Serum Cholesterol Efflux Potential in Postmenopausal Monkeys Treated With Tibolone or Conjugated Estrogens

Tomi S. Mikkola, Mary S. Anthony, Thomas B. Clarkson, and Richard W. St. Clair

Tibolone is a synthetic steroid used for the treatment of climacteric symptoms and the prevention of osteoporosis, but the effect on the cardiovascular system is unclear since tibolone lowers high-density lipoprotein (HDL) levels. We investigated if long-term treatment with tibolone or conventional hormone replacement therapy (HRT) in cynomolgus monkeys could affect their serum cholesterol efflux potential. Surgically postmenopausal cynomolgus monkeys were treated for 2 years with conjugated equine estrogens (CEE), CEE plus medroxyprogesterone acetate (MPA), low-dose tibolone, or high-dose tibolone. Plasma lipid, lipoprotein, and apolipoprotein levels were monitored during the study. The cholesterol efflux potential of the serum from each animal was determined in ^{3}H-cholesterol–labeled Fu5AH cells and skin fibroblasts in culture. Tibolone induced a dose-dependent 30% to 52% reduction in HDL levels. When HDL concentrations were reduced by 30%, as seen in women, there was no reduction in the serum cholesterol efflux potential in Fu5AH cells. With a 52% reduction in HDL, there was a 14% reduction in cholesterol efflux. Although CEE or CEE+MPA had no significant effect on HDL levels, CEE treatment increased serum cholesterol efflux potential by 7%. With the same sera, no changes in cholesterol efflux were seen with fibroblasts. Although our findings suggest that HDL concentration is correlated with cholesterol efflux potential of serum, this relationship is weak, explaining only 16% of the variability. This is emphasized by the fact that despite a 30% lowering of HDL with tibolone, there was no indication of an adverse effect on cellular cholesterol efflux. Other changes in the serum not measured in this study must contribute significantly to the cholesterol efflux potential of serum. Because changes in cholesterol efflux potential of serum were seen only in Fu5AH cells, a cell line rich in SR-B1 receptors, this implies that the changes seen in this study were mediated largely by the SR-B1 pathway.

NUMEROUS OBSERVATIONAL studies indicate that women using postmenopausal estrogen replacement therapy have lower risk for coronary heart disease[1] and osteoporosis,[2] while a slight increase in the risk for breast cancer.[3] However, a randomized and controlled secondary prevention study evaluating the efficacy of postmenopausal hormone replacement therapy (HRT) in reducing coronary heart disease, failed to confirm the previous observational findings.[4] This study has been followed with another secondary prevention study also failing to show beneficial effects of HRT on coronary heart disease progression.[5] The majority of these HRT studies have been in women using conjugated equine estrogens (CEE) alone or combined with medroxyprogesterone acetate (MPA). It has been suggested that coadministration of a progestin, which is necessary for women with an intact uterus to prevent the occurrence of endometrial hyperplasia and cancer, may partially attenuate the estrogen-associated cardioprotection[6] and even further increase breast cancer risk.[7] Because the overall benefits and risks of HRT have not been fully defined, many women choose not to undergo conventional HRT and, therefore, a need for alternative regimens remains.

Tibolone [(7α, 17α)-17 hydroxy-7-methyl-19-norpregnen-5(10)-en-20-yn-3-one; Org OD14; Livial; Organon, Oss, The Netherlands] is a synthetic steroid possessing estrogenic, progestogenic, and androgenic action.[8] Clinically, tibolone has been shown to be effective for the treatment of climacteric symptoms[9] and for the prevention of postmenopausal osteoporosis,[10] without a proliferative effect on the endometrium.[11] Tibolone has also been shown to reduce the formation of atherosclerotic lesions in ovariectomized rabbits,[12] however, in these animals, tibolone markedly reduced low-density lipoprotein (LDL) concentrations and did not reduce high-density lipoprotein-cholesterol (HDLC) levels. In postmenopausal women using tibolone, a reduction in HDLC of approximately 30% is generally reported.[13,14] Because low serum HDLC is a potent predictor of premature coronary heart disease, the HDLC lowering effect of tibolone on the cardiovascular system needed to be evaluated.

HDLs play an important role in reverse cholesterol transport, the mechanism by which peripheral cell cholesterol can be returned through the plasma to the liver for excretion.[15] If this pathway is impaired, more cholesterol is believed to be retained in the arterial wall, and atherosclerosis develops more rapidly. The first step in reverse cholesterol transport involves efflux of cholesterol from cell membranes to an extracellular acceptor. Although HDLs or their precursors are thought to be the most important cholesterol acceptors in vivo, a wide spectrum of lipoproteins and apolipoproteins found in plasma and serum also can act as cholesterol acceptors.[16]

The aim of the present study was to investigate whether long-term postmenopausal tibolone treatment or conventional HRT in surgically postmenopausal cynomolgus monkeys would affect the cholesterol efflux potential of their serum. We used a recently established in vitro serum screening method[17] to determine the cholesterol efflux potential of each animal's serum in 2 cell types that have predominantly different mechanisms for the stimulation of cholesterol efflux.[16] The relative

From the Department of Pathology, Comparative Medicine Clinical Research Center, Wake Forest University School of Medicine, Winston-Salem, NC.

Submitted August 6, 2002; accepted October 4, 2001.

Supported in part by a grant from Organon, Oss, The Netherlands. T.S.M. is supported by a personal grant from The Academy of Finland and The Finnish Medical Foundation.

Address reprint requests to Richard W. St. Clair, PhD, Department of Pathology, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157.

0026-0495/02/5104-0015$35.00/0

doi:10.1053/meta.2002.31331

ability of each animal's serum to promote cholesterol efflux was correlated with lipid, lipoprotein, and apolipoprotein levels measured during the 2-year study. Furthermore, we used multivariate regression modeling to determine variables that are independently associated with cholesterol efflux potential.

MATERIALS AND METHODS

Animals

One hundred fifty-one premenopausal female cynomolgus monkeys (*Macaca fascicularis*) were ovariectomized to make them surgically postmenopausal and randomized into 5 groups of 29 to 31 animals/group. Throughout the 2-year study, the animals were fed a moderately atherogenic diet (0.28 mg cholesterol/kcal). Social groups of approximately 5 animals each were randomly assigned to treatment using a permuted block randomization scheme with a block size of 5. The treatments were as follows: no hormone replacement (control); CEE 0.042 mg/kg, a dose comparable to 0.625 mg of CEE/d in women; CEE+MPA 0.167 mg/kg, a dose comparable to 2.5 mg of MPA/d in women given continuously; low-dose tibolone (lo tib) 0.05 mg/kg, and high-dose tibolone (hi tib) 0.2 mg/kg. Tibolone doses result in plasma levels for the main metabolites comparable to those observed after 1.25 to 2.5 mg/d in women.[18] Hormones were administered in the diet continuously for 24 months. At this time, necropsies were performed, and the severity of coronary artery atherosclerosis (CAA) was evaluated blind to treatment, as described elsewhere.[18] The present cholesterol efflux studies were performed with a 142-animal subset. Nine animals from the original 151 had to be excluded due to technical difficulties in sample collection. The 142 animals were distributed into different groups as follows: control, n = 30; CEE, n = 27; CEE+MPA, n = 26; lo tib, n = 29; and hi tib, n = 30. All procedures involving animals were performed in compliance with state and federal regulations and were approved by the Wake Forest University Animal Care and Use Committee.

Blood Samples

To monitor plasma total cholesterol (TC), triglycerides (TG), HDLC, and LDL- cholesterol (LDLC) concentrations, blood samples from each animal were collected at 3-month intervals throughout the 2-year study, and a final measurement was performed 4 weeks prior to necropsy. The concentrations of apolipoprotein A1 (apo A1), apolipoprotein E (apo E), and lipoprotein a [Lp(a)] were determined on plasma collected at 12-month intervals. Apolipoprotein B (apo B) was measured on plasma collected 4 weeks prior to necropsy. Serum samples for the cholesterol efflux studies and for phospholipid (PL) measurements were collected without anticoagulant within 1 week prior to necropsy. Food was withheld from the animals for 18 hours prior to blood sample collection. Plasma TC was measured by enzymatic techniques based on the methods of Allain et al.[19] Plasma TG levels were determined by the method of Fossati and Prencipe.[20] HDLC concentrations were measured using the heparin-manganese precipitation procedure described in the Manual of Laboratory Operations of the Lipid Research Clinics Program.[21] LDLC concentrations were determined indirectly by subtracting HDLC from TC, thus values represent LDLC plus very–low-density lipoprotein-cholesterol (VLDLC). However, it should be noted that since TG levels were quite low in the cholesterol-fed monkeys, VLDLC represents less than 5% of the LDLC+VLDLC fraction. Apo A1,[22] apo B,[23] apo E,[24] and Lp(a)[25] concentrations were quantified by enzyme-linked immunosorbent assay (ELISA). Serum total PL was measured as described by St.Clair and Leight.[26]

Cell Culture

Stock cultures of Fu5AH cells and normal human skin fibroblasts (GM 970 cells) were grown in T-75 flasks (Costar, Acton, MA) in Eagle's minimum essential medium (MEM) (JRH Biosciences, Lenexa, KS) containing MEM vitamins (JRH Biosciences), 2 mmol/L L-glutamine (Mediatech, Herndon, VA), 100 U/mL penicillin (Mediatech, Herndon, VA), and 100 μg/mL streptomycin (Mediatech). For Fu5AH cells, MEM was supplemented with 5% calf serum (CS) (Sigma Chemical, St Louis, MO) and for GM 970 cells with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) that was heat inactivated at 56°C for 30 minutes before use. Medium was changed every 2 days, and when cells were confluent, they were detached with 0.05% trypsin (Sigma) and split in a 1:20 ratio. For experiments, cells were seeded on 24-well plates (Costar) at 30,000 cells/well for Fu5AH cells, 10,000 cells/well for GM 970 cells, and grown for 2 and 5 days, respectively, to obtain 80% to 90% confluent cell monolayers.

Labeling Cells With ^{3}H-Cholesterol

Cells were labeled with ^{3}H-cholesterol as described for Fu5AH cells by de la Llera Moya et al,[17] with slight modifications. Briefly, radiolabeled cholesterol ([1,2-^{3}H]-cholesterol, DuPont, Boston, MA) was purified by thin-layer chromatography and added in 100 μL ethanol to MEM containing 25% CS, incubated for 2 hours at 37°C, and diluted to a final concentration of 5% CS (1 to 2 μCi/well) before adding it to the Fu5AH cells. For GM 970 cells, radiolabeled cholesterol was added to MEM containing 30% FBS, incubated for 2 hours at 37°C, and diluted to a final concentration of 10% FBS (1 to 2 μCi/well) before adding it to the cells. Cells were grown in the presence of the radiolabeled cholesterol for 2 days. To be certain the ^{3}H-cholesterol had equilibrated with the cellular pools of cholesterol, the labeling medium was replaced with MEM supplemented with 1% bovine serum albumin and incubated for an additional 18 hours prior to using the cells to measure the serum cholesterol efflux potential.

Cholesterol Efflux Assay

The cholesterol efflux potential of each serum sample was assayed as described for Fu5AH cells by de la Llera Moya et al,[17] with slight modifications. Briefly, serum samples that had been stored at –70°C were thawed and diluted to a final concentration of 5% in MEM just before addition to cells. Cells were incubated in triplicate with each serum sample for 4 hours at 37°C in an incubator with 5% CO_2 and 95% air. The efflux phase was ended by placing the plates on ice and removing the serum-containing medium from the wells and transferring it into tubes in an ice bath. This efflux medium was centrifuged at 4°C for 5 minutes at 900 $\times$ g to remove any cells, and an aliquot of the supernatant fluid was taken to measure ^{3}H-cholesterol by liquid scintillation counting. At the end of the efflux phase, the cell monolayers were gently washed once with cold phosphate-buffered saline. The cellular lipids were extracted by adding isopropanol to each dish for 60 minutes at room temperature, and an aliquot of the extract was taken to measure radioactivity. In each individual experiment, serum-free and 5% pooled human serum (Sigma, lot:0739) in MEM were used as controls.

Statistical Analysis

Analyses were performed using Statistical Analysis Software (version 6.08; SAS Institute, Cary, NC) or Primer of Biostatistics (version 4.02; McGraw Hill, New York, NY). Treatment group means and P values were obtained using analysis of variance (ANOVA). T tests were used for posthoc between-group comparisons if the ANOVA P value was significant ($P \leq .05$). All results are expressed as mean $\pm$ SD unless otherwise indicated.

In an effort to better understand associations between cholesterol efflux in Fu5AH and GM970 cells and plasma lipid, lipoprotein, and apolipoprotein measurements, simple associations using Pearson's correlations and multiple regression analyses were performed. Multiple

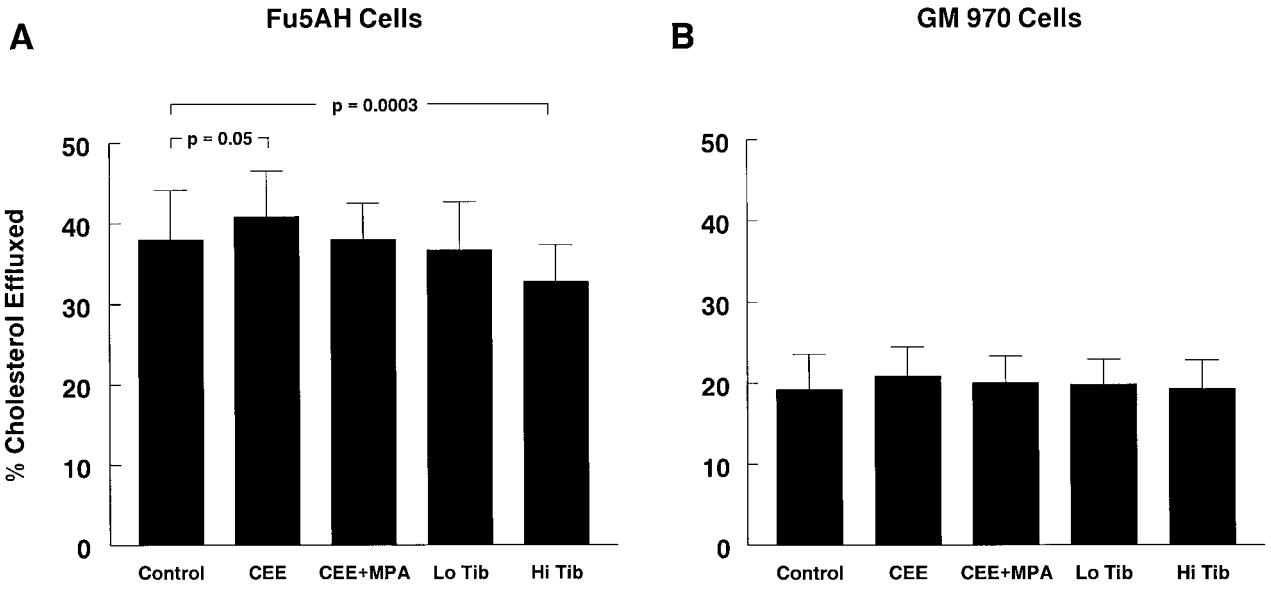

Fig 1. Effect of different hormone treatments on the capacity of serum from cynomolgus monkeys to induce efflux of ^{3}H-cholesterol from Fu5AH cells (A) and GM 970 cells (B). Cells were incubated for 4 hours in culture medium containing 5% serum from individual monkeys. Results are calculated as a percentage of the total ^{3}H-cholesterol in the cells at 0 time that was released into the medium in 4 hours. Results are expressed as the mean ± SD.

regression modeling was performed by backward stepping. The initial models were set up with dummy variables for treatment groups (the control group was the referent group) and all relevant plasma lipid, lipoprotein, and apolipoprotein variables, plus interaction terms between treatment groups and plasma measures.

RESULTS

To compare the cholesterol efflux potential of serum from cynomolgus monkeys treated with CEE, CEE+MPA, or tibolone, we measured the percentage of ^{3}H- cholesterol in cells at 0 time that was released into the medium from Fu5AH cells and GM 970 cells after 4 hours. For this, we used 5% serum from individual animals, as recommended in the study by de la Llera Moya, et al[17] with Fu5AH cells. To provide a direct comparison of results from the 2 different cell types, a 5% serum concentration and a 4-hour incubation time were selected for the GM 970 cells as well. Cholesterol efflux was linear for up to 8 hours with serum having HDLC concentrations ranging from 10 to 62 mg/dL. A 5% pooled human serum was included as an internal control in all cholesterol efflux experiments with the serum samples from the experimental monkeys. The 5% concentration of pooled human serum promoted 29.4% ± 0.6% (n = 3) efflux of ^{3}H-cholesterol from the Fu5AH cells and 14.6% ± 0.7% (n = 3) from the GM 970 cells. Using the 5% concentration of pooled human serum, the intra- and interassay coefficients of variation for Fu5AH cells were 1.4% (n = 6) and 8.7% (n = 6), respectively, and 5.9% (n = 6) and 12% (n = 6), respectively, for GM 970 cells. Normalizing the cholesterol efflux data against the pooled human serum controls did not change the results significantly, and thus, the data are presented without normalization.

The effect of the different treatments on the ability of serum from cynomolgus monkeys to promote cholesterol efflux from Fu5AH and GM 970 cells is shown in Fig 1. Each serum sample's ability to promote cholesterol efflux was determined from the mean of triplicate dishes. In Fu5AH cells, serum from the control subjects promoted 38.0% ± 6.2% cholesterol efflux. Serum from the CEE subjects showed a small, but significant, increase in cholesterol efflux (40.8% ± 5.7%, $P = .05$), while serum from the hi tib subjects promoted a significant decrease in cholesterol efflux (32.7% ± 4.6%, $P = .0003$). None of the other groups were different from controls. In the GM 970 cells, serum from the control group promoted 19.2% ± 4.4% cholesterol efflux, and there were no significant differences in efflux with serum among any of the treatment groups (ANOVA P value = .47).

Average plasma lipid, lipoprotein, and apolipoprotein concentrations within the different treatment groups for the animals used in the cholesterol efflux studies are shown in Table 1. As is typical in most nonhuman primate species, fasting TG levels were low, but were elevated over controls in all treatment groups, whereas there was no significant change in TC or PL levels among the different treatment groups. Tibolone treatment significantly increased LDLC+VLDLC concentration by 22% in the high-dose group and decreased HDLC concentration in both low- and high-dose groups by 30% and 52%, respectively. Consistent with the lowering effect of tibolone on HDLC, there was a 19% and 40% decrease in apo A1 levels in the lo tib and hi tib groups, respectively. Apo B levels were not significantly affected by the treatments, whereas apo E concentrations were decreased in CEE, lo tib, and hi tib groups by 34%, 28%, and 30%, respectively. Lp(a) levels were not significantly affected by the treatments.

The relationship between the potential of serum from individual animals to promote cholesterol efflux from Fu5AH or

Table 1. Plasma Lipid, Lipoprotein, and Apolipoprotein Concentrations (mg/dL) in the Different Treatment Groups During the 2-Year Study

	Control (n = 30)	CEE (n = 27)	CEE + MPA (n = 26)	Lo Tib (n = 29)	Hi Tib (n = 30)
TC	377 ± 128	329 ± 127	348 ± 139	385 ± 148	427 ± 119
TG	37.9 ± 14	58.1 ± 29‡	56.2 ± 26‡	48.3 ± 22†	69.0 ± 33*
PL	281 ± 61	297 ± 78	280 ± 74	297 ± 87	292 ± 78
HDLC	40.7 ± 14	36.5 ± 14	33.5 ± 14	28.6 ± 12*	19.4 ± 7*
LDLC + VLDLC	336 ± 136	293 ± 132	315 ± 148	356 ± 156	408 ± 122†
Apo A1	231 ± 80	221 ± 80	221 ± 71	186 ± 91†	138 ± 46*
ApoB	178 ± 85	186 ± 72	171 ± 61	175 ± 76	224 ± 88
ApoE	10.3 ± 6	6.8 ± 4†	7.8 ± 5	7.2 ± 4†	7.4 ± 4†
Lp(a)	27.8 ± 17	26.4 ± 12	29.4 ± 12	29.3 ± 14	37.0 ± 20

NOTE. Data represent mean values from the repeated measurements during the 2-year study.
Statistical significance calculated against control group values (*$P < .001$, †$P < .05$, ‡$P < .005$).

GM 970 cells and the plasma lipid and lipoprotein concentrations are shown in Figs 2 and 3. The data in these figures are from subjects in all 5 experimental groups, as indicated by the different symbols. There was a negative correlation between plasma TC ($r = -.35$, $P < .0001$), as well as LDLC+VLDLC concentrations and serum cholesterol efflux potential from Fu5AH cells (Fig 2A). A positive correlation was detected between plasma HDLC concentration and serum cholesterol

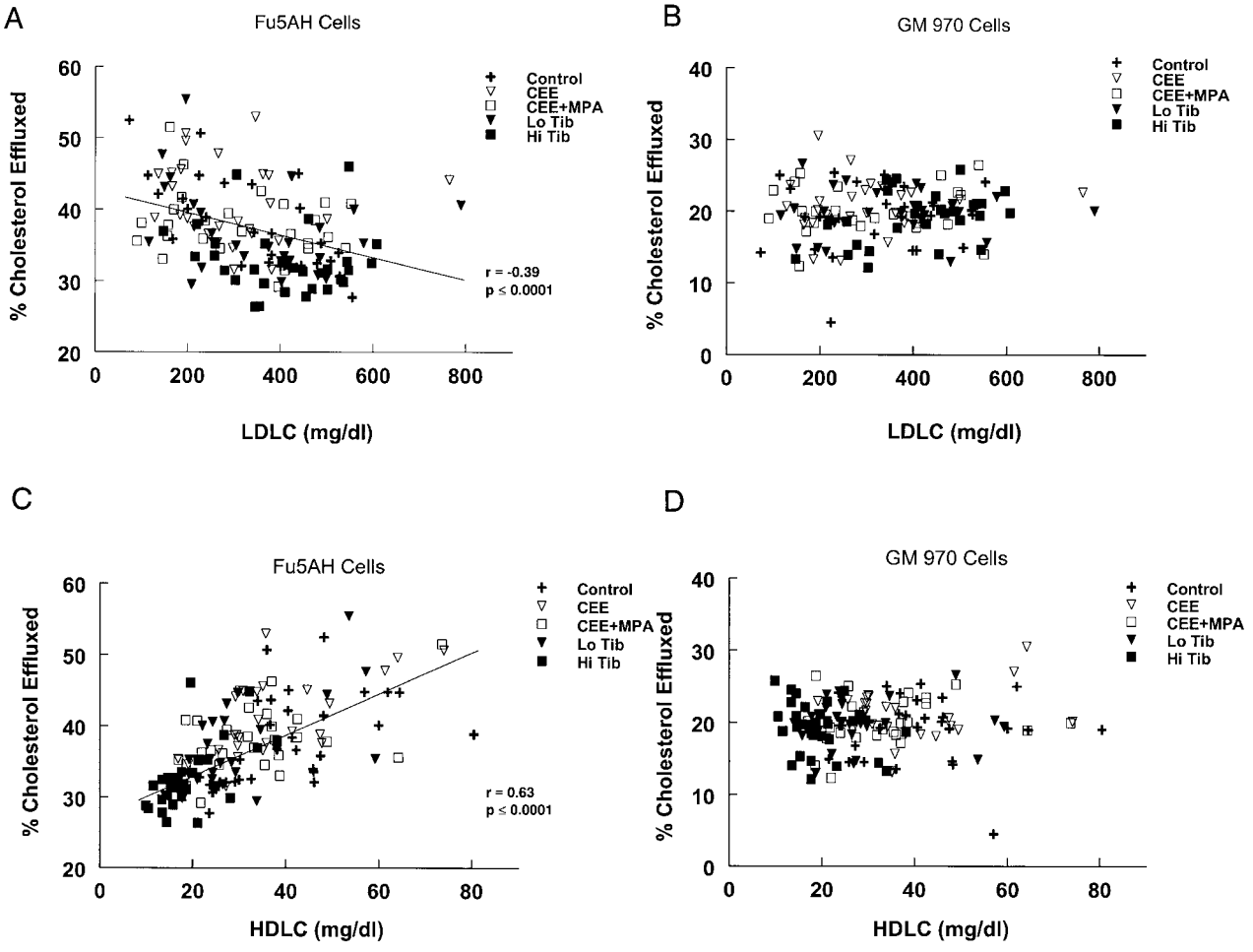

Fig 2. Scatterplot showing the relationship between serum cholesterol efflux potential from Fu5AH cells (A and C) and GM 970 cells (B and D) and plasma LDL + VLDLC (A and B) and HDLC (C and D). Each data point is the average of triplicate efflux determinations expressed as a percentage of total cellular ^{3}H-cholesterol released into the medium. The linear regression lines were calculated by using the least-squares method. Symbols are used for different treatment groups as indicated.

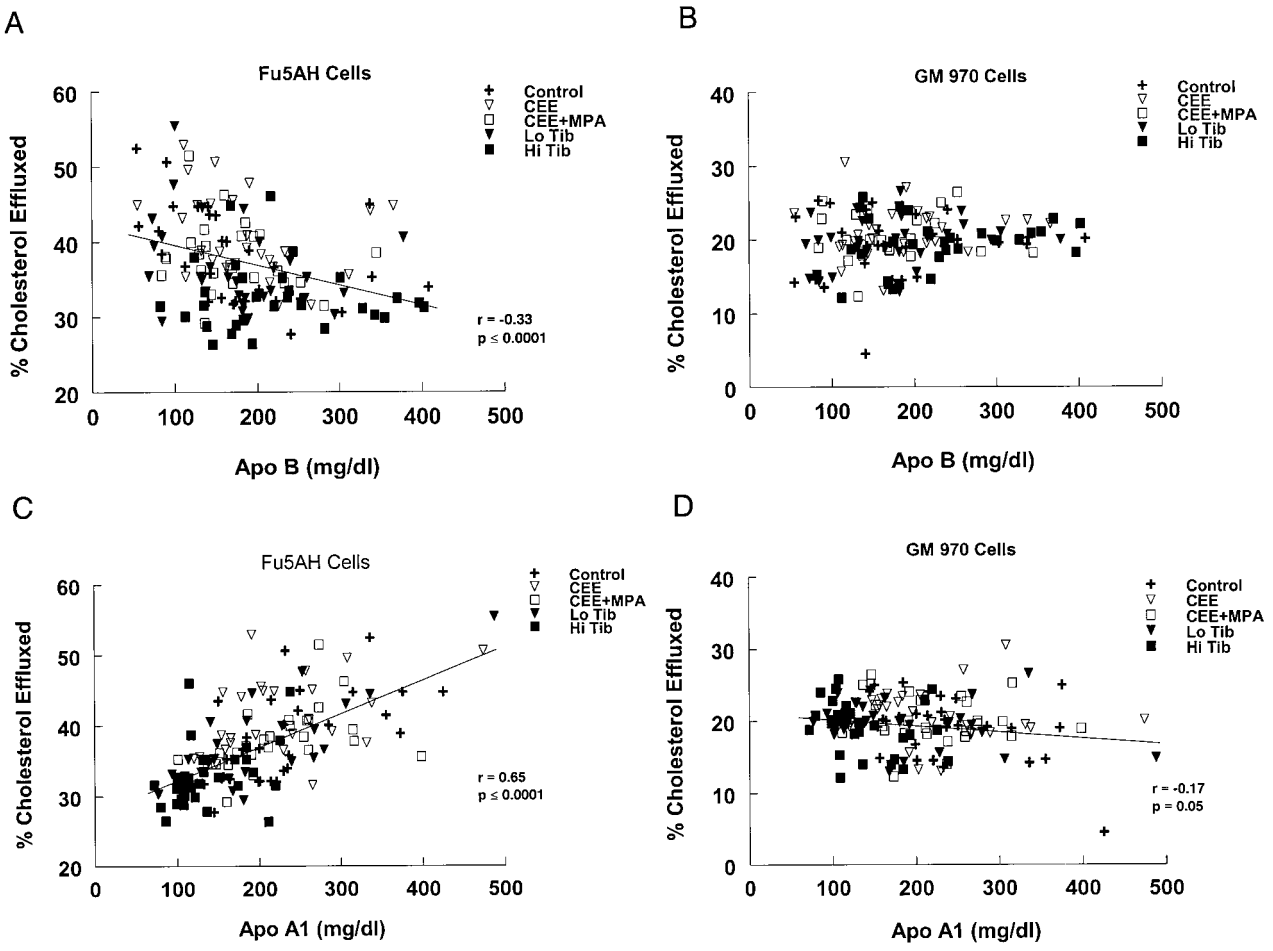

Fig 3. Scatterplot showing the relationship between serum cholesterol efflux potential from Fu5AH cells (A and C) and GM 970 cells (B and D) and plasma apo B (A and B) and apo A1 (C and D). Each data point is the average of triplicate efflux determinations expressed as a percentage of total cellular ^{3}H-cholesterol released into the medium. The linear regression lines were calculated by using the least-squares method. Symbols are used for different treatment groups as indicated.

efflux potential from Fu5AH cells (Fig 2C). Consistent with these results, apo B concentrations showed a negative correlation, while apo A1 concentrations showed a positive correlation with the ability of serum to promote cholesterol efflux from Fu5AH cells (Fig 3A and C). The serum cholesterol efflux potential in GM 970 cells showed a weak negative correlation only with apo A1 concentrations (Fig 3D), but not with any other lipid, lipoprotein, or apolipoprotein variable (Figs 2B and D, 3B).

To get a better idea of the independent variables that affect cholesterol efflux potential of serum, we performed multiple regression analyses (Table 2). HDLC was most highly associated (positive association) with the serum cholesterol efflux potential in the Fu5AH cells, explaining 16.1% of the variability in efflux. Apo A1 was also significantly associated with cholesterol efflux, but since it is highly correlated with HDLC ($r = .81$, $P < .0001$) and was not more strongly associated with cholesterol efflux than HDLC, we chose to use HDLC in the regression models. Also significantly associated with cholesterol efflux were PL (positive association) and LDLC+VLDLC (negative association), explaining about 6.6% and 4.9%, respectively, of the variability. This model includes the unadjusted plus squared term for HDLC to account for the fact that the best association between cholesterol efflux and HDLC was nonlinear with simple correlation analysis. The term indicating the CEE group remained significant in all models, although interaction terms were not significant; suggesting that cholesterol efflux is 2.88% higher in the CEE group, accounting for

Table 2. Variables Significantly Associated With Serum Cholesterol Efflux Potential From Fu5AH Cells in Multiple Regression Modeling

	β Coefficient ± SE	P Value	Partial r^2
y-Intercept	31.96 ± 1.75	.0001	
HDLC + (HDLC)^2	0.0021 ± 0.0004	.0001	.161
LDLC + VLDLC	−0.0103 ± 0.0039	.009	.049
PL	0.0019 ± 0.0006	.002	.066
CEE group	2.88 ± 1.02	.005	.056
Full model		$P < .0001$	Total $r^2 = .424$

5.6% of the variability in the efflux potential, compared with the other groups when plasma concentrations of HDLC, LDLC+VLDLC, and PL are the same. The terms for all other treatment groups were not statistically significant, suggesting that the LDLC+VLDLC, HDLC, and PL explained the association with cholesterol efflux in these groups, and there were not treatment effects independent of these variables.

The same backward stepping regression modeling was performed with serum cholesterol efflux potential in the GM 970 cells. None of the plasma measures was significantly associated with the ability of serum to promote cholesterol efflux from the GM 970 cells using multivariate regression modeling (data not shown).

DISCUSSION

In the present study, we show that long-term tibolone treatment reduces plasma HDLC and apo A1 concentrations in a dose-dependent manner, whereas CEE or CEE+MPA treatment did not have a significant effect on the lipoprotein or apolipoprotein levels. Two different doses of tibolone were used, which were designed to bracket the most commonly used clinical doses (1.25 to 2.5 mg/d) in women.[18] In the lo tib group, HDLC and apo A1 concentrations were reduced by 30% and 19%, respectively, compared with the control group, which is similar to what has been reported in the earlier human studies.[13,14] In this study, the TC and LDLC+VLDLC concentrations were higher than in most human studies due to the use of an atherogenic diet that was needed to promote the formation of atherosclerotic lesions over the 2-year study. The effect of these treatments on CAA are reported separately.[18] We also measured serum total PL, because it has been shown that addition of PL to serum enhances cholesterol efflux.[27] There were no effects of any of the hormone treatments on serum total PL levels. Changes in the PL content of specific lipoproteins were not measured.

For measurement of the cholesterol efflux potential of serum, we used both Fu5AH cells and GM 970 human skin fibroblasts, because these 2 cell types have been shown to have different rates of cholesterol efflux and likely different mechanisms for the stimulation of cholesterol efflux.[16] Fu5AH cells have a fast rate of cholesterol efflux, a high sensitivity to serum PL content,[27] and a high density of scavenger receptor BI (SR-B1)[28] on their cell surface. Skin fibroblasts have a slow rate of cholesterol efflux, are resistant to serum PL content, but they are sensitive to lipid-free apo A1-mediated cholesterol efflux.[16] At least 3 different mechanisms of cellular cholesterol efflux have been described; aqueous diffusion, SR-BI–mediated efflux, and lipid-free apo A1-mediated efflux.[15,16,29] The most basic mechanism is simple aqueous diffusion in which cholesterol from the plasma membranes of cells desorbs into the aqueous environment around the cell and is taken up by acceptor particles. Aqueous diffusion operates in all cell types, and although it is viewed as a relatively inefficient mechanism, its contribution to total cellular cholesterol efflux depends on cell type and acceptor composition. In cells like Fu5AH that express high numbers of SR-B1 receptors,[28] the binding of HDL leads to reorganization of cholesterol within the plasma membrane, which facilitates cholesterol efflux.[28] In contrast, the lipid-free apo A1-mediated efflux appears to be secondary to the binding of apo A1 to the adenosine triphosphate (ATP)-binding cassette 1 (ABCA1) transporter.[30] This ABCA1 transporter is thought to form channels within the plasma membrane through which PLs and cholesterol are transferred and picked up by lipid-free or lipid-poor apolipoproteins.[31] This mechanism has been shown to mediate cholesterol efflux in various cell types, including normal human fibroblasts.[30]

In the present study, the ability of serum to promote cholesterol efflux from Fu5AH cells was not affected in the lo tib group, although there was a 30% reduction in HDLC concentrations. In the hi tib group, with a 52% decrease in HDLC, there was a significant decrease in cholesterol efflux, and in the CEE group, there was a significant increase in efflux. There was no change in cholesterol efflux from the GM 970 fibroblasts with any of the treatments, although apo A1 levels were decreased in both lo tib and hi tib groups by 19% and 40%, respectively. Removal of cholesterol from normal human skin fibroblasts by lipid-free apolipoproteins is well documented,[32] however, the magnitude of this pathway when whole serum is used as an acceptor is not well defined. Our results suggest that differences in cholesterol efflux potential of serum are due primarily to differences in the SR-B1–mediated pathway. The mechanism of SR-B1–mediated cholesterol efflux is unclear. It is possible that SR-B1 acts by enhancing the aqueous diffusion pathway. There is also evidence that binding of HDL to SR-B1 is required for cholesterol efflux to occur, which is not a requirement for aqueous diffusion. If the differences in cholesterol efflux potential of the various sera were due exclusively to effects on the aqueous diffusion pathway, an effect would have been expected in skin fibroblasts as well, which was not the case.

The decrease in HDLC concentration seen in the hi tib group could partly explain the decrease in cholesterol efflux, because serum HDLC is known to be an effective mediator of cholesterol efflux in Fu5AH cells, perhaps secondary to the binding of HDLs to SR-B1.[17] Nevertheless, the 52% reduction in HDLC levels in the hi tib group reduced cholesterol efflux by only 14%, and no reduction in cholesterol efflux was seen in the lo tib group, even though the HDLC concentrations were decreased by 30%. One explanation of this relative insensitivity of cholesterol efflux to decreases in HDLC concentrations is differences in HDL composition. In studies with Fu5AH cells, optimal conditions for efflux are achieved through a synergistic effect of the efficiency of the small particles and the capacity of large PL-rich acceptors.[33] Thus, our findings in the lo tib group could be explained if tibolone treatment results in the production of HDL or other components of serum that are more efficient as cholesterol acceptors. Consistent with this possibility is the observation that if one compares HDLC concentration with cholesterol efflux from Fu5AH cells for the control and lo tib groups, at HDLC concentrations less than 40 mg/dL, nearly all of the lo tib cholesterol efflux values are higher than control values. To answer this question conclusively, however, will require the direct comparison of sera and HDL from tibolone and control subjects with similar HDLC concentrations. Another possibility is that over the 4-hour efflux period there was adequate cholesterol acceptor capacity, even with a 30% reduction in HDLC concentrations, to allow efflux to proceed at

an unaltered rate. However, with a 52% reduction in HDLC concentrations, the cholesterol acceptor capacity was inadequate, and the rate of cholesterol efflux slowed. An argument against this possibility is the fact that in our preliminary experiments, cholesterol efflux was linear for at least 8 hours at HDLC concentrations ranging from 10 to 62 mg/dL (unpublished data). It should be kept in mind that women taking tibolone have a reduction in HDLC of about 30%, and thus their serum would not be expected to have a reduced cholesterol efflux potential.

There is a growing literature from both clinical and experimental studies demonstrating that induced changes in HDLC concentrations are not always associated with expected changes in atherosclerosis.[29] This could be attributed to different mechanisms by which HDLC concentrations are lowered. A reduction in HDLC secondary to enhanced clearance does not appear to increase atherosclerosis, while reductions secondary to reduced synthesis cause enhanced atherosclerosis. The most likely explanation is that with enhanced HDLC turnover there is greater reverse cholesterol transport, even though the steady state levels of HDLC are low. Additional studies are needed to determine the mechanism by which tibolone lowers HDLC.

To determine the key mediators of cholesterol efflux in serum, we first did simple correlation analyses between the ability of serum to promote cholesterol efflux in Fu5AH or GM 970 cells and lipid, lipoprotein, and apolipoprotein levels. This was followed by multiple regression analysis to determine which parameters were independent and the extent to which they could explain the correlations with cholesterol efflux. In Fu5AH cells, there was a strong correlation with HDLC or apo A1 concentrations and cholesterol efflux. This is consistent with studies by others using human serum[17] or serum from apo A1 transgenic mice.[34] In our study, this strong positive correlation remained for both of these parameters when the different treatment groups were analyzed separately. There was also a negative correlation between the ability of serum to promote cholesterol efflux in Fu5AH cells and TC, LDLC+VLDLC, or apo B concentrations. This differs from the results of de la Llera Moya et al,[17] in which no correlation was seen between cholesterol efflux and any apo B containing lipoproteins, although there was a positive correlation with serum TC. This difference may be due to the fact that the sera from the animals in our study were more hypercholesterolemic, containing a greater proportion of apo B containing lipoproteins than HDLs. Thus, the negative correlation with serum TC most likely reflects the contribution of LDLC+VLDLC, whereas the positive correlation with cholesterol efflux and TC concentrations with relatively normolipidemic serum,[17] probably reflects the greater contribution of HDLC.

Multivariate regression analyses confirmed our results with simple correlations indicating that HDLC was most highly associated with the ability of serum to promote cholesterol efflux from Fu5AH cells, accounting for 16.1% of the variability. LDLC+VLDLC accounted for 4.9% of the serum cholesterol efflux potential. Although there was no relationship of serum PL concentrations with cholesterol efflux using simple correlations, with multivariate analysis, PL could explain 6.6% of the variability in cholesterol efflux from Fu5AH cells when simultaneously controlling for HDLC and LDLC+VLDLC concentrations. This is probably largely a reflection of the mix of lipoproteins in the plasma, especially the relative enrichment of PL in HDLs. We did not, however, measure HDL PL concentration, which may have correlated better with cholesterol efflux potential than total PL.[35] Interestingly, CEE treatment accounted for 5.6% of the cholesterol efflux variability by a mechanism independent of changes in any of the lipid or lipoprotein concentrations. We have no explanation for this finding other than to suggest the possibility that CEE and, perhaps tibolone, may modify HDL or HDL precursors in such a way to make them more efficient cholesterol acceptors. Because the regression model with all variables included could explain only about 42% of the variability in cholesterol efflux, there must be other important factors in serum that account for the remaining proportion. These could include other plasma proteins,[36,37] changes in HDL subclass distribution,[29] differences in HDL lipid concentrations,[38,39] the concentration of HDL precursors, such as pre beta HDL,[40] etc.

In conclusion, our study shows that in cynomolgus monkeys, long-term tibolone treatment reduces HDLC concentrations. When HDLC concentrations were reduced by 30%, as seen in women using tibolone, there was no reduction in the serum cholesterol efflux potential, and thus, no indication for adverse effects of tibolone treatment on reverse cholesterol transport. In CEE-treated animals, HDLC levels were not affected, but serum cholesterol efflux potential was increased, suggesting a possible beneficial effect of CEE on reverse cholesterol transport. Multivariate regression analysis indicated that although HDLC was the lipoprotein most highly associated with the cholesterol efflux, HDLC could account for only a relatively small proportion (16.1%) of the total capacity of serum to promote cholesterol efflux.

ACKNOWLEDGMENT

The authors wish to thank Dick Meuleman, PhD (Organon) for his scholarly and thoughtful input at many stages of this study. The authors also thank Mary Leight, Patricia Hester, and Catrina Rankin for excellent technical assistance.

REFERENCES

1. Grady D, Rubin SM, Petitti DB, et al: Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Intern Med 117:1016-1037, 1992

2. Ettinger B, Pressman A, Sklarin P, et al: Associations between low levels of serum estradiol, bone density, and fractures among elderly women: The study of osteoporotic fractures. J Clin Endocrinol Metab 83:2239-2243, 1998

3. Collaborative Group on Hormonal Factors in Breast Cancer: Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 350: 1047-1059, 1997

4. Hulley S, Grady D, Bush T, et al: Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. JAMA 280:605-613, 1998

5. Herrington DM, Reboussin DM, Brosnihan KB, et al: Effects of

estrogen replacement on the progression of coronary-artery atherosclerosis. N Engl J Med 343:522-529, 2000

6. Adams MR, Register TC, Golden DL, et al: Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. Arterioscler Thromb Vasc Biol 17:217-221, 1997

7. Schairer C, Lubin J, Troisi R, et al: Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. JAMA 283:485-491, 2000

8. van der Vies J: Pharmacological studies with (7 alpha,17 alpha)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one (Org OD 14). Maturitas 1:15-24, 1987 (suppl)

9. Egarter C, Sator M, Berghammer P, et al: Efficacy, tolerability, and rare side effects of tibolone treatment in postmenopausal women. Int J Gynaecol Obstet 64:281-286, 1999

10. Bjarnason NH, Bjarnason K, Haarbo J, et al: Tibolone: Prevention of bone loss in late postmenopausal women. J Clin Endocrinol Metab 81:2419-2422, 1996

11. Genazzani AR, Benedek-Jaszmann LJ, Hart DM, et al: Org OD 14 and the endometrium. Maturitas 13:243-251, 1991

12. Zandberg P, Peters JL, Demacker PN, et al: Tibolone prevents atherosclerotic lesion formation in cholesterol-fed, ovariectomized rabbits. Arterioscler Thromb Vasc Biol 18:1844-1854, 1998

13. Crona N, Silfverstolpe G, Samsioe G: A double-blind cross-over study on the effects of ORG OD14 compared to oestradiol valerate and placebo on lipid and carbohydrate metabolism in oophorectomized women. Acta Endocrinol 102:451-455, 1983

14. Bjarnason NH, Bjarnason K, Haarbo J, et al: Tibolone: Influence on markers of cardiovascular disease. J Clin Endocrinol Metab 82: 1752-1756, 1997

15. Fielding CJ, Fielding PE: Molecular physiology of reverse cholesterol transport. J Lipid Res 36:211-228, 1995

16. Rothblat GH, de la Llera Moya M, Atger V, et al: Cell cholesterol efflux: Integration of old and new observations provides new insights. J Lipid Res 40:781-796, 1999

17. de la Llera Moya M, Atger V, Paul JL, et al: A cell culture system for screening human serum for ability to promote cellular cholesterol efflux. Relations between serum components and efflux, esterification, and transfer. Arterioscler Thromb 14:1056-1065, 1994

18. Clarkson TB, Anthony MS, Wagner JD: A comparison of tibolone and conjugated equine estrogens effects on coronary artery atherosclerosis and bone density of postmenopausal monkeys. J Clin Endocrinol Metab 85:5396-5404, 2001

19. Allain CC, Poon LS, Chan CS, et al: Enzymatic determination of total serum cholesterol. Clin Chem 20:470-475, 1974

20. Fossati P, Prencipe L: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 28:2077-2080, 1982

21. Lipid Research Clinics Program: Manual of laboratory operations: Lipid and lipoprotein analysis. Bethesda, MD, National Institutes of Health, DHEW publication 75-628, 1984 (revised)

22. Koritnik DL, Rudel LL: Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). J Lipid Res 24:1639-1645, 1983

23. Sorci-Thomas M, Wilson MD, Johnson FL, et al: Studies on the expression of genes encoding apolipoproteins B100 and B48 and the low density lipoprotein receptor in nonhuman primates. Comparison of dietary fat and cholesterol. J Biol Chem 264:9039-9045, 1989

24. Stevenson SC, Sawyer JK, Rudel LL: Role of apolipoprotein E on cholesteryl ester-enriched low density lipoprotein particles in coronary artery atherosclerosis of hypercholesterolemic nonhuman primates. Arterioscler Thromb 12:28-40, 1992

25. Wagner JD, Adams MR, Schwenke DC, et al: Oral contraceptive treatment decreases arterial low density lipoprotein degradation in female cynomolgus monkeys. Circ Res 72:1300-1307, 1993

26. St Clair RW, Leight MA: Differential effects of isolated lipoproteins from normal and hypercholesterolemic rhesus monkeys on cholesterol esterification and accumulation in arterial smooth muscle cells in culture. Biochim Biophys Acta 530:279-291, 1978

27. Jian B, de la Llera Moya M, Royer L, et al: Modification of the cholesterol efflux properties of human serum by enrichment with phospholipid. J Lipid Res 38:734-744, 1997

28. Ji Y, Jian B, Wang N, et al: Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. J Biol Chem 272:20982-20985, 1997

29. von Eckardstein A, Nofer JR, Assmann G: High density lipoproteins and arteriosclerosis: Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 21:13-27, 2001

30. Bortnick AE, Rothblat GH, Stoudt G, et al: The correlation of ATP-binding cassette 1 mRNA levels with cholesterol efflux from various cell lines. J Biol Chem 275:28634-28640, 2000

31. Wang N, Silver DL, Costet P, et al: Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem 275:33053-33058, 2000

32. Bielicki JK, Johnson WJ, Weinberg RB, et al: Efflux of lipid from fibroblasts to apolipoproteins: Dependence on elevated levels of cellular unesterified cholesterol. J Lipid Res 33:1699-1709, 1992

33. Rodrigueza WV, Williams KJ, Rothblat GH, et al: Remodeling and shuttling: Mechanisms for the synergistic effects between different acceptor particles in the mobilization of cellular cholesterol. Arterioscler Thromb Vasc Biol 17:383-393, 1997

34. Fournier N, de la Llera Moya M, Burkey BF, et al: Role of HDL phospholipid in efflux of cell cholesterol to whole serum: Studies with human apoA-I transgenic rats. J Lipid Res 37:1704-1711, 1996

35. Yancey PG, de la Llera-Moya M, Swarnakar S, et al: High density lipoprotein phospholipid composition is a major determinant the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. J Biol Chem 275:36596-36604, 2000

36. Bartholow LC, Geyer RP: Sterol efflux from mammalian cells induced by human serum albumin complexes. Dependence on phospholipid acyl chain length, degree of net charge. J Biol Chem 257: 3126-3130, 1982

37. Fielding CJ, Moser K: Evidence for the separation of albumin- and apo A-I-dependent mechanisms cholesterol efflux from cultured fibroblasts into human plasma. J Biol Chem 257:10955-10960, 1982

38. Davidson WS, Gillotte KL, Lund-Katz S, et al: The effect of high density lipoproein phospholipid acyl chain composition of cellular free cholesterol. J Biol Chem 270:5882-5890, 1995

39. Fournier N, Atger V, Cogny A, et al: Analysis of the relationship between triglyceridemia and HDL-phospholipid concentrations: Consequences on the efflux capacity of serum in the Fu5AH system. Atherosclerosis 157:315-323, 2001

40. Kawano M, Miida T, Fielding CJ, et al: Quantitation of pre beta-HDL-dependent and nonspecific components of the total efflux of cellular cholesterol and phospholipid. Biochemistry 32:5025-5028, 1993