Activated platelets secrete a protein-like factor that stimulates oxidized-LDL receptor activity in macrophages

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Abstract Platelet secretory products were shown to modulate the interaction between lipoproteins and their receptors on macrophages. Preincubation of macrophages for 2 h at 37°C with platelet conditioned medium (PCM), followed by its removal and a further 5-h incubation in the presence of oxidized-LDL (Ox-LDL), resulted in increased cellular degradation of Ox-LDL (34%), stimulation of cellular cholesterol esterification (31%), and mass accumulation of esterified and nonesterified cholesterol (25% and 41%, respectively). These effects were found to be the result of a PCM-mediated increase in the number of Ox-LDL receptors on macrophages. PCM was shown to interact with the macrophage scavenger receptor. Enhanced Ox-LDL uptake by macrophages preincubated with PCM could not be reproduced when PCM remained in the incubation medium. Maintenance of PCM in the incubation medium reduced Ox-LDL uptake by macrophages (40%) and was shown to be PCM dose-dependent. Whereas incubation at 37°C demonstrated enhanced uptake of Ox-LDL, preincubation of macrophages with PCM at 4°C exhibited a 64% reduction in Ox-LDL-mediated cellular cholesterol esterification. Thus, PCM internalization by macrophages after its binding to the scavenger receptor is required to promote the enhancing effect of PCM on Ox-LDL uptake by macrophages. PCM activity was associated with platelet degranulation, and was recovered in the protein fraction of PCM. It was found to be heat- and trypsin-labile with a molecular weight greater than 25,000. PCM obtained from platelets derived from a patient with alpha granules deficiency failed to enhance the uptake of Ox-LDL by macrophages, suggesting that the active protein-like factor in PCM originated from platelet alpha granules. These results indicate that a platelet-secreted protein-like factor can modulate macrophage uptake of Ox-LDL with subsequent effect on foam cell formation.—**Fuhrman, B., G. J. Brook, and M. Aviram.** Activated platelets secrete a protein-like factor that stimulates oxidized-LDL receptor activity in macrophages. *J. Lipid Res.* 1991. **32:** 1113–1123

Supplementary key words cholesterol • scavenger receptor

Lipid-laden macrophages are characteristic constituents of the atherosclerotic plaque (1, 2). Macrophages accumulate cholesterol after incubation with chemically (3, 4) or biologically (5) modified LDL, but not with native LDL (6). Modification of LDL by oxidative processes results in its enhanced uptake by macrophages (7). LDL oxidation in vitro can be achieved by incubation in cell-free medium under oxidative conditions (8) and by incubation with endothelial cells (8, 9), smooth muscle cells (10, 11), or monocyte derived macrophages (12). Thus, LDL oxidation may occur within the arterial wall, and indeed, evidence for the in vivo existence of Ox-LDL has been presented (13, 14).

Recently platelets were shown to enhance in vitro oxidation of LDL (15). Blood platelets play an important role in the development of atherosclerosis (16, 17) and they are found in close association with foam cells in the developing atherosclerotic lesion (18). Platelet aggregation and release may be implicated early in atherogenesis as well as in the more advanced stages. In early lesions platelets adhere to regions of the arterial wall where blood flow is disturbed (19). The latter also permits the accumulation of agents that can injure the endothelium, which may in turn cause further platelet activation and degranulation (20–22). Platelet involvement in the more advanced atherosclerotic lesion may occur subsequent to oxidation of LDL in the subendothelial space where the concentration of antioxidants is low. Ox-LDL can be highly cytotoxic, causing loss of endothelial cells overlying the fatty streak (20), thus leading to platelet adherence and activation. The secretory products then released may react with macrophages with subsequent effect on cellular uptake of Ox-LDL. Activated platelets release a variety of substances that have been shown to enhance cholesterol es-

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidized LDL; PCM, platelet conditioned medium; MPM, mouse peritoneal macrophages; HMDM, human monocyte-derived macrophages; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; MDA, malondialdehyde.

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terification in murine macrophages (23) and in human monocyte-derived macrophages (24), to modify LDL to a form taken up rapidly by macrophages (25), and also to affect the uptake of lipoproteins via the scavenger receptor (26, 27). Recent study in our laboratory revealed that preincubation of macrophages with PCM resulted in up to 40% inhibition in cellular uptake of native LDL (Fuhrman, B., J. G. Brook, and M. Aviram, unpublished data).

Since Ox-LDL may exist in vivo and can lead to cholesterol accumulation in macrophages, the present study was undertaken to elucidate the effect of platelet secretory products on Ox-LDL uptake by macrophages.

METHODS

Materials

Tissue culture media, penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from Biological Industries, Beth Haemek, Israel. Sodium ^{125}I-iodide (carrier-free in 0.1 M NaOH) and sodium [^{3}H]oleate were purchased from Amersham (Amersham International, Bucks., England). Bovine serum albumin (BSA), trypsin, trypsin inhibitor (type I-S from soybean), and adenosine -5'-diphosphate (ADP) were purchased from Sigma Chemical Co. (St. Louis, MO). Thrombin was purchased from Parke-Davis (Paramus, NY). Collagen was purchased from Hormon Chemie (Munich, Germany). A monoclonal antibody that reacts with the human LDL receptor, IgG C-7, was derived from the supernatant of a hybridoma purchased from American Tissue Culture Collection (ATCC) (Rockville, MD).

Cells

A J-774 macrophage cell line obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD) was maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and fetal calf serum (FCS) 10%, heat-inactivated at 56°C for 30 min.

Human monocyte-derived macrophages (HMDM) were prepared from human monocytes isolated by density gradient centrifugation from blood derived from fasting normolipidemic subjects (28). Twenty ml of blood drawn into heparinized syringes (5 units/ml) was layered over 15 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) in Leucosep tubes (Assaf Pharmaceutical Industries, Israel) and centrifuged at 500 *g* for 30 min at 23°C. The mixed mononuclear cell band was removed by aspiration and the cells were washed twice in RPMI-1640 culture medium containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated at 3×10^5 monocytes per 16-mm dish (Primaria brand, Falcon labware, Becton Dickinson, Oxnard, CA). Non-adherent cells were removed by three washes with serum-free medium supplemented with 20% autologous serum. Monocyte-derived macrophages were used within 8–10 days of plating.

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal fluid of Balb/c mice, 4 days after intraperitoneal injection of 3 ml of 4% thioglycolate in saline (29). Cells were pooled in phosphate-buffered saline (PBS), washed once with DMEM, and plated at 5×10^5 cells per 16-mm dish with DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 15% horse serum (heat-inactivated at 56°C for 30 min). Non-adherent cells were removed after 2-h of incubation (37°C, 5% CO_2) by washing with DMEM. Adherent MPM were then incubated under similar conditions.

Lipoproteins

Human LDL was separated from venous blood drawn into 1 mg/ml disodium EDTA, by discontinuous density gradient ultracentrifugation (d 1.019–1.063 g/ml) (30). The separated LDL was extensively dialyzed against HEPES buffer (5 mM HEPES, 137 mM sodium chloride, 2.7 mM potassium chloride, 1.2 mM magnesium chloride, 12 mM sodium bicarbonate, 0.4 mM sodium biphosphate, and 0.6 mM glucose, pH 7.4) in the presence of 0.3 mM EDTA. LDL was radioiodinated by the iodine monochloride method as modified for lipoproteins (31).

LDL was oxidatively modified in a cell-free system (8, 32). Briefly, LDL was diluted to 300 μg protein /ml with nutrient mixture F-10, dialyzed against PBS, and incubated with 5 μM $CuSO_4$ for 24 h at 37°C. Oxidation was terminated by refrigeration and addition of 0.1 mM EDTA. Oxidative modification was confirmed by measurement of the lipid peroxide content of Ox-LDL by a modification of the thiobarbituric acid-reacting substance (TBARS) assay (33). LDL oxidation was expressed as MDA equivalents and values of 0.9 ± 0.5 and 35 ± 7 nmol MDA/mg protein were obtained for LDL and Ox-LDL, respectively.

Platelet conditioned medium (PCM) preparation

Platelets were isolated from platelet-rich plasma (PRP) of healthy donors by centrifugation at 1000 *g* for 10 min. The platelet pellet was washed once with HEPES buffer, centrifuged at 1000 *g* for 10 min, and resuspended at a concentration of 10^9 platelets/ml in DMEM. Platelet conditioned medium was obtained by activating the platelet suspension with thrombin (0.5 U/ml) for 5 min while stirring, followed by centrifugation at 1000 *g* for 15 min at 4°C, and supernatant recovery. PCM was also prepared for one set of experiments by high-speed centrifugation (100,000 *g* for 60 min at 4°C). PCM cholesterol content was measured by a modification of the ferric chloride method (34). PCM was subjected to gel filtration on Sephadex G-25 mini-column (10 × 2 cm) using HEPES

buffer as the eluant and 1-ml fractions were collected.

PCM was delipidated with chloroform–methanol 2:1 (v/v) in a volume ratio of 1:10. The lipid phase was dried under nitrogen and redissolved in ethanol. The non-lipid fraction was lyophilized and reconstituted to the initial volume with DMEM.

[^{14}C]Serotonin release by platelets was measured as described elsewhere (35). Briefly, PRP was incubated for 30 min with 0.2 μCi/ml [^{14}C]serotonin (50.7 mCi/mmol, New England Nuclear, Boston, MA, NEC-225). Excess free [^{14}C]serotonin was removed by centrifugation; the platelet pellet was resuspended in HEPES buffer, placed in a siliconized cuvette in the aggregometer and activated with 0.5 U/ml thrombin for 5 min. The suspension was then centrifuged and the supernatant was counted in vials containing 15 ml scintillation fluid. A control sample with no aggregating agent was included.

Experimental procedure

Cells were plated in 16-mm multiwell dishes in DMEM supplemented with 10% FCS. When 90% confluency was reached, the medium was removed and cells were incubated in fresh medium containing 0.2% BSA (control) or PCM (15 μg protein/ml) for 2 h at 37°C. The cells were further incubated with Ox-LDL at 37°C under experimental conditions specified for each experiment.

Cellular metabolism of Ox-LDL was determined by the following methods. Ox-LDL degradation was measured after 5 h incubation of the cells with ^{125}I-labeled Ox-LDL (sp act 80 cpm/ng protein) at 37°C. Ox-LDL cellular degradation was measured in the collected medium as the trichloroacetic acid (TCA)-soluble non-lipid radioactivity that was not due to free iodide (36). Lipoprotein degradation in a cell-free system, which was measured under identical conditions, was minimal (less than 10%) and was subtracted from total degradation. The remaining cells were washed 3 times with cold PBS and dissolved in 0.1 N NaOH for protein determination and for cellular associated ^{125}I-labeled Ox-LDL.

Cellular binding of ^{125}I-labeled Ox-LDL was determined after incubation of the cells with increasing concentrations of ^{125}I-labeled Ox-LDL in the presence or absence of 20-fold unlabeled Ox-LDL at 4°C for 4 h. The cells were washed with cold PBS on ice, dissolved in 0.1 N NaOH from which a sample was taken to measure radioactivity. Nonspecific binding was subtracted from total binding, giving the specific binding. Cellular cholesterol esterification rate was analyzed after incubation of cells in the presence of Ox-LDL at the indicated concentrations for 18 h at 37°C. For the last 2 h of incubation, [^{3}H]oleic acid complexed with albumin (2.7 mM, 83 nmol oleate/mg BSA, 10 μCi/ml) was added to the medium, after which cells were washed 3 times with cold PBS. Lipids were extracted in situ by hexane–isopropanol 3:2 (v/v) at room temperature (37). The lipid extract was dried under nitrogen and lipids dissolved in chloroform–methanol 2:1 (v/v) were separated by thin-layer chromatography (TLC) on silica gel plates developed in hexane–diethyl ether–acetic acid 130:40:1.5 (v/v/v). Cholesteryl ester spots were visualized by iodine vapor, scraped into scintillation vials, and counted in a beta scintillation counter. Cellular cholesterol esterification rate was estimated as incorporation of [^{3}H]oleic acid into cholesteryl [^{3}H]oleate (38). Cellular cholesterol mass was determined after incubation of the cells with Ox-LDL for 18 h at 37°C. Cells were washed (× 3) with cold PBS and cellular lipids were extracted using 1 ml (× 3) hexane–isopropanol 3:1 (37). Unesterified and esterified cholesterol were separated by TLC as described above, scraped, and cholesterol mass was measured by the ferric chloride method (34).

Other assays

Protein was measured by the method of Lowry et al. (39) using bovine serum albumin as standard.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the protein moiety of PCM was performed with 3–25% gradient gels under reducing conditions using β-mercaptoethanol, as previously described (40). Protein bands were identified after electrophoresis by staining with 0.1% Coomassie brilliant blue R (Sigma Chemical Co.).

Statistical analysis

Each separate experiment was performed in triplicate. Statistical analyses were performed using paired Student's *t*-test. Results are given as mean ± S.D.

RESULTS

J-774 A.1 macrophage-like cells that were preincubated with platelet conditioned medium (PCM, 15 μg protein/ml) for 2 h at 37°C, followed by its removal, demonstrated enhanced uptake of Ox-LDL as shown by increased cellular degradation of the lipoprotein (34%) and by lipoprotein-mediated increased cellular cholesterol esterification (27%) ($P < 0.01$, n = 10) (**Table 1**).

In another set of experiments, PCM was prepared after ultracentrifugation at 100,000 *g* (PCM 100,000 *g*). This preparation was found to contain amounts of protein and cholesterol similar to the original PCM preparation (PCM 1,000 *g*). PCM 1,000 *g* and PCM 100,000 *g* contributed to cellular cholesterol accumulation (30% and 13% elevation in esterified cholesterol mass, respectively, and 12% and 13% elevation in unesterified cholesterol mass, respectively (**Table 2**). Ox-LDL stimulation of cellular cholesterol mass accumulation was enhanced by pretreatment of the cells with both PCM preparations. This effect was due to an increment in both cellular esterified and

TABLE 1. Effect of platelet conditioned medium (PCM) on Ox-LDL uptake by macrophages as manifested by degradation and cholesterol esterification

	Control	+ PCM	% Stimulation
Degradation (μg/mg cell protein/5 h)	5.20 ± 0.30	7.00 ± 0.45*	34
Cholesterol esterification (nmol/mg cell protein/2 h)	0.55 ± 0.04	0.70 ± 0.05*	27

Macrophages were preincubated for 2 h at 37°C in DMEM in the absence (control) or presence of PCM (15 μg protein/ml) followed by its washout. Then cells were incubated in the presence of ^{125}I-labeled Ox-LDL (10 μg protein/ml) for 5 h at 37°C and lipoprotein degradation was determined. Cholesterol esterification was measured after 18 h of cell incubation with Ox-LDL (25 μg protein/ml). [^{3}H]Oleic acid (0.27 mM, 10 μCi/ml) complexed with albumin was added for the last 2 h of incubation, after which cellular cholesteryl [^{3}H]oleate was measured. In the absence of Ox-LDL, cellular cholesterol esterification rate in control cells was 0.19 ± 0.02 compared to 0.29 ± 0.02 in cells incubated with PCM ($P < 0.01$, n = 7). Values are expressed as means ± SD (n = 7); *$P < 0.01$ (vs. control).

unesterified cholesterol mass by 25% and 41%, respectively (Table 2). Ox-LDL stimulation of cellular cholesterol esterification was increased by 30% (from 0.59 ± 0.05 to 0.77 ± 0.06 nmol/mg cell protein) in cells preincubated in the presence of PCM 100,000 *g*. Upon macrophage incubation with increasing amounts of PCM, followed by its replacement with fresh medium, a dose-dependent effect in Ox-LDL-mediated increased cellular cholesterol esterification was observed (**Fig. 1**). In order to substantiate the potential physiologic relevance of these observations, we performed similar experiments on macrophages derived from peripheral blood monocytes (HMDM, human monocyte-derived macrophages) and on mouse peritoneal macrophages (MPM). As demonstrated in **Table 3**, pretreatment of MPM and HMDM with PCM was followed by 25% and 22% enhanced Ox-LDL degradation, respectively.

The stimulatory effect of PCM on Ox-LDL degradation was observed at all lipoprotein concentrations studied (**Fig. 2**). Analysis of these data by a linearization technique (41) revealed that PCM stimulated macrophage Ox-LDL uptake by increasing the number of Ox-LDL receptors (increment in "Apparent V_{max}" by 25%) (**Fig. 3**). Only a minimal effect on lipoprotein affinity for its receptor was observed (Fig. 3). Since the extrapolation from the cellular degradation data could affect receptor number as well as rate of degradation, rate of internalization, rate of degradation and/or release of degradation products to the media, a direct binding study at 4°C was also performed. **Fig. 4** demonstrates that PCM increased the binding of Ox-LDL to macrophages in a dose-dependent pattern. Scatchard analysis revealed that the PCM-treated cells express more binding sites than non-treated cells.

PCM induction of macrophage Ox-LDL receptor activity was found to be dependent on de novo cellular protein synthesis. Preincubation of the cells with PCM for 2 h at 37°C in the presence of 0.4 μM actinomycin D, an RNA synthesis inhibitor, or 2.5 μM cycloheximide, a protein synthesis inhibitor, prevented the enhanced cellular degradation of Ox-LDL observed in the cells that were preincubated with PCM. In cells preincubated with PCM, 6.35 μg protein of Ox-LDL was degraded per mg cell protein, whereas 4.85 and 4.45 μg protein of Ox-LDL were degraded per mg cell protein in cells incubated in the presence of actinomycin D and cycloheximide, respectively. In control cells incubated without PCM, 4.60 μg protein of Ox-LDL was degraded per mg cell protein.

When preincubation of macrophages with PCM was not followed by medium removal, the subsequent addition of Ox-LDL to the incubation medium (which contained the PCM) for a further 5 h of incubation at 37°C, did not result in enhanced uptake of Ox-LDL (measured as cellular cholesterol esterification rate). On the contrary, macrophage uptake of Ox-LDL was reduced by 40% in comparison to cells incubated without PCM (**Fig. 5**). Further studies revealed that the extent of PCM-mediated

TABLE 2. Effect of PCM on Ox-LDL-mediated increased cholesterol accumulation in J-774 macrophages

	Cellular Cholesterol	
Macrophages	Esterified Cholesterol	Unesterified Cholesterol
	μg/mg cell protein	
Without Ox-LDL		
Control	13.0 ± 0.5	16.7 ± 0.7
PCM (1,000 *g*)	17.0 ± 1.5	18.4 ± 0.8
PCM (100,000 *g*)	15.0 ± 0.7	19.0 ± 0.8
With Ox-LDL		
Control	39 ± 6	45 ± 5
PCM (1,000 *g*)	51 ± 6*	56 ± 4*
PCM (100,000 *g*)	61 ± 5*	54 ± 5*

J-774 cells were preincubated for 2 h without (control) or with PCM 1,000 *g* or PCM 100,000 *g* (15 μg protein/ml) followed by removal of medium. Then cells were further incubated in fresh medium without or with Ox-LDL (100 μg protein/ml) for 18 h at 37°C. Cellular cholesterol content was then determined as described in Methods. Data are means ± SD from two experiments performed in triplicate; *$P < 0.01$ (vs. control).

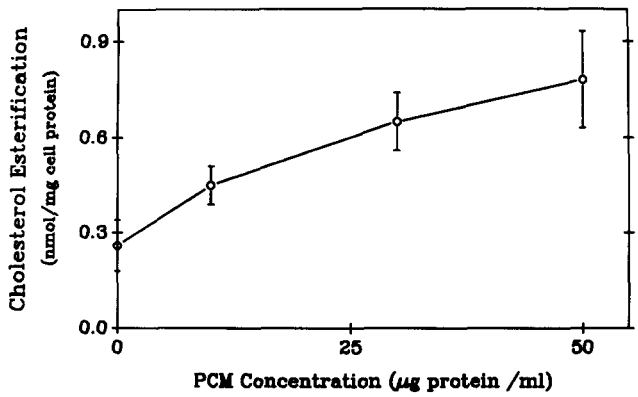

Fig. 1. Dose effect of platelet conditioned medium (PCM) on Ox-LDL-mediated increased cholesterol esterification in macrophages. J-774 A.1 macrophages were preincubated for 2 h at 37°C with increasing concentrations of PCM, followed by its washout and a further cell incubation in the presence of Ox-LDL (25 μg protein/ml) for 18 h at 37°C after which cellular content of cholesteryl [^{3}H]oleate was determined as described in Methods. Data are means ± SD of three separate experiments performed in triplicate.

reduction in Ox-LDL degradation was positively related [r^2 = 0.92 as calculated from second order regression of the PCM curve (42)] to PCM concentration (**Fig. 6**). A 36% reduction in cellular degradation of Ox-LDL was achieved by 50 μg of PCM protein/ml, whereas 100 μg of PCM protein/ml reduced Ox-LDL degradation by 48%, thus demonstrating a typical competitive pattern. In a control study using unlabeled Ox-LDL instead of PCM, up to 67% reduction in Ox-LDL degradation was achieved. Unlabeled LDL, however, only minimally (25%) competed with ^{125}I-labeled Ox-LDL for cellular degradation (Fig. 6). These results suggest that the effect of PCM on Ox-LDL uptake by macrophages is mediated via the scavenger receptor. Under these circumstances we tested the possibility that PCM reacts with the scavenger receptor. The ability of PCM to stimulate cholesterol esterification in macrophages was abolished in the presence of fucoidin or polyinosinic acid, two negatively charged compounds known to compete for binding to the scavenger receptor. Addition of the monoclonal antibody towards LDL receptor, IgGC-7, however, did not affect PCM stimulation of cellular cholesterol esterification (**Table 4**). In order to study whether expression of PCM activity required its internalization into the macrophages, we performed incubation studies at both 4°C and 37°C. When preincubation of macrophages with PCM was performed at 37°C, Ox-LDL-mediated cholesterol esterification was enhanced by 27%, whereas at 4°C, when PCM internalization is inhibited, a 64% reduction was observed (**Table 5**). If, however, subsequent to the 2-h incubation of the cells with PCM at 4°C, the medium was changed and cells were left for a further 2 h at 37°C prior to the addition of Ox-LDL (thus enabling the PCM active factor(s) that were bound to the cell surface receptors to internalize), a 31% elevation in cellular Ox-LDL-mediated cholesterol esterification occurred (from 0.64 in control cells to 0.86 nmol/mg cell protein in PCM-treated cells). Study of the active component in PCM that was responsible for stimulation of Ox-LDL uptake by macrophages revealed that it was secreted only from activated platelets. Platelet activation in response to 0.5 U/ml thrombin resulted in [^{14}C]serotonin release of 82% ± 5 (n = 5). PCM obtained from thrombin (0.5 U/ml)- or collagen (1 μg/ml)-stimulated platelets enhanced Ox-LDL degradation by macrophages (22%) in comparison to PCM obtained from nonactivated platelets (PCM-control). When thrombin was mixed with hirudin (1 U/ml) prior to its addition to the platelets, or when platelets were pretreated with aspirin (200 μM) prior to collagen addition, no significant activity of PCM was observed (**Fig. 7**). Thus, the

TABLE 3. Effect of PCM on Ox-LDL degradation in MPM and HMDM

	^{125}I-Labeled Ox-LDL Degradation	
	MPM	HMDM
	μg/mg cell protein	
Control	0.88 ± 0.08	0.57 ± 0.05
PCM	1.18 ± 0.12*	0.70 ± 0.06*
Stimulation (%)	24	22

MPM and HMDM were incubated for 2 h at 37°C with PCM (15 μg protein/ml). Then the cell monolayers were washed and incubated in the presence of ^{125}I-labeled Ox-LDL (10 μg protein/ml) for 5 h at 37°C, at the end of which lipoprotein degradation was determined in the collected medium. Data are expressed as means ± SD of three separate experiments; *$P < 0.01$ (vs. control).

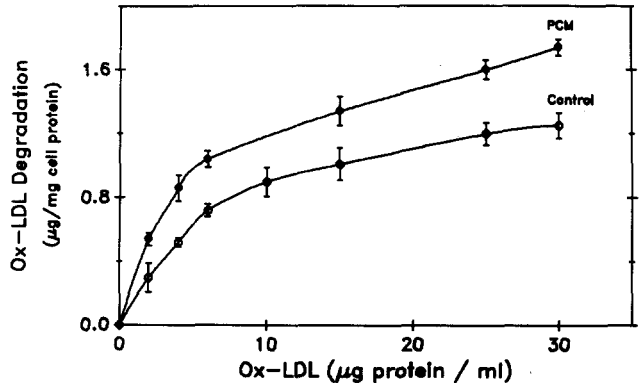

Fig. 2. Effect of platelet conditioned medium (PCM) on Ox-LDL degradation by macrophages: lipoprotein concentration study. Cells were preincubated for 2 h without (control) or with PCM (15 μg protein/ml) then washed (× 3) with serum-free DMEM followed by cell incubation with increasing concentrations of ^{125}I-labeled OX-LDL for 5 h at 37°C. Lipoprotein degradation was measured as described in Methods. Results represent means ± SD of three experiments performed in triplicate. For each lipoprotein concentration tested, a statistically significant difference ($P < 0.01$) was found between the two curves.

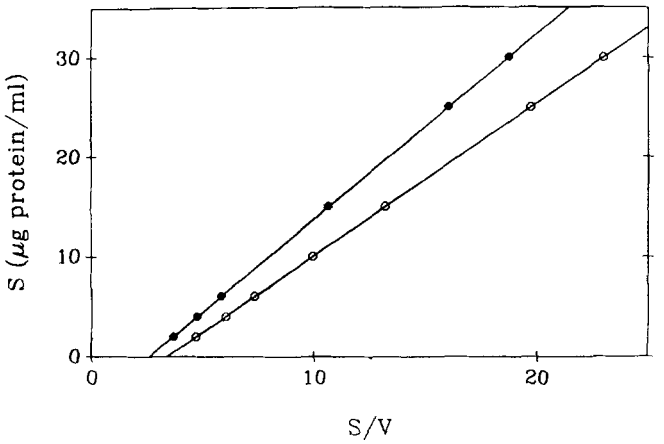

Fig. 3. Linearization plot of the data in Fig. 2. The y axis represents lipoprotein concentration (S), and the x axis represents S/v, where v is the lipoprotein degradation rate. The "apparent V_{max}" is the slope of the line and the "apparent K_m" is the point of intersection with the y axis.

presence of an active component in PCM was associated with platelet degranulation.

Chromatography of PCM on a Sephadex G-25 mini-column (10 × 2 cm) resulted in elution of the active PCM component in the void volume of the column. This fraction was found to contain 95 ± 15 and 15 ± 3 μg of protein and cholesterol per 10^9 platelets, respectively. After SDS-PAGE of the protein moiety of the delipidated PCM, 17 bands of different proteins were visualized, with molecular weights between 25,000 and 140,000 (**Fig. 8**).

In order to study whether the active component of PCM required both its protein and lipid fractions, we performed incubation studies with delipidated fractions of PCM. Preincubation of macrophages with the PCM protein fraction resulted in enhanced Ox-LDL-mediated cholesterol esterification (by 60%) similar to the effect of whole PCM (71%), whereas no effect was observed in cells preincubated with the lipid fraction of PCM (**Fig. 9**).

Further characterization revealed that the active component of PCM was heat-labile as well as trypsin-sensitive (**Fig. 10**) Preincubation of macrophages with PCM that was heated at 100°C for 10 min, or with PCM that was pretreated with trypsin (0.2 mg/ml) for 15 min at 37°C, unlike untreated PCM, did not demonstrate enhanced Ox-LDL-mediated cellular cholesterol esterification (Fig. 10). These results suggest that the active component in PCM possesses the characteristics of a protein.

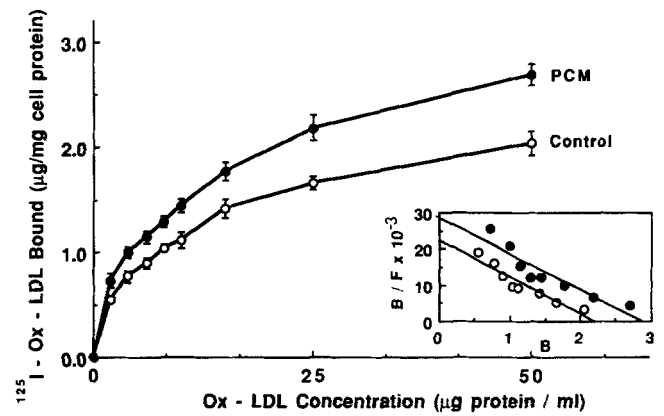

Fig. 4. Binding of Ox-LDL to macrophages: effect of PCM. Cells were preincubated for 2 h at 37°C in the absence (control) or presence of PCM (15 μg protein/ml). Then the cells were cooled to 4°C, the medium was washed, and cells were further incubated at 4°C for 4 h with increasing concentrations of ^{125}I-labeled Ox-LDL in the presence or absence of 20-fold excess of unlabeled Ox-LDL. At the end of this incubation the medium was collected and the unbound lipoprotein was counted. The cultures were washed with cold PBS (on ice) and dissolved in 0.1 N NaOH from which samples were taken for assay of radioactivity and protein determination. The specific binding data represent the average of three experiments ± SD. The inset shows the Scatchard plot of the mean of the averaged data.

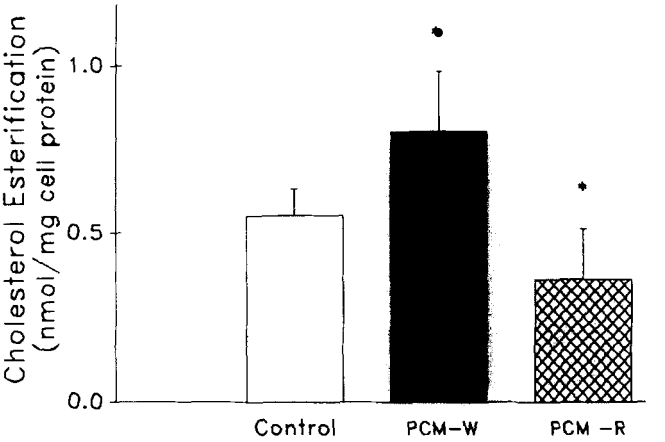

Fig. 5. Effect of the presence of PCM in the incubation medium on Ox-LDL-mediated increased cholesterol esterification in macrophages. Macrophages were preincubated with PCM (15 μg protein/ml) for 2 h at 37°C followed by its washout (PCM-W) or retention (PCM-R). Then cells were incubated with Ox-LDL (25 μg protein/ml) for 18 h at 37°C. Cellular cholesterol esterification was determined as incorporation of [^{3}H]oleic acid into cholesteryl [^{3}H]oleate. Values are expressed as means ± SD (n = 4); *P < 0.01 (vs. control).

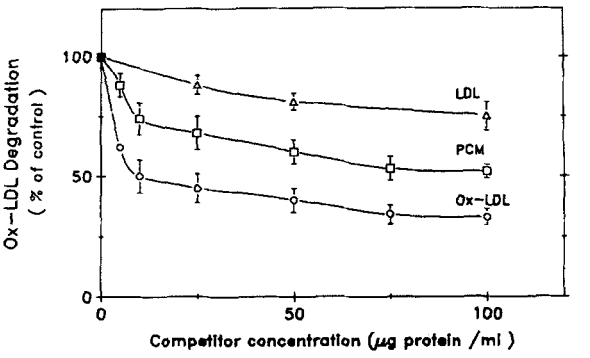

Fig. 6. PCM competitive inhibition of Ox-LDL uptake by macrophages. Cells were incubated with ^{125}I-labeled Ox-LDL (10 μg/ml) in the presence of increasing concentrations of PCM, Ox-LDL, and LDL, for 5 h at 37°C. Then ^{125}I-labeled Ox-LDL degradation was measured in the collected media. Results are means ± SD (triplicate determinations).

TABLE 4. PCM interaction with the macrophage scavenger receptor

Addition to Medium	Cholesterol Esterification			
	Control	Polyinosinic Acid (20 μg/ml)	Fucoidin (10 μg/ml)	IgGC-7 (10%, v/v)
	nmol/mg cell protein			
No addition	0.20 ± 0.02	0.18 ± 0.03	0.21 ± 0.02	0.20 ± 0.02
PCM	0.30 ± 0.02*	0.16 ± 0.02	0.18 ± 0.02	0.29 ± 0.03*
Acetyl-LDL	1.70 ± 0.15*	0.17 ± 0.04	0.19 ± 0.05	1.50 ± 0.17*
LDL	0.45 ± 0.05*	0.43 ± 0.04*	0.45 ± 0.05*	0.15 ± 0.02

Cells were incubated with no additions, PCM (15 μg protein/ml), acetyl-LDL (25 μg protein/ml), or LDL (25 μg protein/ml) in the absence (control) or presence of polyinosinic acid, fucoidin, or IgGC-7. Cellular cholesterol esterification rate was determined as described in Methods. Results represent means ± SD (n = 4); $^*P < 0.01$ (vs. control).

Platelet alpha granules possess specific proteins with different functions. We prepared PCM from a patient with defined "Gray Platelet Syndrome" that was characterized by platelet alpha granule deficiency (43). The patient's PCM was tenfold poorer than normal PCM in its protein content (20 μg/ml compared to 205 μg/ml, respectively) but contained similar amounts of cholesterol (10 μg/ml). As demonstrated in **Fig. 11**, PCM from the patient's platelets (P-PCM), unlike normal PCM (N-PCM), lacked the ability to stimulate Ox-LDL mediated cellular cholesterol esterification.

DISCUSSION

The early atherosclerotic lesion is characterized by foam cells derived mostly from macrophages loaded with cholesteryl ester (1, 44). Platelets that are intimately involved in the atherosclerotic process (17, 45–47) can possibly affect macrophage cholesterol accumulation.

The present study demonstrates that activated platelets secrete a protein-like factor which, subsequent to its uptake via the macrophage scavenger receptor, can modulate Ox-LDL receptor activity. Exposure of macrophages to this factor for a limited duration results in enhanced cellular uptake and degradation of Ox-LDL. This stimulatory effect was demonstrated in three different types of macrophages: J-774A.1 macrophage-like cell, MPM, and HMDM. Since PCM increased the number of Ox-LDL binding sites on macrophages, the scavenger receptor may be subject to regulation, as was also recently demonstrated for acetyl-LDL receptor up-regulation by platelet secretory products in rabbit fibroblasts and smooth muscle cells (48). Our results show that the active factor in PCM is secreted only from activated platelets

TABLE 5. Effect of incubation temperature on PCM ability to stimulate Ox-LDL-mediated cellular cholesterol esterification

	Cholesterol Esterification	
	37°C	4°C
	nmol/mg cell protein	
Control	0.60 ± 0.02	0.64 ± 0.02
+ PCM	0.76 ± 0.03*	0.23 ± 0.02*

Cells were preincubated without (control) or with PCM (15 μg protein/ml at 37°C or at 4°C for 2 h. The cells were washed (× 2) with serum-free medium and further incubated in the presence of Ox-LDL (25 μg protein/ml) for 18 h at 37°C, after which cellular cholesterol esterification was determined as described in Methods. Values are means ± SD, n = 3; $^*P < 0.01$ (PCM vs. control).

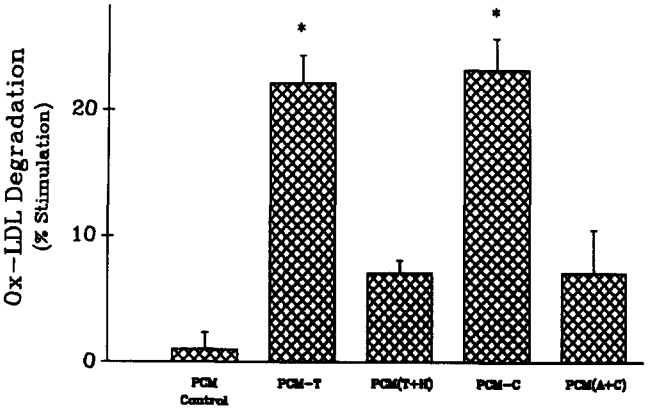

Fig. 7. Effect of platelet activation on ability of PCM to stimulate Ox-LDL degradation by macrophages. Cells were preincubated for 2 h with the indicated PCM preparation (15 μg protein/ml) followed by its washout and a further incubation with ^{125}I-labeled Ox-LDL (10 μg protein/ml) for 5 h at 37°C. Lipoprotein degradation was determined in the collected media. Conditions for PCM preparation: PCM control, non-activated platelets; PCM-T, platelets activated with 0.5 U/ml thrombin; PCM (T + H), thrombin (0.5 U/ml) was mixed with hirudin (1 U/ml) before addition to platelets; PCM-C, platelets were activated with 1 μg/ml collagen; PCM (A + C), platelets were incubated for 15 min with 200 μM aspirin prior to collagen (1 μg/ml) addition. Results represent means ± SD (n = 3); $^*P < 0.01$ (vs. control).

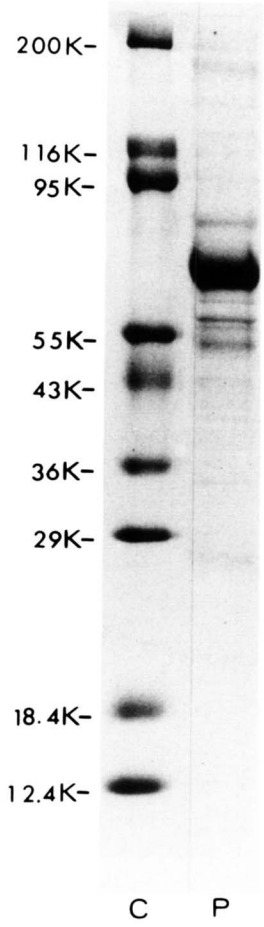

Fig. 8. Polyacrylamide gel electrophoresis of protein moiety of PCM. Electrophoresis was performed in 3%–25% SDS polyacrylamide gels under reducing conditions using β-mercaptoethanol. Lane C (control), molecular weight standards; lane P (proteins of PCM).

and possesses characteristics of a protein; it is recovered in the protein fraction of delipidated PCM, is heat- and trypsin-labile, and is recovered in the 100,000 *g* supernate of PCM. It probably originates from the platelet alpha granules since PCM obtained from a patient with platelet alpha deficiency was not active. Recognition and internalization of the protein-like factor in PCM by the macrophage scavenger receptor was necessary for expressing its effect on cellular uptake of Ox-LDL. First, we showed that PCM stimulation of cholesterol accumulation in macrophages is abolished when the negatively charged compounds fucoidin and polyinosinic acid are included in the incubation medium. Second, we observed that when PCM is present in the incubation medium together with Ox-LDL, which was shown to be taken up by the scavenger receptor (9, 49), inhibition of cellular Ox-LDL uptake occurs. When macrophages were incubated with Ox-LDL in the presence of increasing concentrations of PCM, Ox-LDL binding to the cells was inhibited in a competitive manner. These results suggest

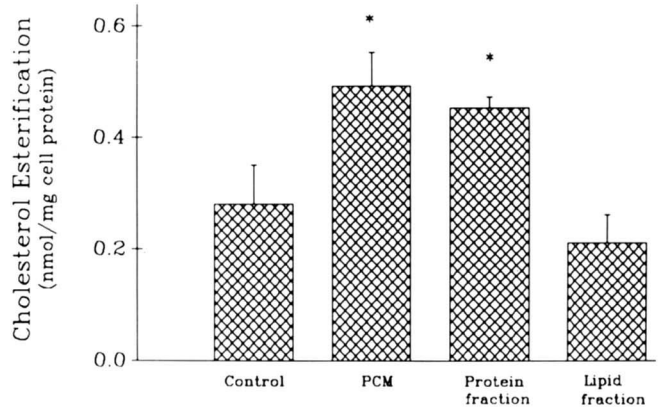

Fig. 9. Effect of the lipid and protein fractions of PCM on Ox-LDL-mediated cholesterol esterification in macrophages. Cells were preincubated for 2 h with the lipid or the protein fraction of PCM. Cells were washed (× 3) with serum-free DMEM and incubated a second time in the presence of Ox-LDL (25 μg protein/ml) for 18 h at 37°C, after which cholesterol esterification rate was determined as described in Methods. Results represent means ± SD (n = 3); $^*P < 0.01$ (vs. control).

that PCM interacts with the macrophage scavenger receptor, which also mediates the uptake of Ox-LDL (50). Lipid vesicles containing acidic phospholipids are also recognized by the scavenger receptors on the surface of macrophages that are also responsible for Ox-LDL cellular uptake (51). Phillips, Arnold, and Innerarity (26) have reported the binding of a platelet secretory product to the scavenger receptor with subsequent inhibition of binding and uptake of aceto-acetyl LDL by the macrophages.

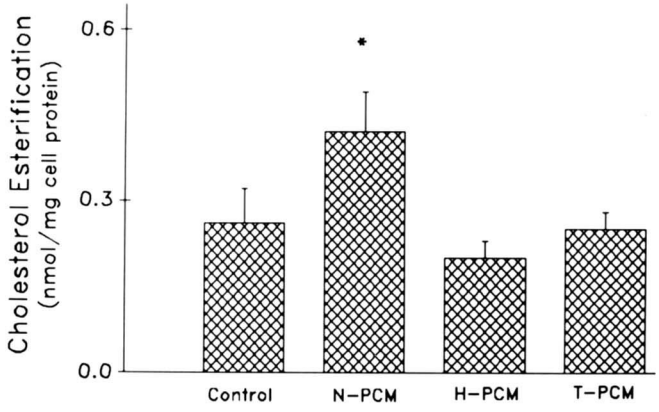

Fig. 10. Effect of heating and trypsin treatment of PCM on its ability to stimulate Ox-LDL-mediated macrophage cholesterol esterification. Cells were preincubated for 2 h at 37°C with PCM (15 μg protein/ml), with PCM that was heated for 10 min at 100°C (H-PCM), or with PCM that had been treated with trypsin (T-PCM). Cells were then washed once with serum-free DMEM and incubated for a further 18 h in the presece of Ox-LDL (25 μg protein/ml). Cholesterol esterification was then determined as described in Methods. Results represent means ± SD (n = 3); $^*P < 0.01$ (PCM vs. control).

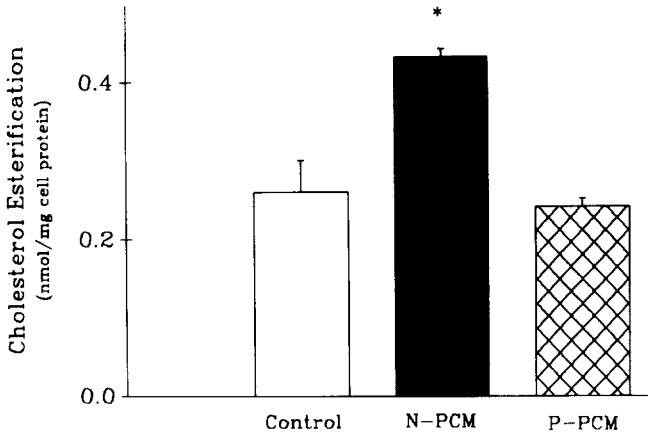

Fig. 11. Effect of PCM derived from a patient with Gray Platelet Syndrome on Ox-LDL-mediated cholesterol esterification. Cells were preincubated for 2 h with 10% PCM (v/v) derived from normal volunteers (N-PCM) or from a patient with platelet alpha granules deficiency (P-PCM). Cells were then washed and incubated for a further 18 h in the presence of Ox-LDL (25 μg protein/ml), at the end of which cellular cholesterol esterification rate was determined. Results represent means ± SD of three experiments performed in triplicate; $^*P < 0.01$ (vs. control).

Binding to the macrophage scavenger receptor is necessary but insufficient for the up-regulation of Ox-LDL receptor activity by PCM, since we showed that the protein-like factor in PCM must be internalized for any effect to be achieved. At 4°C, binding of lipoproteins and other molecules to cell surface receptors occurs without internalization (6). Preincubation of cells with PCM at 4°C, which allows binding but not internalization of the PCM factor, was not followed by enhanced macrophage uptake of Ox-LDL. When cells that were preincubated at 4°C in the presence of PCM were washed, followed by the addition of fresh medium for 2 h at 37°C (thus allowing the protein-like factor that was bound to the scavenger receptor to internalize), the stimulatory effect of PCM on Ox-LDL uptake by macrophages was obtained. Thus, PCM may have a dual effect on Ox-LDL uptake by macrophages. It can compete with Ox-LDL for binding to the scavenger receptor, resulting in reduced Ox-LDL uptake. After cellular uptake of the PCM factor, it increased scavenger receptor synthesis, resulting in enhanced uptake of Ox-LDL. We tend to believe that PCM uptake by macrophages is a rapid process and if PCM binding to macrophages is followed by its rapid uptake, then there is not enough time for the presence of PCM to inhibit cellular binding of Ox-LDL. Thus, the internalized PCM factor may lead, in vivo, to enhanced Ox-LDL receptor activity.

Platelet secretory products have been shown to interact with the scavenger receptor (26, 27). Individual substances known to be secreted from activated platelets have been also shown to affect macrophage receptor activity (52). The purified proteins studied (fibrinogen, fibronectin, and platelet derived growth factor, PDGF) affected the affinity of lipoproteins towards their specific receptors without any significant effect on the number of receptors. This would indicate that the protein-like factor present in PCM, which is responsible for the stimulatory effect on Ox-LDL uptake in our study, is different from these proteins. An increase in the number of lipoprotein receptors also has been obtained after incubation of macrophages with secretory products from cells other than platelets. Secretory products derived from cells of the arterial wall stimulated both acetylated and native LDL degradation subsequent to increase in the number of receptors on human monocyte-derived macrophages (53) and in smooth muscle cells (54). Cytokines released by arterial wall cells were also shown to modulate the acetyl-LDL receptor pathway and to influence the rate of foam cell generation. Interferon gamma was identified as the cytokine responsible for inhibition of the acetyl-LDL receptor pathway at the level of intracellular transport of the internalized lipoprotein (55). Thus, the scavenger receptor may also be subjected to regulation by substances that are probably present at the site of the atherosclerotic lesion.

Further studies are in progress to isolate and further characterize the factor in PCM responsible for Ox-LDL receptor modulation in macrophages.

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