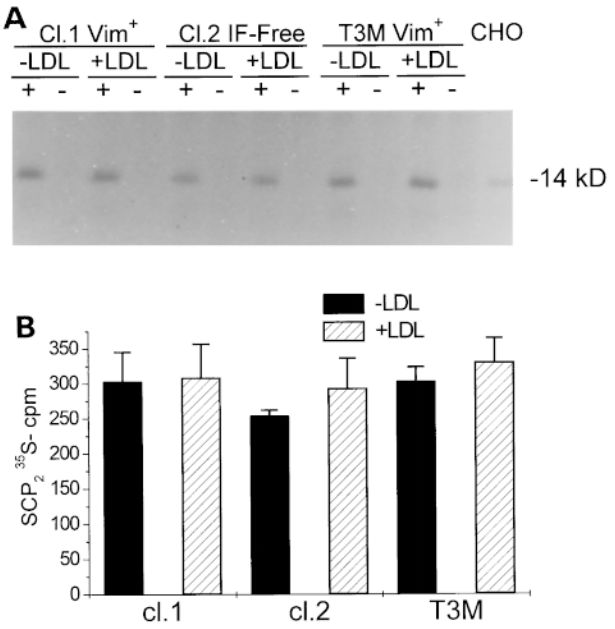


Fig. 8. Expression of SCP_2 in SW-13 cell lines. SW-13 cl.1 vim^+, IF-free cl.2 and T3M vim^+ were grown either in serum-free medium (−LDL) or in medium with 50 μg/ml LDL (+LDL) for 20 h. The cells were then labeled with [^{35}S]methionine for 24 h in medium ± 50 μg/ml LDL and anti-SCP_2 immunoprecipitates were prepared with material from equivalent numbers of cells. (A) Fluorograph of a 12.5% acrylamide gel showing the ^{35}S-labeled proteins that were precipitated from each sample with anti-SCP_2 (+) and non-immune serum (−). The position of a 14 kD marker is shown. (B) The gels were directly scanned for ^{35}S and the cpm incorporated into the 13.2 kD SCP_2 band were determined as described in the Methods section. The values represent the mean of immunoprecipitated ^{35}S-SCP_2 from triplicate cultures ± SE.

esterify LDL-cholesterol is not associated with a difference in caveolin-1 or -2.

DISCUSSION

In addition to the effect of vimentin on the capacity of these cells to utilize lipoprotein derived cholesterol, Gillard et al. (58) have shown that the metabolism of glycosphingolipids is also affected by vimentin expression in SW-13 cells. While the effect of vimentin expression on sterol and glycolipid metabolism may involve trafficking events, it is certainly not clear whether this effect is direct or indirect. Only a small number of specific molecules have been identified as potentially involved in the intracellular trafficking of cholesterol. Because both apoE and SCP_2 are adrenal proteins that can affect cholesterol availability for steroidogenesis, the possibility that differences in expression or distribution of these proteins might play a role in a process affected by vimentin IFs was intriguing. Similarly, caveolin-1 is reported to bind cholesterol and is associated with transport vesicles known to carry glycolipids (34). Nevertheless, it seems clear from our studies that SW-13 cells express little or no caveolin and the effects of vimentin expression on cholesterol metabolism in these

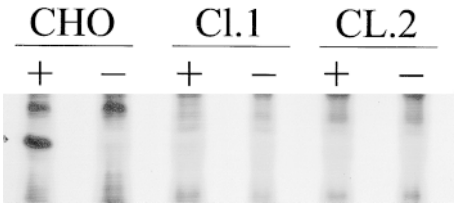


Fig. 9. Expression of caveolin-1 in CHO and SW-13 cells. SW-13 cl.1 vim^+, IF-free cl.2 and CHO- K1 cells were grown in medium with 50 μg/ml LDL (+LDL) for 48 h. The cells were labeled with [^{35}S]methionine for 4 h in medium containing 50 μg/ml LDL and anti-caveolin-1 immunoprecipitates were prepared from count-matched samples. The figure shows the fluorograph of a 10% acrylamide gel of the ^{35}S-labeled proteins that were precipitated with anti-caveolin-1 (+) or rabbit anti-mouse antiserum alone (−). The position of the ~22 kD caveolin band is indicated.

cells is not associated with a difference in the level of expression of SCP_2. In addition, while individual SW-13 cell lines may characteristically differ in levels of apoE expression, this is not directly associated with the presence or absence of a vimentin filament system. IF-free SW-13 cells have a decreased capacity to esterify lysosomal cholesterol but secrete significant amounts of apoE, while SW-13 cell lines that spontaneously contain human vimentin have a greater capacity to esterify lysosomal cholesterol, but secrete very little apoE. However, in cell lines derived from these IF-free cells that stably express a vimentin transgene, the increased capacity to esterify lysosomal cholesterol that is associated with vimentin expression was not linked to any reduction in the level of apoE expression. This indicates that in SW-13 cells, not only is apoE expression not directly associated with the presence or absence of vimentin filaments, there does not appear to be a simple relationship between the level of apoE expression and the capacity of these cells to utilize LDL-cholesterol. These results suggest that while apoE expression may affect cholesterol utilization for steroidogenesis in Y-1 adrenal cells (13), the differences in the level of apoE expression observed in these non-steroidogenic cells did not have a similar effect on those aspects of cholesterol metabolism required for esterification.

The most surprising aspect of these studies was the apparent lack of association between apoE mRNA levels and apoE expression in some of the SW-13 cell lines. While the cl.1 and cl.2 cell lines differed significantly in the level of apoE synthesis, they had similar levels of apoE mRNA, indicating that the difference in apoE expression in these cells primarily involves post-transcriptional events. In contrast, the difference in apoE secretion between the parental cl.2 cells and the T3M vimentin transfectant cell line that exhibited the highest level of secretion was found to be associated with a change in the level of apoE mRNA. Transcriptional regulation of apoE expression has been described in adrenal tissue (59) and has been reported to account for the increased apoE synthesis observed in cholesterol-loaded macrophages (47). However, other studies have indicated that post-transcriptional events may also be involved in regulating apoE expression. Wyne et

al. (52) have reported a lack of concordance between changes in apoE mRNA and apoE expression in ovarian granulosa cells. Similarly, studies by Dory (46) have shown that inhibition of cholesteryl esterification in cholesterol-loaded macrophages stimulated apoE synthesis but had little effect on the level of apoE mRNA. These observations led the authors to suggest that apoE expression is regulated at both the transcriptional and post-transcriptional levels. The mechanism(s) responsible for this post-transcriptional regulation of apoE synthesis has not been characterized, but the large difference in the capacity of SW-13 cell lines to secrete apoE would seem to provide a unique model to study this aspect of the regulation of apoE expression.

Addition of LDL to the culture medium stimulated apoE secretion in the cells that lack endogenous vimentin IFs. Although studies with a variety of cell types have demonstrated a relationship between cell cholesterol content and apoE secretion (for example see refs. 45, 60), there are a number of reasons that make it unlikely that the differences in the level of apoE expression observed in the SW-13 cell lines are related to the differences in cholesterol metabolism in these cells. First, after treatment with LDL, these cell lines do not consistently differ in free cholesterol content (6) (T. A. Holwell and R. M. Evans, unpublished observation). Second, although we have previously shown that these cell lines do differ in rates of cholesterol synthesis (6), this correlates with vimentin expression and not with the expression of apoE. Finally, while most previous work has indicated that cholesterol effects on apoE expression are characterized by changes in apoE mRNA levels (20, 45, 47), the differences in apoE expression in SW-13 cell lines appear to be largely independent of mRNA levels.

Previous studies had suggested that there was some similarity between the cholesterol metabolism of SW-13 cells that lack vimentin IFs and CHO cell mutants that lack peroxisomes and are deficient in the 13.2 kD SCP_2 protein. Van Heusden et al. (30) reported increased cholesterol synthesis and cholesterol efflux in SCP_2-deficient CHO cells, although uptake and hydrolysis of LDL-cholesterol was not affected. We had observed an increase in cholesterol synthesis and efflux in IF-free SW-13 cells without apparent effects on receptor-mediated endocytosis of LDL (6). Although SW-13 cells were found to express SCP_2 at levels that were comparable to that found in CHO cells, there was no difference in the level of SCP_2 expression in cells that contain or lack vimentin IFs. Therefore, while SCP_2 may well play a significant role in the intracellular movement of cholesterol, it does not appear to be responsible for the impaired capacity of IF-free cells to utilize lipoprotein-derived cholesterol.

A number of studies have indicated that there is an interaction between vimentin and various protein kinases, including isotypes of PKC (reviewed in ref. 61). Although the significance of these IF–kinase interactions is not understood, recent studies have suggested that there is a relationship between vimentin IFs and signal transduction. Terzi et al. (49) have reported that partial nephrectomy of vimentin knockout mice was lethal within 72 h, due to end-stage renal failure under conditions that resulted in no lethality in wild-type littermates. An increased level of renal endothelin and decreased nitric oxide in the vimentin-null animals suggested that the renal failure was associated with an imbalance between these bioactive molecules that regulate vascular tone. Administration of the endothelin receptor antagonist bosetan totally prevented this lethality in the vimentin-null mice (49). Experiments measuring the resistance of isolated perfused mesenteric arteries to pressure and flow indicated that the absence of vimentin selectively decreased the dilation response of resistance arteries to flow, suggesting a role for vimentin in the mechanotransduction of shear stress in vascular endothelial cells (50). As a result of these observations we considered the possibility that the decreased capacity of IF-free SW-13 adrenal tumor cells to utilize lysosomal cholesterol might reflect an impaired signalling pathway in these cells. Our initial studies indicated that there was no difference in the effects of agents that alter PKA or PKC activity on the rate of cholesterol esterification or triglyceride synthesis in the vimentin expressing and IF-free cells. These results suggest that the impaired ability of IF-free cells to esterify LDL-cholesterol is not simply associated with the capacity of the cells to elevate cAMP or PKC activity. Because the signalling pathways that affect the post-lysosomal trafficking of cholesterol have yet to be identified, it cannot be concluded that the role of vimentin in SW-13 cells is not related to some signalling function.

SW-13 cells were originally isolated from a human adrenal carcinoma (62) and the lack of significant caveolin-1 or -2 in these cell lines may simply be a reflection of the level of caveolin in adrenal tissue. Although the level of caveolin expression in the adrenal tissue has not been reported, another cell line of adrenal origin, PC-12, has been shown to lack detectable caveolin (63). The absence of detectable caveolin in SW-13 cells could also be related to the observation that caveolin and caveolae can be reduced in neoplastically transformed cells (64). Regardless of the significance of the lack of caveolin expression in SW-13 cells, our results show that neither caveolin-1 nor caveolin-2 is required for these cells to utilize lipoprotein derived cholesterol.

The results of these studies indicate that the impaired capacity of IF-free SW-13 cells to utilize LDL-cholesterol is not related to the level of expression of three proteins that have been suggested to be involved in some aspect of the intracellular availability of cholesterol. Therefore, it is apparent that another mechanism is likely to be involved. Based on what is currently known about lysosomal cholesterol transport, there seem to be two likely alternatives. It is possible that vimentin affects, directly or indirectly, some carrier protein other than SCP_2. Recent studies by Underwood et al. (11) have indicated that agents that affect vesicular trafficking inhibit LDL-cholesterol transport in CHO cells. Another potential mechanism is that transport of lysosomal cholesterol to the endoplasmic reticulum involves caveolin-indepen-

dent vesicular transport which is influenced by vimentin expression.

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