Suppression of interleukin-1β and LDL scavenger receptor expression in macrophages by a selective protein kinase C inhibitor

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Abstract A human monocytic cell line, THP-1, stimulated with 40 nM phorbol myristate acetate (PMA), differentiated to macrophage-like cells, and exhibited increased expression and release of interleukin-1β and expression of acetylated low density lipoprotein (ac-LDL) receptors. A selective inhibitor, MDL 29,152 (4-propyl-5-(4-quinolinyl)-2(3H)-oxazolone) was used to show that this induction required activation of protein kinase C. MDL 29,152 acts in the catalytic domain of protein kinase C and is at least 200-fold selective for protein kinase C over cAMP-dependent protein kinase in THP-1 cells. MDL 29,152 (50 μM) reduced levels of interleukin-1β mRNA in PMA-stimulated cells by 76% and eliminated detectable interleukin-1β in the media. Flow cytometric analysis showed that 48 h after THP-1 activation, approximately 50% of the cells expressed ac-LDL receptors, while in the presence of 100 μM MDL 29,152, less than 5% of the cells expressed receptors. The relationship between THP-1 differentiation and protein kinase C activation was determined by following the expression of the cell surface antigen MO-1. Expression of MO-1 antigen increases as monocytes differentiate to macrophages. After 48 h of phorbol activation, 90% of the THP-1 population was MO-1-positive; less than 16% of the population was MO-1-positive when 100 μM MDL 29,152 was present. By dual analysis, it was found that within the differentiated, MO-1-positive population, only approximately 50% of the cells also expressed ac-LDL receptors. Based on these findings, we conclude that protein kinase C promotes processes important in THP-1 activation and differentiation to macrophage-like cells including interleukin-1β expression and secretion, ac-LDL receptor and MO-1 expression.—**Akeson, A. L., K. Schroeder, C. Woods, C. J. Schmidt, and W. D. Jones.** Suppression of interleukin-1β and LDL scavenger receptor expression in macrophages by a selective protein kinase C inhibitor. *J. Lipid Res.* 1991. **32:** 1699–1707.

Supplementary key words phorbol ester • MO-1 antigen • THP-1 cells

The Ca^{2+}/phospholipid protein kinase, protein kinase C, is involved in a variety of cellular responses related to differentiation and proliferation (1, 2). Protein kinase C-dependent pathways regulate cellular responses in concert with other pathways including those regulated by cAMP-dependent protein kinase and tyrosine protein kinase. Analysis of the role of protein kinase C in cellular activation and differentiation has been hampered by the lack of selective noncytotoxic inhibitors. We have developed an inhibitor MDL 29,152 (4-propyl-5-(4-quinolinyl)-2(3H)-oxazolone) that we believe will be useful in analysis of activation pathways.

Short-term incubation of cells with specific activators or inhibitors is frequently used to study the physiological role of protein kinase C. Some phorbol esters, including phorbol myristate acetate (PMA), are potent agonists of protein kinase C and are thought to substitute for the endogenous activator, diacylglycerol, in the direct activation of this signal pathway (1). However, phorbol esters also affect other cellular processes (3, 4). Therefore, use of selective inhibitors is important for confirmation of the role of protein kinase C in any phorbol-induced response. In general, protein kinase C inhibitors can be classified into two groups depending on whether they react with the catalytic or regulatory domains of the protein. Inhibitors targeted toward the regulatory domain including sphingolipids (5) and calphostin (6) have a high degree of specificity for protein kinase C. However, the effective use of this class of inhibitors in measurement of cellular responses has been limited by cytotoxicity and the ability to deliver the inhibitors to the cells (7). In contrast, inhibitors interacting with the catalytic domain, including staurosporine and H-7 (1-(5-isoquinolinylsulfonyl)-2 methylpiperazine), are not selective for protein kinase C. Staurosporine, the most potent kinase inhibitor, has IC50 (concentration of

Abbreviations: IL-1β, interleukin-1β; PMA, phorbol myristate acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; ac-LDL, acetylated low density lipoprotein; MDL 29,152, 4-propyl-5(4-quinolinyl)-2(3H)-oxazolone; FITC, fluorscein isothiocyanate; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

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half maximal inhibition) values of 2.7 nM for protein kinase C, 8.2 nM for cAMP-dependent protein kinase, and 6.4 nM for tyrosine protein kinase (8). The inhibitory effects of staurosporine and its analogs correlated with their cytotoxicities (7). H-7 (9) also has no selectivity for protein kinase C with an IC50 of 15 μM for protein kinase C and 13 μM for cAMP-dependent protein kinase. Although less potent, H-7 has the advantage of low cytotoxicity. In this report we demonstrate the selectivity of MDL 29,152 for protein kinase C and characterize the site of inhibition in the catalytic subunit of the protein. The biological effects of MDL 29,152 were tested on the phorbol-induced activation and differentiation of the human monocytic cell line THP-1.

The differentiation of THP-1 cells resembles the conversion of human monocytes to macrophages in several ways such as morphological changes, expression of membrane antigens and receptors, production of secretory products, and expression of genes involved in lipid metabolism (10–12). We believe these traits make THP-1 cells a good model for the role of monocyte/macrophages in atherosclerotic plaque formation. Of particular interest to us is characterization of the intracellular signal transduction pathways involved in induction of these traits. In this study the selective kinase inhibitor, MDL 29,152, was used to analyze the role of the protein kinase C in phorbol-induced THP-1 differentiation, IL-1β secretion, and scavenger LDL receptor expression.

METHODS AND MATERIALS

Cell culture

THP-1 (TIB202) cells were obtained from the American Type Culture Collection and were maintained at 1–2 $\times$ 10^5 cells/ml in RPMI1640 (Whitaker) containing 50 μM 2-mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS) (HYclone), 2 mM supplemental glutamine, and antibiotics plus fungizone (Gibco/BRL). PMA (phorbol 12-myristate 13-acetate) (Sigma) was prepared as a 2 mM solution in ethanol and diluted 1:100 in RPMI and stored as single-use aliquots at −80°C. Kinase inhibitors, H-7 (1-(5-isoquinolinylsulfonyl)-2 methylpiperazine) (Sigma) and MDL 29,152 (4-propyl-5-(4-quinolinyl)-2(3H)-oxazolone) (Marion Merrell Dow, available on request) were dissolved in dimethylsulfoxide at a concentration of 50 mM and used in cultures at 0.5% or less dimethylsulfoxide. Control cultures received equivalent dimethylsulfoxide. Cells (2 $\times$ 10^5 cell/ml) were transferred to RPMI-2% FCS or -10% lipoprotein-deficient serum (13) and cultured overnight (37°C, 95% air–5% CO_2). Cells were plated at 1–2 $\times$ 10^6 cells/ml in 24-well plates (Falcon), the inhibitors were added, and cells were incubated for 2 h before 40 nM PMA was added.

Cell viability was determined by trypan blue exclusion. The viabilities of unstimulated cells were 98 ± 0.5% for control cells, and for cells treated with 100 μM H-7 or MDL 29,152, 96 ± 1.0% and 95 ± 2%, respectively. Because PMA-treated cells no longer proliferate, there is a reduced viability in PMA-treated cultures to 80 ± 5%. With addition of 100 μM H-7 or MDL 29,152, cell viabilities were 72–80% and 74–81%, respectively.

In vitro analysis of protein kinase C and cAMP-dependent protein kinase

Partially purified protein kinase C was prepared from rat brain cytosol as described by Salama (14) and stored in 0.5% Triton and 10% glycerol at −70°C. Enzyme activity was determined at 37°C by the phosphocellulose paper method of Sahal and Fujita-Yamaguchi (15). The final incubation contained 30 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$, 40 μg/ml phosphatidylserine, 4 μg/ml diolein, 3 mg/ml histone III-S (Sigma), and 50 μM [γ-^{32}P]ATP (70 dpm/pmol) (New England Nuclear) in a volume of 80 μl. The reaction was terminated after 5 min by the addition of ice-cold 75 mM H_3PO_4 after which an aliquot of the mixture was spotted onto Whatman P81 paper. After several washes in 75 mM H_3PO_4, the sheets were sectioned and counted by liquid scintillation spectrometry. Levels of protein kinase C activity in whole cell homogenates of THP-1 cells were measured using 50 μM [γ-^{32}P]ATP and the Amersham protein kinase C enzyme assay system which determines phosphorylation of a peptide that is specific for protein kinase C. For activation of protein kinase C by calpain, the kinase was first incubated for 5 min with the protease plus $CaCl_2$ (2.2 mM) at 20°C to allow cleavage of the regulatory and catalytic domains to occur. Prior to the second incubation at 37°C, $MgCl_2$, EGTA, and ATP were added to the assay. Calpain was partially purified from chicken gizzard and was the kind gift from Dr. S. Mehdi (Marion Merrell Dow). cAMP-dependent protein kinase was assayed as above using partially purified catalytic subunit (Sigma). cAMP-dependent protein kinase activity of THP-1 cells was measured using an assay system from Gibco BRL which detects the phosphorylation of the cAMP-dependent protein kinase-specific peptide, Kemptide.

RNA isolation and analysis

Total RNA was isolated from 1 $\times$ 10^7 cells using guanidine thiocyanate and phenol–chloroform extractions by the method of Chomczynski and Sacchi (16). The relative cellular levels of IL-1β were determined by slot blot analysis. Either 2 or 10 μg of total RNA was denatured in 37% formaldehyde in 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0, and 67°C for 15 min and transferred by

vacuum to Nytran (Schleicher and Schuell). An EcoRI/XbaI, 480 base pair portion of the mature IL-1β cDNA (Beckman) or a PstI/XbaI, 516 base pair portion of human skeletal muscle actin (17) were used consecutively as hybridization probes and were radiolabeled by random priming with [^{32}P]dCTP (18). The blots were exposed to X-OMAT film (Kodak) and the relative band intensities were determined by densitometry.

IL-1β quantitation

For each sample, media were collected, residual cells were separated by centrifugation, and media were stored at −80°C until analysis. Secreted IL-1β was measured using an enzyme-linked, immunosorbent assay (Cistron). This assay is specific for human IL-1β and reliably detects as low as 20 pg/ml.

MO-1 antigen expression

To release differentiated cells, the tissue culture plates were transferred to 4°C for 30 min. Both adherent and nonadherent cells were removed with vigorous pipetting and washed 3 times with cold phosphate-buffered saline. Cells (10^5) were labeled for 30 min at 4°C with fluorescein isothiocyanate-labeled anti-MO-1 antibody (Coulter Immunology) or, as a control, fluorescein isothiocyanate-labeled IgM, washed with cold phosphate-buffered saline, and stored at 4°C in 0.05% paraformaldehyde in saline. Labeled cells were analyzed on a Coulter EPICS C flow cytometer equipped with an argon ion laser at 488 nm. A minimum of 10^4 cells per sample was analyzed by flow cytometry.

DiIacLDL uptake

Five hours before collection of cells, DiI-acLDL (acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) (Biomedical Technologies, Inc.) was added to the cultures in RPMI at a final concentration of 5 μg/ml. After 5 h incubation, the cells were released from the plates at 4°C, washed twice with cold phosphate-buffered saline, and stored in 0.05% paraformaldehyde at 4°C until analysis by flow cytometry.

RESULTS

Mechanisms of protein kinase C inhibition by MDL 29,152

We have recently developed a non-cytotoxic kinase inhibitor, MDL 29,152, with a clear selectivity for protein kinase C. Using rat brain, a rich source of protein kinase, we determined the concentration of half maximal inhibition or IC50 for MDL 29,152 and H-7 (**Table 1**). In rat brain, MDL 29,152 was about as potent an inhibitor of protein kinase C as H-7, with IC 50s of 40 μM and 37 μM, respectively. But unlike H-7, MDL 29,152 was 10-fold more selective for protein kinase C than cAMP-dependent protein kinase.

TABLE 1. In vitro analysis of kinase inhibitors

Source	Activity	IC50 H-7	IC50 MDL 29,152
		μM	
Brain	PKC	37	40
Brain	PKA	56	431
THP-1	PKC	22	2
THP-1	PKA	45–75	>400

Protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) activities determined for rat brain preparations or THP-1 cell lysates as described in Methods and Materials.

The effects of these inhibitors were also tested in THP-1 cell lysates by following the phosphorylation of a protein kinase C-specific peptide (Amersham). In this assay, the IC50 for H-7 was 22 μM, similar to the inhibition in rat brain (Table 1). However, at the same ATP concentration, the IC50 for MDL 29,152 was 2 μM, approximately 20-fold more potent than as measured in rat brain. For cAMP-dependent protein kinase in THP-1 cells, the IC50 for H-7 was 45–70 μM and for MDL 29,152 was greater than 400 μM. In THP-1 cell lysates, MDL 29,152 had at least a 200-fold selectivity for protein kinase C over cAMP-dependent protein kinase.

Previous studies have demonstrated that several oxazolones related to MDL 29,152 inhibit protein kinase C activity by competition with ATP (19). To verify that the site of action of MDL 29,152 was also associated with this domain of the enzyme, a catalytically active fragment of protein kinase C was generated by proteolysis using calpain (20, 21). The inset of **Fig. 1** shows that before digestion with calpain, maximal protein kinase C activity was achieved only when Ca^{2+}, phosphatidylserine, and diacylglycerol were present. After calpain treatment, kinase activity of the catalytic domain of protein kinase C became independent of all three regulators and required only the presence of protein substrate and ATP (inset, Fig. 1). As illustrated in Fig. 1, MDL 29,152 produced a concentration-dependent inhibition of the calpain-treated form of the enzyme, consistent with a site of action on the catalytic domain.

Protein kinase C inhibitors reduce PMA-induced IL-1β

PMA activates THP-1 cells inducing expression and optimal secretion of IL-1β by 24 h (22, 23). Secreted IL-1β was measured 24 h after activation of THP-1 cells with 40 nM PMA (**Fig. 2**) with or without MDL 29,152 or H-7. MDL 29,152 was a more potent inhibitor of IL-1β secretion than H-7 with IC50 values of 5 μM and 20 μM, respectively. At 50 μM, MDL 29,152 eliminated IL-1β in

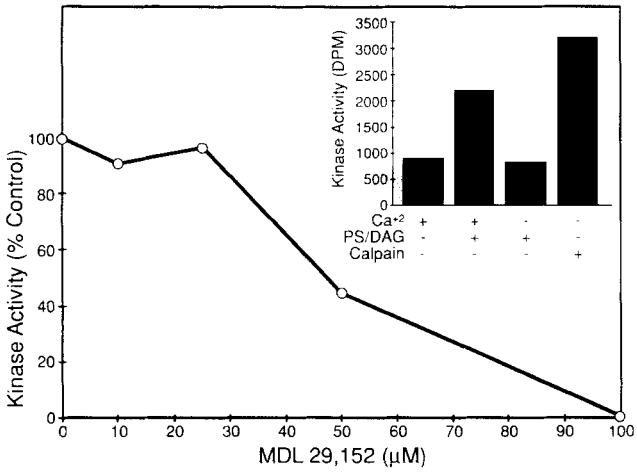

Fig. 1. MDL 29,152 acts at the catalytic domain of protein kinase C. The catalytic domain of protein kinase C was generated by proteolysis with calpain and protein kinase C activity was determined as described in Methods and Materials. To show independence from the regulatory domain, the catalytic fragment was incubated with or without 1.1 mM $CaCl_2$, 40 μg/ml (PS) phosphatidylserine, and 4 μg/ml diolein as diacylglycerol (DAG) activator (inset). Activity for the catalytic domain was then determined in the presence of 10–100 μM MDL 29,152. Kinase activity is shown as the percent of activity determined without inhibitor.

the media, while 50 μM H-7 reduced IL-1β levels by only 70%. Increasing H-7 concentrations to 100 μM did not further reduce IL-1β levels.

We examined the effects of 50 μM MDL 29,152 and H-7 on IL-1β mRNA levels (**Fig. 3**). Either 2 or 10 μg of total RNA was denatured and analyzed. The blots were first hybridized with a cDNA probe for human IL-1β, the probe was stripped, and the blot was hybridized with a cDNA probe for actin. The relative IL-1/actin band intensities showed that after 24 h of PMA stimulation, 50 μM MDL 29,152 and H-7 significantly reduced IL-1β mRNA levels without altering total RNA as indicated by the constant level of actin mRNA. IL-1β mRNA levels were reduced to 53% of PMA-induced levels by H-7 and to 24% by MDL 29,152. Unstimulated THP-1 cells have no detectable IL-1β mRNA (22).

Phorbol esters induce differentiation of THP-1 cells

PMA induces THP-1 cells to differentiate to cells that are macrophage-like. In the differentiated state, the cells stop proliferating and become adherent, granular, and irregular in shape (10). Adherence of monocytes and the THP-1 cell line is regulated, at least in part, by the protein kinase C-dependent expression of the cell surface antigen MO-1 or Mac-1 (24). As a marker for differentiation, the number of cells expressing MO-1 was examined by flow cytometry.

Fig. 4 shows representative histograms of cells for up to 72 h after PMA stimulation. Twenty-four h after culturing, unstimulated labeled cells showed a peak channel at 22 (Fig. 4A) and only 3% of the cells were in channels above 50, the limit set to calculate the percent of MO-1-positive cells in each population. The percent of unstimulated cells expressing MO-1 increased slightly with culture time. At 72 h, 15% of the control cells were MO-1-positive, but the peak channel remained between 22 and 28 (data not shown). At 24 h after activation with PMA, the peak channel had shifted to 62, and 35% of the cells were MO-1-positive. By 48 h, virtually all of the cells (89%) expressed MO-1 (peak channel 110). At 72 h, 95% of the cells were MO-1-positive. These data show that PMA increases both the number of cells positive for MO-1 and the density of MO-1 on each cell.

Protein kinase C inhibitors alter phorbol-induced differentiation of THP-1 cells

The level of MO-1 expression was followed for 72 h after PMA induction in the presence of 100 μM H-7 or MDL 29,152. At 24 h in the presence of H-7, only 10% of the total cells (adherent and nonadherent) were MO-1-positive and the peak channel remained at 23 (Fig. 4B). At 48 h, 58% of the cells were MO-1-positive with a peak channel for this differentiated population of 91. A portion of the cells remained undifferentiated, as shown by the negative peak at approximately channel 28. At 72 h, the number of MO-1-positive cells treated with PMA and H-7 increased to 64%, still 30% fewer MO-1-positive cells than in populations treated with PMA alone (Fig. 4A). This indicated that there was a considerable portion of the population that remained undifferentiated. Concentrations of H-7 above 100 μM did not further affect levels of MO-1 expression, and concentrations above 200 μM reduced cell viability.

The addition of 100 μM MDL 29,152 dramatically reduced PMA-induced MO-1 expression. At 24 h, expression of MO-1 in the presence of MDL 29,152 was less than 5% (Fig. 4B), similar to unstimulated cells. By 48 h, only

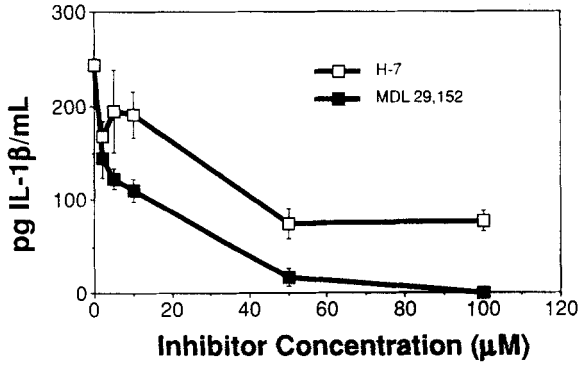

Fig. 2. Protein kinase C inhibitors reduce IL-1β secretion. A. Cells (1×10^6/ml) in RPMI-2% FCS were stimulated with PMA (40 nM) in the presence of increasing concentrations of H-7 (open squares) and MDL 29,152 (closed squares). The release of IL-1β into the media was assayed after 24 h of culture by enzyme-linked, immunosorbent assay as described in Methods.

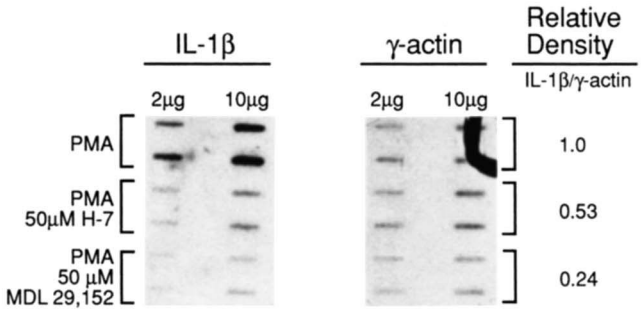

Fig. 3. Protein kinase C inhibitors reduce IL-1β mRNA levels. Cells were cultured for 24 h as in Fig. 2 and RNA was isolated from 1×10^7 cells as indicated in Methods. Total RNA (2 and 10 μg) was transferred to Nytran and the blot was hybridized first with a cDNA probe for human IL-1β and then with a cDNA probe for human γ-actin. The relative band intensities were determined by densitometry and expressed as relative density of IL-1β to actin.

16% of the MDL 29,152-treated cells expressed MO-1 (peak channel 22). Not until 72 h did a significant number of cells (45%) become MO-1-positive. At 72 h, more than half the cells treated with MDL 29,152 remained in channels lower than 50, indicating low MO-1 expression.

Expression of receptors for acetylated LDL are regulated by protein kinase C

PMA-treated THP-1 cells, like macrophages, have increased expression of the scavenger receptor for modified lipoproteins (12, 25). This is identified by specific receptor uptake of acetylated low density lipoprotein (**Fig. 5A**). DiI, a lipophilic fluorescent probe, can be used to label acLDL (26). DiIacLDL is recognized by acLDL receptor or scavenger receptors on cells. DiIacLDL taken up via the acLDL receptor is degraded by lysosomes and the intracellular DiI that accumulates within the lysosomal membranes of the cells (27) can then be quantitated by flow cytometry. The percent of DiI-positive cells can be used as an indirect measurement of the number of cells with receptors for acLDL. Under the culture conditions used, RPMI-10% lipoprotein-deficient serum, less than 4% of untreated cells had taken up DiIacLDL even after 72 h. Maximum uptake of DiIacLDL occurred 48 h after PMA treatment with 52% of the cells positive (Fig. 5A). Via et al. (12) using the same technique reported similar results: 44% of THP-1 cells positive for DiIacLDL uptake 96 h after treatment with PMA. The level of DiIacLDL-positive cells was reduced to 25% with 100 μM H-7 and

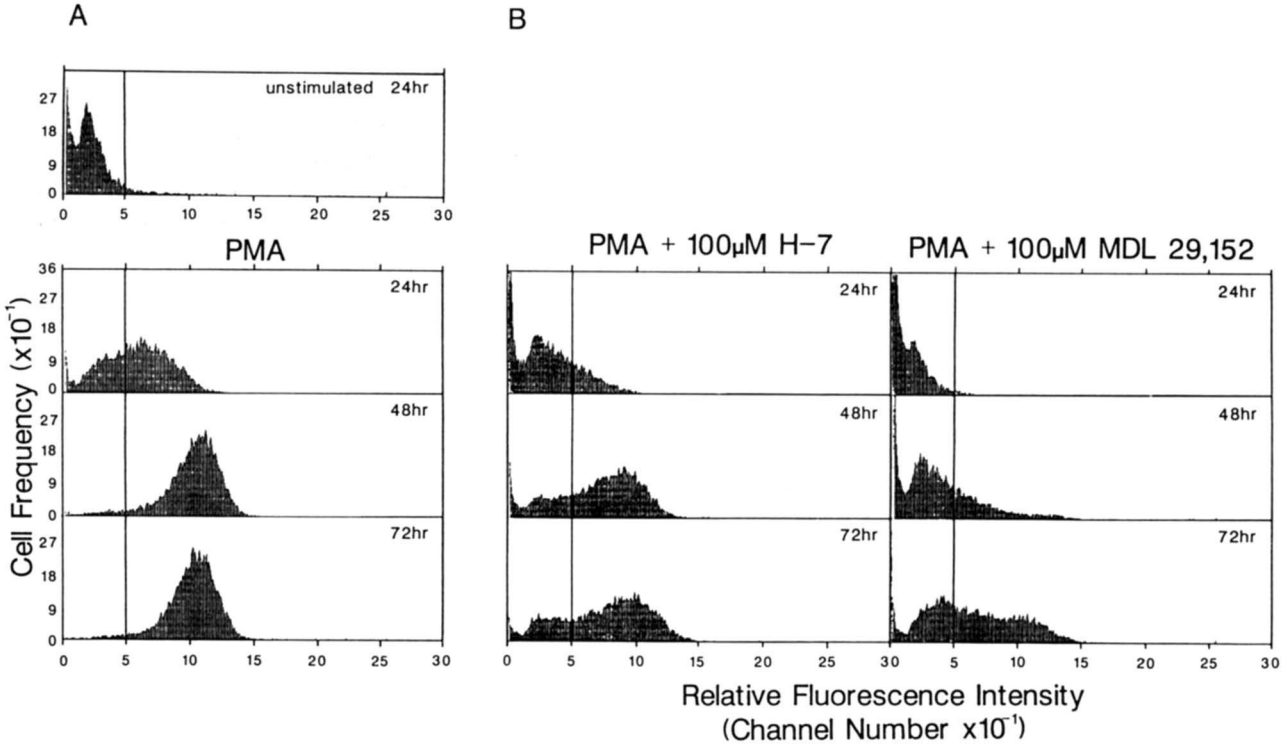

Fig. 4. Protein kinase C inhibitors reduce THP-1 differentiation in response to PMA. Cells were cultured in RPMI-10% lipoprotein-deficient serum with (A) no inhibitor or (B) either 100 μM H-7 or 100 μM MDL 29,152 and collected 24, 48, and 72 h after PMA stimulation. Unstimulated control cells were collected at 24 h. After collection of both adherent and nonadherent populations, cells were stained with anti-MO-1 as indicated in Methods and the fixed cells were analyzed with an EPICS C flow cytometer. The histograms are shown as relative intensity (frequency × 10) versus channel number (×10). The line in each histogram indicates the window used for determination of percent MO-1+ cells.

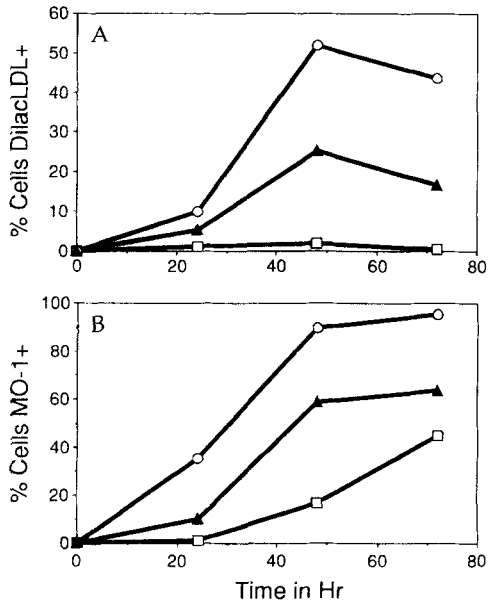

Fig. 5. The effects of the protein kinase C inhibitors on acLDL receptor expression and THP-1 differentiation. Cells and media from the experiment described in Fig. 4 were analyzed for percent cells positive for DiIacLDL uptake (A) or percent cells positive for MO-1 (B) as described in Methods. The cells were cultured with no inhibitor (open circles), 100 μM H-7 (solid triangles), or 100 μM MDL 29,152 (open squares).

to less than 5% with 100 μM MDL 29,152 (Fig. 5A). The uptake was specific for acLDL as 100-fold excess unlabeled acLDL, but not 100-fold excess LDL, reduced the number of cells DiI-positive to less than 10% of the population (data not shown). However, because this is an indirect method for measurement of acLDL receptor activity, it cannot be determined whether the inhibitors are having a direct effect on receptor expression versus effects on receptor internalization or receptor affinity.

In this experiment, 72 h after PMA activation, 95% of the cells were MO-1-positive (Fig. 5B) while only 42% of the cells were DiI-positive (Fig. 5A). This result suggests that under these experimental conditions not all differentiated THP-1 cells express receptors for modified LDLs. This conclusion was confirmed by dual analysis of acLDL receptor and MO-1. In the experiment shown in **Fig. 6,** THP-1 cells were activated with PMA, and 72 h later DiIacLDL was added. After a further 4 h, the cells were collected and simultaneously analyzed for DiIacLDL uptake and MO-1 expression. The majority of the cells were MO-1-positive (75%). Sixty-one percent of the cells were DiI-positive and only 52% of the cells were both DiI- and MO-1-positive. In the differentiated, MO-1-positive population, approximately 30% of the cells did not take up DiIacLDL, indicating that these cells did not express acLDL receptor.

DISCUSSION

To better understand the role of protein kinase C and other kinases in activation and differentiation, we have developed a new selective inhibitor of protein kinase C, MDL 29,152. The selectivity of MDL 29,152 was confirmed by in vitro analysis of protein kinase C and cAMP-dependent protein kinase in both rat brain and THP-1 cell preparations. In both cases, MDL 29,152 was a better inhibitor of protein kinase C than of cAMP-dependent protein kinase.

Like H-7 (9), MDL 29,152 was shown to be competitive at the ATP-binding site in the catalytic domain of protein kinase C. When protein kinase C activity was measured in THP-1 cell lysate, MDL 29,152 was 20-fold more potent than in rat brain. This difference in potency may be due to the reaction conditions used for in vitro analysis in the two systems. In the rat brain assay, protein kinase C was activated by diacylglycerol, the substrate was histone, and the ATP concentration was 50 μM. For analysis in THP-1 cells, protein kinase C was activated with PMA, the substrate was a protein kinase C-specific peptide, and the ATP concentration was also 50 μM. Alternatively, changes in potency may be the result of the specific protein kinase C isotypes active in the two systems.

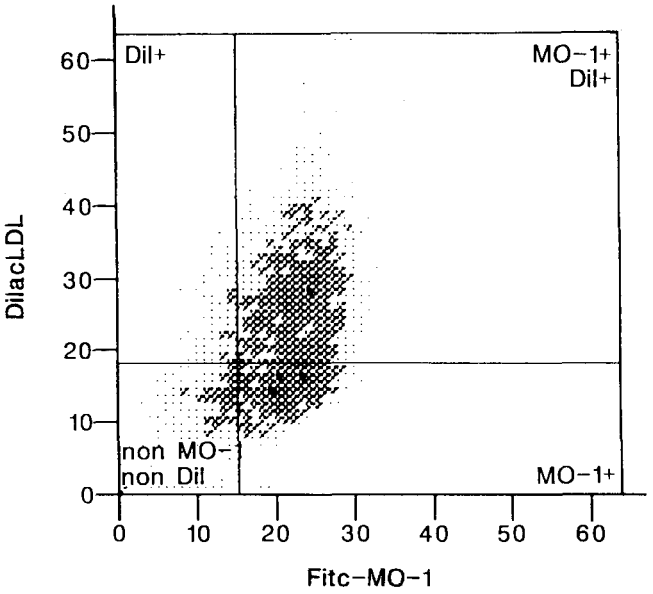

Fig. 6. Expression of acLDL receptor by differentiated THP-1 cells. Cells (1×10^6 cells/ml) in RPMI-10% lipoprotein-deficient serum were activated with PMA (40 nM) and cultured for 72 h. DiIacLDL was added to the cultures and 4 h later all cells were collected, stained with anti-MO-1, and analyzed by flow cytometry. The two-parameter histogram depicts FITC-MO-1-positive cells along the x-axis and DiIacLDL positive cells along the y-axis. The number of positive cells, as a percentage of the total population, are: nonMO-1, nonDiI, 16%; DiI+, 8.5%; MO-1+, DiI+, 52.5%; MO-1+, 23%.

Protein kinase C is a family of enzymes with at least seven isotypes (α, βI, βII, γ, δ, ϵ and ζ) that exhibit distinct tissue specific patterns of expression (1). The brain is a rich source of protein kinase C-γ with isotypes α, βI, βII also present (1). While the protein kinase C isotypes in THP-1 cells are as yet unreported, in another human promyelocytic cell line HL-60, protein kinase C-α and -β are the principle isotype (28). Strulovici et al. (29) have shown that protein kinase C-α plays a potential role in PMA-induced differentiation of another human monocytic cell line, U-937. Each protein kinase C isotype has slightly different modes of activation and kinetic properties (1). For instance, protein kinase C-γ activation by arachidonic acid does not require Ca^{2+}, while protein kinase C-α activation by arachidonic acid does require Ca^{2+}. Therefore, inhibitors that compete for the various protein kinase C substrates and cofactors are also likely to have different reactivity with each isotype. While we have not yet determined the effect of the inhibitor on different protein kinase C isotypes, the use of selective inhibitors like MDL 29,152 may help to determine the relative role of protein kinase C isotypes in biological events.

Of particular interest to us is the role of protein kinase C in the activation and differentiation of monocyte/macrophages. In this study we followed the effects of MDL 29,152 on the phorbol-induced activation and differentiation of the human monocytic cell line THP-1. The level of expression of the surface antigen MO-1 was used as a marker for monocyte differentiation (24). PMA-induced differentiation was only partially suppressed by MDL 29,152. Within the differentiated cell population, the inhibition of protein kinase C also reduced the level of MO-1 expression/cell as indicated by the shifts in peak channel. The failure to completely block phorbol-induced differentiation with the selective protein kinase C inhibitor suggests that in this system THP-1 differentiation is induced by multiple pathways. There has been some work to indicate that phorbol esters can affect other kinases (3) as well as Ca^{2+} channels (4). It may be phorbol-induced activation of these alternate pathways that allows differentiation of a portion of the THP-1 population. Kreutter, Caldwell, and Morin (3), have shown that PMA-induced maturation of the promyelocytic cell line HL-60 can be dissociated from protein kinase C. Together these data suggest that activation of protein kinase C pathways may not always be required for monocyte differentiation. MDL 29,152 is currently being used to investigate protein kinase C pathways in activation and differentiation of peripheral blood monocytes. Also of interest is determination of biologically relevant protein kinase C activators. Recent work indicates that oxidized LDL induces IL-1β secretion (30) and differentiation and adhesion of monocytes (31). The selective inhibitor, MDL 29,152, is currently being used in studies to determine whether protein kinase C is involved in monocyte activation and differentiation induced by oxidized LDL.

In contrast to the partial suppression of differentiation, phorbol-induced expression of the acLDL receptor by THP-1 cells, as indicated by the number of cells that were able to take up DiIacLDL, was completely blocked by 100 μM of MDL 29,152. Further, as suggested by the results of Via et al. (12), we show by dual analysis that 72 h after PMA stimulation approximately 50% of the population was acLDL receptor-positive, while virtually all of the population was MO-1-positive. Thus it appears that differentiated THP-1 cells do not all necessarily acquire the ability to take up modified LDLs.

THP-1 cells, as circulating monocytes, can be induced to secrete a variety of factors including IL-1 (11, 22, 23). In this study, after PMA activation, IL-1β levels in the media were effectively eliminated by 50 μM MDL 29,152, while H-7, at even 100 μM, reduced IL-1β levels by only 70%. IL-1β mRNA levels were measured to determine whether the decreased levels of IL-1β in the media were the result of decreased induction of the gene. IL-1β mRNA levels were reduced by 76% with 50 μM MDL 29,152 and by 47% with 50 μM H-7. While this supports the possibility of decreased IL-1β transcription, the method of RNA analysis used here cannot distinguish between effects on transcription rates and/or turnover of IL-1β mRNA. Although mRNA levels were reduced, it did not account for the very low levels of IL-1β detected in the media when the cells were incubated with MDL 29,152. It is likely that protein kinase C influences not only the levels of IL-1β mRNA, but also translation, processing, and release of the protein by the cell. There is a unique mechanism for the processing and secretion of IL-1β. The protein is synthesized as a large precursor molecule, which is processed by a specific, inducible protease to the mature form (32). This occurs either before or during release of IL-1β from the cell by a novel mechanism (33). Inhibition of these processes could account for the differences between levels of IL-1β mRNA and IL-1β in the media.

This work and that of others shows that events associated with protein kinase C pathways appear to play a role in regulating receptor-mediated metabolism of both LDL and modified LDL. Hara et al. (25) and Auwerx (11) have shown that PMA causes a reduction in LDL receptors and a concomitant increase in acLDL receptors in THP-1 cells. A popular hypothesis for the cellular basis of atherosclerosis suggests that the appearance of scavenger LDL receptors on monocyte/macrophages trapped below the endothelial cell layer of the artery leads to accumulation of intracellular lipids and subsequent foam cell formation (34, 35). Evidence from Ross et al. (36) and Gerrity et al. (37) also suggests that the terminal tissue-specific differentiation of macrophages and proliferation of smooth muscle cells within the artery wall are influenced by interacting mediators including IL-1 and platelet-derived growth factor. In vivo analysis has shown that IL-1β increases monocyte adhesion to endothelial cells (38) and

induces proliferation of smooth muscle cells (39, 41). Like monocytes, THP-1 cells have the capacity to take up modified LDL. Further THP-1 cells, as well as monocytes, can be induced to secrete a variety of mediators including IL-1β. Therefore, as others have proposed (11), we believe that THP-1 cells are a useful model system for studying the role of monocyte/macrophages in the pathogenesis of atherosclerosis.

We have shown here that MDL 29,152 is a selective in vitro inhibitor of protein kinase C. Further, MDL 29,152 was shown to be a more effective inhibitor of phorbol-induced THP-1 cell activation and differentiation than the nonselective kinase inhibitor H-7. The selectivity of MDL 29,152 needs to be confirmed by analysis of its effect on other kinase activities including tyrosine protein kinase. Also, the potency of MDL 29,152 needs to be verified in other biological systems. For instance, we are currently using MDL 29,152 to analyze the role of protein kinase C in platelet-derived growth factor-induced smooth muscle cell proliferation. The precise mechanism of protein kinase C activation of lipoprotein receptor expression IL-1 expression and secretion, and monocyte differentiation remains to be elucidated. However, MDL 29,152 may prove to be a powerful tool in furthering our understanding of the mechanisms of kinase activation pathways and their relationship to these and other biological responses.

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REFERENCES

1. Kikkawa, U., A. Kishimoto, and Y. Nishizuka. 1989. The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* **58:** 31–44.
2. Pelech, S. L., J. S. Sanghera, and M. Daya-Makin. 1990. Protein kinase cascades in meiotic and mitotic cell cycle control. *Biochem. Cell Biol.* **68:** 1297–1330.
3. Kreutter, D., A. B. Caldwell, and M. J. Morin. 1986. Dissociation of protein kinase C activation from phorbol ester-induced maturation of HL-60 leukemia cells. *J. Biol. Chem.* **260:** 5979–5984.
4. Hockberger, P., M. Toselli, D. Swandulla, and H. D. Lux. 1989. A diacylglycerol anologue reduces neuronal calcium currents independently of protein kinase C activation. *Nature.* **338:** 340–342.
5. Merrill, Jr., A. H., A. M. Sereni, V. L. Stevens, Y. A. Hannun, R. M. Bell, and J. M. Kinkade, Jr. 1986. Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. *J. Biol. Chem.* **261:** 12610–12615.
6. Kobayashi, E., H. Nakano, M. Morimoto, and T. Tamaoki. 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159,** 548–553.
7. Tamaoki, T., and H. Nakano. 1990. Potent and specific inhibitors of protein kinase C of microbial origin. *Biotechnology.* **8:** 732–735.
8. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135:** 397–402.
9. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry.* **23:** 5036–5041.
10. Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada. 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* **42:** 1530–1536.
11. Auwerx, J. 1991. The human leukemia cell line, THP-1: a multifacetted model for study of monocyte-macrophage differentiation. *Experientia.* **47:** 22–31.
12. Via, D. P., L. Pons, D. K. Dennison, A. E. Fanslow, and F. Bernini. 1989. Induction of acetyl-LDL receptor activity by phorbol ester in human monocyte cell line THP-1. *J. Lipid Res.* **30:** 1515–1524.
13. Brown, M. S., J. R. Foust, and J. L. Goldstein. 1975. Role of LDL receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.* **55:** 783–793.
14. Salama, S. E. 1986. A rapid preparation of platelet calcium-activated phospholipid dependent protein kinase. *Thromb. Res.* **44:** 649–660.
15. Sahal, D., and Y. Fujita-Yamaguchi. 1987. Protein kinase assay by trichloroacetic acid method: high performance by using phosphocellulose paper and washing an ensemble of samples on flat sheets. *Anal. Biochem.* **167:** 23–30.
16. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
17. Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α-, β- and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell Biol.* **3:** 787–795.
18. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.
19. Robinson, P. J., H. C. Cheng, C. K. Black, C. J. Schmidt, T. Kariya, W. D. Jones, and R. C. Dage. 1990. MDL 27,032 [4-propyl-5-(4-pyridinyl)-oxazolone], an active site-directed inhibitor of protein kinase C and cyclic AMP-dependent protein kinase that relaxes vascular smooth muscle. *J. Pharmacol. Exp. Ther.* **255:** 1392–1398.
20. Loomis, C. R., and R. B. Bell. 1988. Sangivamycin, a nucleoside analogue, is a potent inhibitor of protein kinase C. *J. Biol. Chem.* **263:** 1682–1692.
21. Nakadate, T., A. Y. Jeng, and P. M. Blumberg. 1988. Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochem. Pharmacol.* **37:** 1541–1545.
22. Akeson, A. L., C. W. Woods, L. B. Mosher, C. E. Thomas, and R. L. Jackson. 1991. Inhibition of IL-1β expression in THP-1 cells by probucol and tocopherol. *Atherosclerosis.* **86:** 261–270.
23. Fenton, M. J., M. W. Vermeulen, B. D. Clark, A. C. Webb, and P. E. Auron. 1988. Human proIL-1β gene expression in monocytic cells is regulated by two distinct pathways. *J. Immunol.* **140:** 2267–2273.

24. Gladwin, A., D. G. Hassal, J. F. Martin, and R. F. G. Booth. 1990. MAC-1 mediates adherence of human monocytes to endothelium via a protein kinase C-dependent mechanism. *Biochim. Biophys. Acta.* **1052:** 166–172.
25. Hara, H., H. Tanishita, S. Yokoyama, S. Tajima, and A. Yamamoto. 1987. Induction of acetylated low density lipoprotein receptor and suppression of low density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell). *Biochem. Biophys. Res. Commun.* **146:** 802–808.
26. Pitas, R. E., T. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* **1:** 177–185.
27. Suzuki, K., N. Sakata, A. Kitani, M. Hara, T. Hirose, W. Hirose, K. Norioka, M. Harigai, M. Kawagoe, and H. Nakamura. 1990. Characterization of human monocytic cell line, U937, in taking up acetylated low-density lipoprotein and cholesteryl ester accumulation. A flow cytometric and HPLC study. *Biochim. Biophys. Acta.* **1042:** 210–216.
28. Hocevar, B. A., and A. P. Fields. 1991. Selective translocation of β_{II}-protein kinase C to the nucleus of human promyelocytic (HL60) leukemia cells. *J. Biol. Chem.* **266:** 28–33.
29. Strulovici, B., S. Daniel-Issakani, E. Oto, J. Nestor, Jr., H. Chan, and A. Tsou. 1989. Activation of distinct protein kinase C isozymes by phorbol esters: correlation with induction of interleukin-1β gene expression. *Biochemistry.* **28:** 3569–3576.
30. Ku, G., and R. L. Jackson. 1990. An active role of lipid peroxidation products in macrophage-modified low density lipoprotein and a passive role of scavenger receptors in the induction of interleukin 1 release. *FASEB J.* **4:** A1745.
31. Frostegard, J., J. Nilsson, A. Haegerstrand, A. Hamsten, H. Wigzell, and M. Gidlund. 1990. Oxidized low density lipoprotein induces differentiation and adhesion of human monocytes and the monocytic cell line U937. *Proc. Natl. Acad. Sci. USA.* **87:** 904–908.
32. Black, R. A., S. R. Kronheim, J. E. Merriam, C. J. March, and T. P. Hopp. 1989. A pre-aspartate-specific protease from human leukocytes that cleaves pro-interleukin-1β. *J. Biol. Chem.* **264:** 5323–5326.
33. Suttles, J., J. G. Giri, and S. B. Mizel. 1990. IL-1 secretion by macrophages: enhancement of IL-1 secretion and processing by calcium ionophores. *J. Immunol.* **144:** 175–182.
34. Kovanen, P. T. 1990. Atheroma formation: defective control in the intimal round-trip of cholesterol. *Eur. Heart J.* **II (Suppl. E):** 238–246.
35. Matsumoto, A., M. Naito, H. Itakura, S. Ikemoto, H. Asaoka, I. Hayakawa, H. Kanamori, H. Aburatani, F. Takaku, H. Suzuki, Y. Kobari, T. Miyai, K. Takahashi, E. H. Cohen, R. Wydro, D. E. Housman, and T. Kodama. 1990. Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **87:** 9133–9137.
36. Ross, R., J. Masuda, E. W. Raines, A. M. Gown, S. Katsuda, M. Sasahara, L. T. Malden, H. Masuko, and H. Sato. 1990. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science.* **248:** 1009–1012.
37. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
38. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone, Jr. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. *J. Clin. Invest.* **76:** 2003–2011.
39. Libby, P., D. J. Wyler, M. W. Janicka, and C. A. Dinarello. 1985. Differential effects of human interleukin-1 on growth of human fibroblasts and vascular smooth muscle cells. *Arteriosclerosis.* **5:** 186–191.
40. Ku, G., N. S. Doherty, J. A. Wolos, and R. L. Jackson. 1988. Inhibition by probucol of interleukin-1 secretion and its implication in atherosclerosis. *Am. J. Cardiol.* **62:** 77B–81B.
41. Raines, E. W., S. K. Dower, and R. Ross. 1989. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. *Science.* **243:** 393–396.