# Platelet-enhanced apolipoprotein E production by human macrophages: a possible role in atherosclerosis

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**Abstract** Cholesterol-loaded human monocyte-derived macrophages increase their production of apolipoprotein E (apoE). Although cholesterol loading is often achieved with modified plasma lipoproteins, macrophages can be loaded also by coculture with platelets. Therefore, the relationship between platelet-mediated cholesteryl ester accumulation and apoE secretion was examined. Macrophages were isolated by adherence and cultured for 6 days in serum-free medium. Secreted apoE was measured with a sensitive solid-phase radioimmunoassay. Maximum apoE secretion by the adherent macrophages from $5 \times 10^6$ peripheral blood mononuclear cells was obtained with $3 \times 10^8$ platelets and was ten-fold greater than control cells cultured in the absence of platelets. Platelet-mediated apoE secretion was consistently greater than that obtained by culture with either native or acetylated low density lipoproteins. Whereas the 1000 *g* supernatants of unstimulated platelets were poor inducers of apoE secretion, substances rich in cholesterol that were shed from thrombin-stimulated platelets and recovered in the 1000 *g* supernatants were almost as active as intact platelets. ■ In all studies, platelet-induced secretion of apoE paralleled the capacity of platelets to induce macrophage cholesterol accumulation, indicating that macrophage apoE secretion was readily influenced by macrophage cholesterol metabolism.—**Takagi, Y., C. A. Dyer, and L. K. Curtiss.** Platelet-enhanced apolipoprotein E production by human macrophages: a possible role in atherosclerosis. *J. Lipid Res.* 1988. **29:** 859–867.

**Supplementary key words** cholesteryl ester accumulation • apoE radioimmunoassay • acetylated LDL • thrombin

The association of platelets with the lesions of atherosclerosis has been repeatedly documented (1–4). In fact, platelet antigens are detected within the foam cells of these lesions (5). Our laboratory has recently demonstrated that platelets have a remarkable capacity to induce cholesteryl ester (CE) accumulation in cultured human monocyte-derived macrophages (6). We demonstrated that thrombin-activated platelets or substances shed by activated platelets increase both the rate of esterification of cholesterol and the accumulation of CE in these cells. These observations suggest that platelets may contribute to lesion progression in atherosclerosis by enhancing foam cell formation. Furthermore, because substances shed by activated platelets are equally active (6), platelets may contribute to lesion progression at local sites of thrombus formation and platelet aggregation even when the endothelium is intact.

A consequence of an increase in the CE content of macrophages is a dramatic increase in their synthesis and secretion of apolipoprotein E (apoE). Basu et al. (7–9) demonstrated that, in response to cholesterol loading, both mouse peritoneal macrophages and human monocyte-derived macrophages increased their production and secretion of apoE. In these studies CE loading of the cells was accomplished by culture of the cells with the modified lipoprotein, acetylated low density lipoprotein (LDL). Furthermore, Kayden, Maschio, and Traber (10) have shown that both native LDL and acetylated LDL can induce macrophage cholesterol esterification and apoE production. Additional studies by Werb and Chin (11, 12) have demonstrated that the production and secretion of apoE by macrophages is sensitive as well to a number of immunologic stimuli and have shown that the exposure of macrophages to certain activators, such as endotoxin, resulted in a decrease in apoE production. Together, these observations establish that the production of apoE by macrophages is sensitive to a number of diverse stimuli.

Although the role of macrophage-produced apoE in atherosclerosis has not been identified, at least two func-

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Abbreviations: PBM, peripheral blood mononuclear cells; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoprotein; CE, cholesteryl ester; RBC, red blood cells; PRP, platelet-rich plasma; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; IgG, gamma immunoglobulin; BBS, borate-buffered saline; OGB, octyl glucoside buffer.

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tions of apoE could be relevant. ApoE plays an important role in mediating the cellular binding of cholesterol-rich lipoproteins (13). Therefore, apoE is believed to be an important participant in "reverse cholesterol transport," whereby excess cellular cholesterol is transported from peripheral tissues to the liver (8). ApoE is also an immunoregulatory monokine, which has potent suppressive activity for lymphocyte stimulation (14). ApoE, therefore, could influence local inflammatory and immune processes.

Both lipid deposition and inflammatory events are believed to contribute to the progression of a fatty streak lesion to an advanced fibrous plaque. Because the local production of apoE by monocyte/macrophages could be vital to these events, we assessed the influence of platelets on the production and secretion of apoE.

## MATERIALS AND METHODS

### Lipoproteins

VLDL and LDL were isolated from plasma of normal fasting subjects and characterized as described (6). Acetylated LDL also was prepared and characterized as described (6).

### Monocyte and platelet isolation and culture

Monocytes were isolated as described (15) with some modifications. Human peripheral blood was drawn from healthy donors into heparin (5 U/ml). After removing the platelet-rich plasma (PRP) by centrifugation at 400 *g* for 15 min, the pelleted cells were diluted to twice their original blood volume with RPMI buffer (RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, and 0.05 mg/ml gentamycin sulfate, pH 7.2) and were layered over Ficoll-Hypaque (d 1.074 g/ml). The tubes were centrifuged at 900 *g* for 20 min at 20°C. The peripheral blood mononuclear cells (PBM) were collected from the interface, washed 3 times with RPMI buffer, and then were resuspended in RPMI medium (RPMI 1640 supplemented with HEPES, L-glutamine, and antibiotics as above, as well as 1 mM sodium pyruvate and 1% Nutridoma HU from Boehringer Mannheim Chemicals, Indianapolis). The PBM were adjusted to $5 \times 10^6$/ml in the RPMI medium and 1.0 ml was dispensed per 2.3-cm well of a 12-well plastic culture plate (Costar, Cambridge, MA). The cells were incubated for 18–20 hr at 37°C in 5% $CO_2$–95% air to allow the monocytes to adhere. After aspiration of the nonadherent cells, the wells were washed vigorously twice with RPMI buffer and refed with 1.0 ml of RPMI medium containing the added platelets or lipoproteins. Greater than 95% of the adherent cells contained nonspecific esterase (16).

Platelets were isolated from the PRP that was obtained during the PBM isolation and had been stored overnight at room temperature in the presence of 1 mM theophylline and 1µg/ml of $PGE_1$. The PRP was centrifuged at 1000 *g* for 15 min. The platelet pellet was washed twice in Tyrode's buffer containing theophylline and $PGE_1$ and finally resuspended in RPMI medium for culture. Platelets were counted in a Coulter counter. Platelet supernatants were obtained by incubating platelets at $1 \times 10^9$/ml for 3 hr in Tyrode's buffer at 37°C in the presence of 1 U/ml of α-thrombin and 1 mM $CaCl_2$. The reaction mixtures were spun at 1000 *g* for 15 min and the supernatants were collected. When appropriate, the platelet supernatants were characterized further by filtering them or centrifuging them at higher speeds.

### Harvesting the cultures

The macrophage culture supernatants were collected for assay of apoE and the cells were harvested for assay of CE accumulation and DNA. At the times indicated, the culture medium was aspirated from the cells, transferred to 1.2-ml micro test tubes (Brinkmann Instrument Co., Westbury, NY) and centrifuged at 8000 *g* for 5 min in a Beckman Microfuge 11. The supernatants were collected and stored at −20°C before they were assayed for apoE. The adherent cells were washed 3 times with PBS, scraped from the plastic, incubated for 30 min at room temperature with 1 ml of absolute ethanol, and centrifuged at 8000 *g* for 5 min. The ethanol supernatant was transferred to glass tubes (Falcon, Oxford, CA) for determination of cholesterol, and the cell debris was resuspended in 0.3 ml of distilled water for determination of cellular DNA.

### Radioimmunoassay for secreted apoE

ApoE in the culture medium was measured in a sensitive solid phase radioimmunoassay. Assays were performed in flexible roundbottom polyvinyl chloride 96-well microtiter plates (Dynatech Inc., Alexandria, VA). The wells were coated for 30 min at 20°C with Protein A (*Staphylococcus aureus*, Calbiochem-Boehring, La Jolla, CA) by adding 0.1 ml/well of 500 ng/ml Protein A in borate-buffered saline (BBS), pH 8.4, and then incubated for 30 min with 0.1 ml/well of a dilution of ascites fluid containing an apoE-specific monoclonal antibody designated 1E. The antibody was diluted in octyl glucoside buffer (OGB) (BBS that contained 4 mM n-octyl β-D-glucopyranoside (Behring Diagnostic, La Jolla, CA) and 3% bovine albumin). After washing the wells, the culture supernatants diluted in OGB were added to the wells and the plate was incubated at 4°C for 18–20 hr. Plasma apoE isolated from VLDL (14) was used as a standard. After washing 4 times with the same washing buffer, 0.1 ml/well of $^{125}$I-apoE (250,000 cpm/well) was added to the wells and the plate was incubated at 4°C for 3 hr. The $^{125}$I-labeled apoE was radioiodinated enzymatically using immobilized lactoperoxidase and glucose



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oxidase (Enzymobeads, Bio-Rad, Richmond, CA) to specific activities of 7-12 μCi/ng. $^{125}$I-Labeled apoE was used within 7 days of radioiodination. After the final wash and dry, individual wells were removed and counted for $^{125}$I. The data were expressed as B/Bo where Bo was the amount of $^{125}$I-labeled apoE bound in the absence of competitors. Following logit transformation, the standard curves obtained with purified plasma apoE were linear between 130 and 5000 ng/ml of apoE and the coefficient of variation of intraassay measurements was consistently less than 7.7%. Because the acetylated LDL added to the cultured cells contained detectable levels of apoE (> 0.1 μg/100 μg of acetylated LDL), the levels of apoE produced by cells cultured in the presence of acetylated LDL were obtained by subtracting the levels of apoE measured in similar cultures containing no macrophages. Because the platelets contained no detectable apoE, similar calculations were not necessary when the macrophages were cultured with platelets.

### DNA assay

DNA in the cell extracts was measured by the method of Burton (17) with some modifications. After resuspending the cell debris in 0.3 ml of distilled water, 0.03 ml of 4.4 N perchloric acid was added and the tubes were mixed thoroughly. The reaction mixtures were incubated for 5 min at 90-95°C to solubilize DNA and developed with the diphenylamine reagent which contained 17.1 N acetic acid, 0.529 N sulfuric acid, 0.08 mg/ml of acetaldehyde, and 15 mg/ml of diphenylamine (Sigma, St. Louis, MO). After incubation for 20 hr at room temperature, the absorbance at 620 nm was measured. The concentration of DNA was calculated from a standard curve generated with calf thymus DNA (Sigma Chemicals, St. Louis, MO).

### Cholesterol assay

Cholesterol in the cultured cells was measured enzymatically. After drying, the ethanol extracts were solubilized in 1.5 ml of hexane. Free and esterified cholesterol were separated by affinity column chromatography on aminopropyl Bond Elut columns (Analytichem International, Harbor City, CA) (18). Cholesteryl esters (CE), which were not retained on the columns, were washed off with 4 ml of hexane. The bound free cholesterol was eluted with 20 ml of 15% ethyl acetate in hexane. After drying, the CE and free cholesterol fractions were redissolved in 0.2 ml of absolute ethanol and 0.015-ml aliquots were assayed for cholesterol enzymatically as previously described (19) in the presence of 0.1 U/ml cholesteryl ester hydroxylase. Flourescence was measured in a fluorescence spectrophotometer (Perkin-Elmer, Oak Brook, IL) with excitation at 325 nm and emission at 415 nm. The cholesterol value was calculated by reference to a standard curve prepared with cholesterol (Sigma, St. Louis, MO) as standard.

### Polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was performed in 3-20% gradient polyacrylamide slab gels containing 0.1% SDS as described (20). Electrophoresis was carried out at 12 mA per slab gel at room temperature for 18-20 hr. For protein staining, the gel was stained with Coomassie blue R-250 and destained with methanol-acetic acid. For reaction with antibody, the proteins were transferred to a nitrocellulose membrane by electrophoresis as described (20). After transfer, the nitrocellulose membranes were incubated with 15 μCi of $^{125}$I-labeled monoclonal antibody 1E and antibody binding was visualized by autoradiography.

### Intrinsic labeling of apoE

Monocytes, isolated as described above, were cultured for 3 days in RPMI medium in the absence or presence of 25 × $10^7$/ml autologous platelets. Following removal of the platelets or medium, the macrophages were cultured for an additional 24 hr at 37°C in methionine-free medium (deficient DME, Irvine Scientific, supplemented with 10 mM HEPES, 2 mM L-glutamine, 5μg/ml insulin, 10 μg/ml polymixine B, and 50 μg/ml gentamycin sulfate) that contained 50 μCi/ml of [$^{35}$S]methionine. The apoE in the macrophage culture supernatants was isolated by ultracentrifugation at a density of 1.2 g/ml as described (7).

## RESULTS

Using intrinsic labeling with [$^{35}$S]methionine, a number of investigators have demonstrated that human monocyte-derived macrophages synthesize and secrete apoE (8, 10). However, because in all cases separation of apoE by ultracentrifugation was required, the secretion of apoE was not strictly quantitated. Therefore, we chose to eliminate the need for ultracentrifugation and its associated losses (8) by measuring apoE with a sensitive radioimmunoassay. Secreted apoE was measured in supernatants of human monocyte-derived macrophages cultured for 6 days **(Fig. 1)**. The macrophages were cultured in serum-free defined medium that did not contain apoE; therefore all detectable apoE was of cellular origin. Control cells in buffer produced approximately 200 ng of apoE/μg of DNA, whereas cells cultured in the presence of 100 μg/ml of acetylated LDL produced approximately 600 ng of apoE/μg of DNA. Culture of the cells in the presence of native LDL enhanced the accumulation of secreted apoE as well, but as expected (10) the levels attained were consistently equal to or less than that obtained with acetylated LDL (Fig. 1). However, extensive accumulation was observed with culture of the cells in the presence of autologous platelets. In 6 days comparable numbers of human monocyte-derived macrophages cultured in the presence of 25 × $10^7$ platelets secreted greater than 1500 ng of apoE/μg of DNA.

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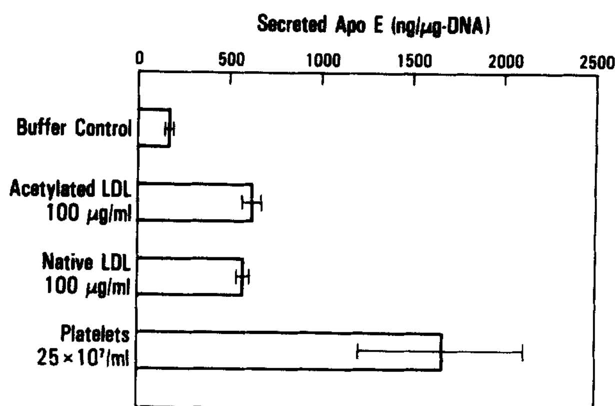


**Fig. 1.** The effect of platelets and lipoproteins on macrophage apoE secretion. The adherent cells from 5 × $10^6$ PBM were cultured at 37°C in quadruplicate in 1 ml of serum-free RPMI 1640 medium for 6 days with 100 μg/ml of acetylated LDL, 100 μg/ml of native LDL, or 25 × $10^7$ autologous platelets. Accumulated apoE was measured by RIA in the macrophage culture supernatants, and the macrophages were harvested for determination of DNA.

Platelets cultured in the absence of macrophages contained no detectable apoE and less than 1 μg of DNA/$10^9$ platelets (6). In additional experiments heterologous platelets were found to be equally active.

Verification was obtained by SDS polyacrylamide gel electrophoresis that the apoE measured immunochemically was intact apoE and that its size was comparable to that of plasma VLDL-derived apoE (**Fig. 2**). The supernatants of macrophages cultured in the presence of 25 × $10^7$ autologous platelets were applied to polyacrylamide gradient slab gels and electrophoresed in the presence of 1% SDS. The separated proteins were either stained for protein or were immediately electrophoretically blotted onto nitrocellulose. ApoE in the culture supernatants, as well as in plasma VLDL, was detected on the Western blots by incubation with the radioiodinated human apoE-specific monoclonal antibody 1E (Fig. 2). Similar molecular weight species were identified in macrophage culture supernatants and in plasma VLDL and these same species were not found in the supernatants of platelets cultured in the absence of macrophages. Although both apoE species reacted with this antibody, a slight difference in mobility was observed between macrophage-derived apoE and plasma VLDL-derived apoE. This is in agreement with differences in mobility previously reported (7), and is thought to be due to differences in the degree of glycosylation.

To verify that the immunochemially detected apoE was newly synthesized, secreted proteins were labeled with [$^{35}$S]methionine. A labeled species having a molecular weight comparable to that of plasma apoE was isolated in a d < 1.25 g/ml fraction of the culture supernatant following ultracentrifugation, indicating that at least a portion of the apoE measured immunochemically in the macrophage/platelet culture supernatants was synthesized de novo. As expected, macrophages cultured in the presence of platelets synthesized greater amounts of apoE than control cells.

To examine the kinetics of apoE secretion by monocyte-derived macrophages cultured in the presence of platelets, the adherent cells from 5 × $10^6$ PBM were cultured with 25 × $10^7$ autologous platelets in 1- ml cultures. ApoE accumulation was measured after 1, 3, 6, and 9 days of culture. Cells cultured in the absence of platelets secreted minimal amounts of apoE **(Fig. 3).** However, cells cultured in the presence of platelets produced detectable amounts of apoE within 6 days of culture, and this production was continued for at least 9 days. In additional studies it was found that macrophages grown in serum-free medium in the presence of platelets will produce apoE for up to 6 weeks of culture. The kinetics of CE accumulation by these cells has been reported previously (6). Because neither esterified cholesterol nor DNA was detectable in 25 × $10^7$ platelets, all the CE and DNA measured were macrophage-derived. As reported, CE accumulation by macrophages cultured with comparable numbers of platelets was less than 10 ng/μg DNA on day 1, but was detectable at 100 ng/μg DNA by the second day of culture. Maximum CE accumulation of 400 ng/μg DNA was apparent by day 3 (6). In contrast, no difference was observed in the accumulation of secreted apoE between platelet-exposed and control macrophages until after day 3 (Fig. 3). Because CE accumulation was maximum by day 3, whereas apoE production was not yet detectable by day 3, CE accumulation by these cells preceded the accumulation of secreted apoE.

The dependence of apoE production on platelet number was examined as illustrated in **Fig. 4.** Increasing numbers of platelets from 7.5 to 120 × $10^7$/ml were added to 1.0-ml cultures of the adherent cells from 5 × $10^6$ PBM. After 6 days the accumulated apoE as well as the CE content of the cells was measured. Maximum accumulation of apoE was observed with 30 × $10^7$ platelets/ml and this same dose of platelets facilitated maximum CE accumulation. Thus, both apoE secretion and CE accumulation occurred with similar doses of platelets.

To determine whether the capacity of platelets to induce apoE secretion was merely a reflection of their capacity to increase the CE content of the macrophages, we compared the amount of apoE produced by platelet-exposed cells to the amount of apoE produced by acetylated LDL-exposed cells. As shown in **Table 1,** comparable levels of CE accumulation (~ 100 ng/μg of DNA) could be achieved by culture of the macrophages for 6 days in the presence of either 30 μg/ml of acetylated LDL or 10 × $10^7$ platelets, yet the platelet-exposed cells secreted over 3 times as much apoE. Furthermore, whereas maximal apoE production was 400-500 ng/μg of DNA with acetylated LDL, it was greater than 1400 ng/μg of DNA with platelets. Therefore, no close correlation was observed between CE loading and apoE production.



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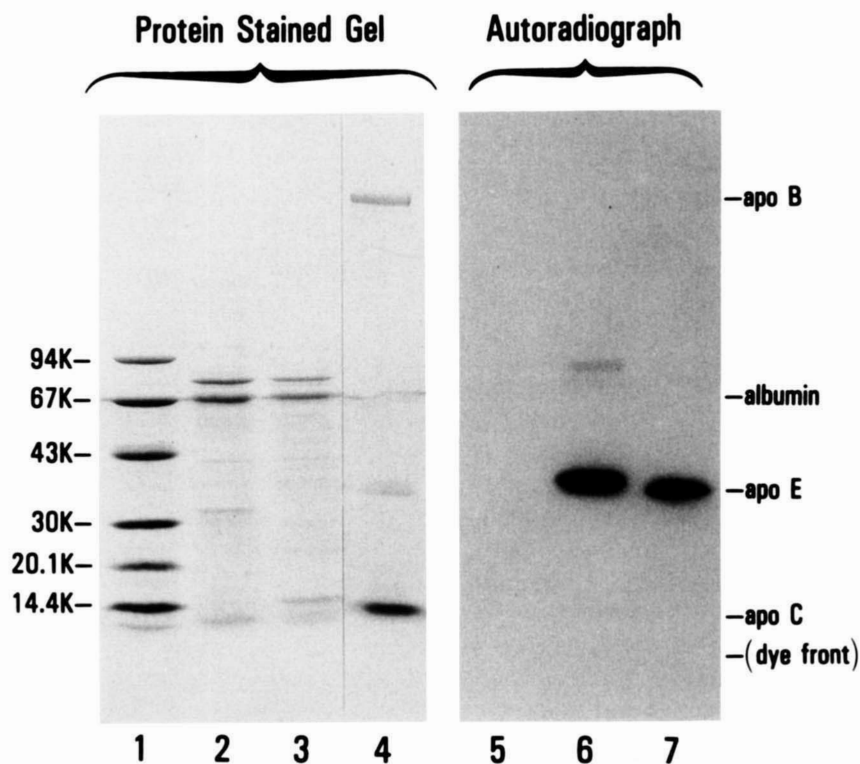


**Fig. 2.** Polyacrylamide gel electrophoresis of macrophage-synthesized apoE. The 3–20% polyacrylamide gradient gels contained 1% SDS, but did not contain a reducing agent. The panel on the left was stained for protein with Coomassie Brilliant Blue R250, whereas the panel on the right was blotted onto nitrocellulose for reaction with antibody. The samples electrophoresed were: lane 1, molecular weight standards; lane 2, 0.1 ml of unfractionated culture supernatant from $25 \times 10^7$ platelets cultured for 6 days in serum-free medium; lane 3, 0.1 ml of unfractionated culture supernatant from the adherent cells of $5 \times 10^6$ PBM cocultured for 6 days with $25 \times 10^7$ platelets; and lane 4, 20 μg of isolated plasma VLDL. Lanes 5, 6, and 7 are Western blots of lanes 2, 3, and 4, respectively. ApoE was detected on the blots by incubation with a radioiodinated apoE-specific monoclonal antibody, IE. The protein in lane 6 of molecular weight greater than 67k was probably an apoE dimer because reduction of the samples before electrophoresis abolished this species.

In all preceding studies, the platelets were isolated from PRP to which theophylline and $PGE_1$ had been added during isolation to inhibit platelet activation. However, because the platelets were subsequently cultured for 6 days with monocytes at 37°C in serum-free medium in the absence of any inhibitors, activation of the platelets during the culture period was likely. Therefore, the effect of platelet activation on apoE production was specifically addressed. Isolated autologous platelets ($1 \times 10^9$/ml), were incubated in the absence or presence of 1 U/ml of the specific platelet activator, α-thrombin. Following a 3-hr incubation at 37°C, the platelets were spun at 1000 $g$ for 15 min and the supernatant or pellet was added separately to the macrophages. Macrophage exposure was continued for 6 days after which the accumulation of apoE was measured. Platelets incubated for 3 hr in the absence of α-thrombin and in the presence of theophylline and $PGE_1$ were fully capable of enhancing macrophage apoE accumulation measured on day 6 **(Fig. 5)**. However, the supernatant from these platelets was not active. In the absence of platelet stimuli, the activity remained with the intact platelet pellet. However, when platelets were incubated for 3 hr at 37°C with α-thrombin, activity was recovered in this thrombin-stimulated platelet supernatant as well. The supernatant from $1 \times 10^9$ platelets contained activity, which was approximately half that of the resuspended unstimulated platelets (Fig. 5). Furthermore, this activity was not pelleted by centrifugation at 8000 $g$ for 2.5 min or retained on a 0.45-μm sterile filter, suggesting that it was not associated with intact platelets. The results indicated that platelet activation was required for the shed-



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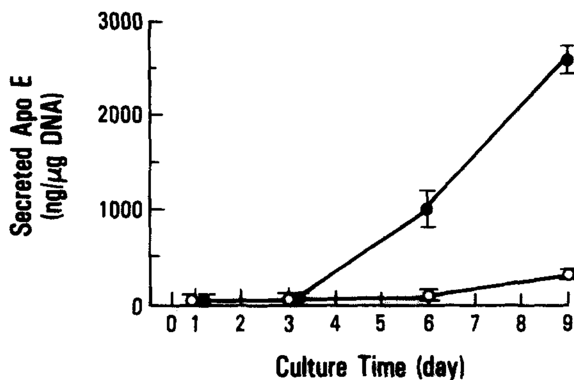


**Fig. 3.** Kinetics of the accumulation of secreted apoE. The adherent cells from 5 × $10^6$ PBM were cultured in quadruplicate in 1 ml in the absence (○) or presence (●) of 25 × $10^7$ platelets. On days 1, 3, 6, and 9, the accumulation of secreted apoE was measured in the culture supernatants by RIA. As reported previously (see reference 6), these macrophages contained <10, <10, 105, 395, and 410 ng of CE/μg of DNA on days 0, 1, 2, 3, and 6, respectively.

ding of active substances from platelets and suggested that the activity observed in the 6-day macrophage cultures with unstimulated intact platelets may have resulted from nonspecific platelet activation, which occurred during the culture period. Furthermore, the ability of platelets to enhance macrophage apoE production was contained within substances that were shed by activated platelets.

## DISCUSSION

Others, using intrinsic labeling of apoE, have demonstrated that human monocyte-derived macrophages synthesize and secrete apoE (8, 10). In this study we have confirmed this observation using a sensitive and precise competitive RIA to quantitate secreted apoE. Studies of various factors that can influence macrophage production of apoE have indicated that the constitutive synthesis of apoE is readily influenced by a number of factors including specific plasma lipoproteins and macrophage activators (7–12). As expected, the modified lipoprotein, acetylated LDL, enhanced apoE secretion (Fig. 1). The native lipoprotein was active as well and this observation is in agreement with the report of Kayden et al. (10). In all instances where apoE production was increased, the macrophages were cultured under conditions that would enhance their content of CE. It was reported that platelets could contribute at least qualitatively to CE accumulation in smooth muscle cells (21). Therefore, we added platelets to the cultured macrophages. A striking increase in CE accumulation to a level that exceeded the level obtained with plasma lipoproteins (6) was observed. Furthermore, this increased CE accumulation induced an increase in apoE production.

The capacity of platelets to increase apoE production was dose-dependent. Optimum platelet doses for apoE and CE accumulation were comparable (Fig. 4) and were well within a physiologically significant ratio of platelets to macrophages. However, when the comparison was made between acetylated LDL and platelets to facilitate CE accumulation and induce apoE secretion, acetylated LDL and platelets did not have identical effects (i.e., comparable levels of CE loading did not lead to comparable levels of apoE production). This observation suggested that either platelets contributed additional signals for apoE production or that apoE production was not regulated by cellular CE. Evidence in favor of the latter possibility was recently published by Mazzone et al. (22). Their studies of the relationship between macrophage cholesterol content and apoE synthesis suggested that it is the free cholesterol rather than the esterified cholesterol that may regulate apoE production. In their studies, inhibition of acyl CoA:cholesterol acyltransferase did not lead to inhibition of apoE production. Unfortunately, direct measurements of the free cholesterol content of platelet-cultured macrophages could not be made in our studies because the platelets and monocytes could not be separated, so only macrophage-free cholesterol was reliably measured. We are now in the

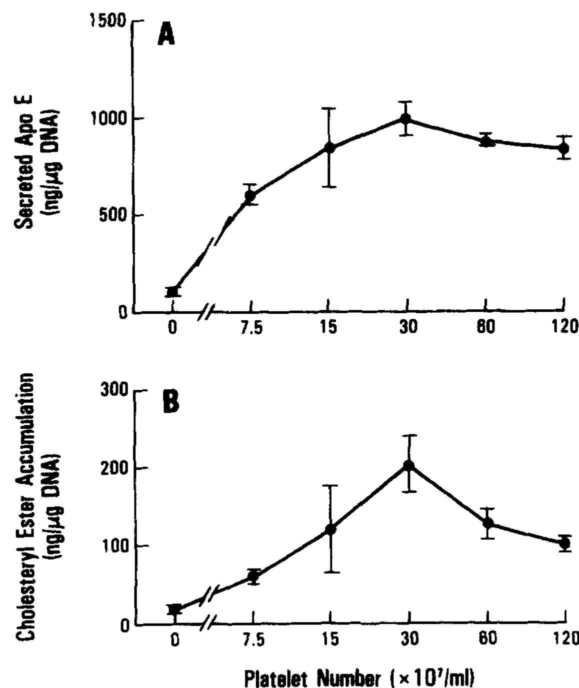


**Fig. 4.** Platelet induction of apoE secretion and macrophage cholesteryl ester accumulation is dose-dependent. Increasing numbers of platelets from 7.5 to 120 × $10^7$ were added to quadruplicate 1-ml cultures of the adherent cells from 5 × $10^6$ PBM. The accumulation of secreted apoE on day 6 was measured in the culture supernatants by RIA. CE accumulation on day 6 was measured in the cells following the separation of free cholesterol on Bond Elut columns as described.



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TABLE 1. Comparison of platelet-mediated versus acetylated LDL-mediated apoE secretion[a]

| Culture Condition | CE | ApoE |
|---|---|---|
| | *ng/μg DNA* | *ng/μg DNA* |
| Buffer control | < 10 | 6 ± 12 |
| Acetylated LDL (μg/ml) | | |
| 3 | < 10 | 97 ± 4 |
| 10 | 54 ± 13 | 107 ± 3 |
| 30 | 103 ± 8 | 281 ± 12 |
| 100 | 139 ± 7 | 420 ± 56 |
| 300 | 124 ± 10 | 432 ± 38 |
| Platelets (cells/ml) | | |
| $1 \times 10^7$ | < 10 | 102 ± 2 |
| $3 \times 10^7$ | < 10 | 207 ± 22 |
| $10 \times 10^7$ | 102 ± 14 | 876 ± 31 |
| $30 \times 10^7$ | 298 ± 34 | 1407 ± 97 |
| $100 \times 10^7$ | 129 ± 5 | 1304 ± 127 |

[a]The adherent cells from $5 \times 10^6$ PBM were cultured for 6 days. CE accumulation and apoE production were assayed as described. Numbers represent the mean ± SD of quadruplicate cultures.

process of determining whether a cholesterol-free stimulus for apoE production can be found, so the relationship between apoE production and cellular-free cholesterol can be studied directly.

What platelet constituents influence apoE production by cultured macrophages? Some insight was obtained by identifying that the supernatants of thrombin-stimulated, but not unstimulated platelets, were active. This suggested that platelet stimulation facilitated the generation of the platelet-associated activity. Active platelet constituents were shed from thrombin-stimulated platelets. These shed constituents were not pelleted by centrifugation at 8000 *g* for 2.5 min and were not retained on a 0.45-μm filter, indicating that intact platelets were not required. However, it cannot be concluded from these studies that platelet stimulation, as defined by shape change and the secretion of alpha and dense granule components, is required or even sufficient for the generation of an active fraction (6). Membrane blebbing and release of lipid vesicles have been described in thrombin-activated platelets (23), and we observed the shedding of free cholesterol upon platelet stimulation (6). In fact, a direct correlation was found between the capacity of the supernatants of activated platelets to induce CE loading and their content of free cholesterol (6). Nevertheless, the capacity of platelets to induce CE accumulation is specific because free cholesterol added as red blood cells (RBC), RBC ghosts, sonicated RBC ghosts, or opsonized RBC is not active (6). Whereas the accumulation of CE was different (6), it was shown that protein synthesis as assessed by the incorporation of [$^3$H]leucine into total trichloroacetic acid-precipitable protein was similar for cells exposed to either thrombin-stimulated platelet supernatants or sonicated RBC. This suggests that the capacity of platelets to induce macrophage apoE secretion is not just a reflection of a general increase in protein synthesis. Rather it suggests that a specific interaction between macrophages and platelets (or platelet constituents) may be required for platelet-induced C or CE accumulation and the subsequent production of apoE. Selective binding of platelets to human monocytes has been described (24), which implies that specific adhesive proteins and their receptors may facilitate the platelet/macrophage interaction that leads to macrophage foam cell formation and apoE production.

What is the physiologic importance of platelet-induced apoE production by macrophages? Secreted apoE was not detectable until after 3 days of culture, whereas significant CE accumulation was observed after 1 day of culture and was maximum at 3 days. Therefore, macrophage apoE production was increased in response to CE accumulation or cholesterol was a trigger for apoE production. Although the two events do not appear to have an obligatory relationship (9), Basu et al. (8) has proposed that apoE secretion by macrophages is concerned with "reverse cholesterol transport" or the transfer of cholesterol from macrophages to the liver, and that it functions with lecithin:cholesterol acyltransferase and HDL to convert cholesterol-poor HDL into cholesterol-rich, apoE-rich HDL for delivery to the liver. Support for this hypothesis comes from the demonstration that apoE plays a compulsory role in the formation of large cholesterol-rich and receptor-active HDL (25).

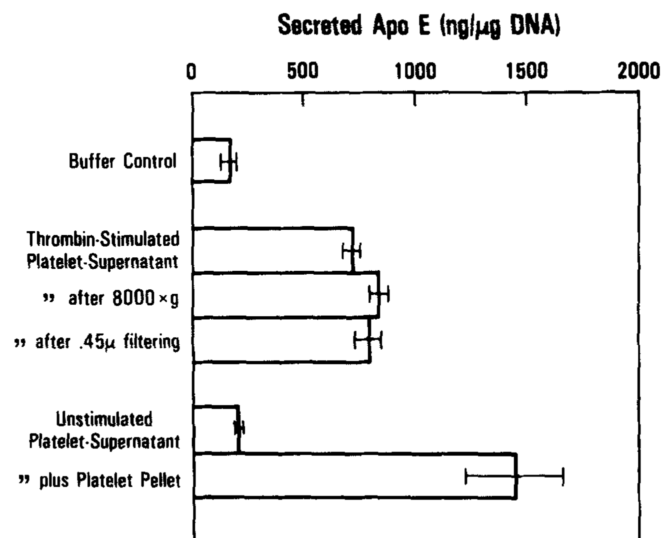


**Fig. 5.** Stimulated platelets release substances that enhance macrophage apoE secretion. Isolated platelets were resuspended to $1 \times 10^9$/ml and incubated in the presence of theophylline and $PGE_1$, and in the absence or presence of 2 U/ml of α-thrombin and 1 mM $CaCl_2$. After 3 hr at 37°C, the platelet supernatants were obtained by centrifuging the incubation mixtures at 1,000 *g* for 15 min. The supernatants from thrombin-stimulated platelets and the supernatants from unstimulated platelets were assayed for activity by adding them in quadruplicate to 1-ml cultures of adherent cells from $5 \times 10^6$ PBM and assaying for secreted apoE on day 6. The thrombin-stimulated platelet supernatant was reassayed also on macrophages after it had been centrifuged at 8,000 *g* for 2.5 min in a Beckman microfuge or filtered through a 0.45-μm filter.



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If platelet-induced macrophage CE accumulation contributes to the generation of foam cells of atherosclerotic fatty streak lesions, the apoE response of the macrophage may play an important role in preventing even greater subendothelial accumulation of cholesterol within the lesion by providing a vehicle for transfer of the cholesterol out of the arterial wall.

We wish to propose an additional role for macrophage-secreted apoE in the developing fatty streak lesion. ApoE-rich lipoproteins are capable of inhibiting lymphocyte proliferation (26), and recently lipid-free apoE has been shown to be the important, active constituent of these immunoregulatory lipoproteins (14). More recently, we have observed that human macrophage-generated apoE was able to inhibit mitogen-induced lymphocyte stimulation as well (27). Lymphocytes, monocytes, and IgG are observed in the subendothelial space (3, 28, 29), and immunologic injury can contribute to the development or progression of atherosclerosis (30–32). Therefore, local increases in the production of apoE by macrophage/foam cells may prevent further lymphocyte-induced immunologic injury by suppressing lymphocyte activation. The ultimate identification of a physiologic role for macrophage/foam cell-generated apoE in the prevention or regression of the fatty streak lesion will require further examination of the specific functional properties of macrophage-produced apoE.

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