Effects of Cardiac Natriuretic Peptides on Oxidized Low-Density Lipoprotein– and Lysophosphatidylcholine-Induced Human Mesangial Cell Migration

Masakazu Kohno, Kenichi Yasunari, Kensaku Maeda, Hiroaki Kano, Mieko Minami, Takao Hanehira, Junichi Yoshikawa

Abstract—The objectives of the present study were (1) to determine whether oxidized LDL and lysophosphatidylcholine (lyso-PtdCho), a major phospholipid component of oxidized LDL, stimulate the migration of cultured human mesangial cells and (2) to investigate the possible effects on mesangial cell migration of the cardiac natriuretic peptides atrial and brain natriuretic peptide (ANP and BNP). Oxidized LDL (10 and 100 μg/mL) and lyso-PtdCho (10^{-7} to 10^{-5} mol/L) stimulated migration in a concentration-dependent manner. In contrast, the effects of native LDL and phosphatidylcholine were modest or nonexistent. Protein kinase C (PKC) inhibitor and downregulation of PKC activity by phorbol ester inhibited oxidized LDL– and lyso-PtdCho–induced migration. Human ANP(1-28) and human BNP-32 significantly inhibited oxidized LDL– and lyso-PtdCho–induced migration in a concentration-dependent manner. C-ANF (des-[Glu18, Ser19, Gly20, Leu21, Gly22]ANP(4-23)), a specific ligand for ANP clearance receptors, could not inhibit oxidized LDL– and lyso-PtdCho–induced migration. Inhibition by ANP and BNP of lyso-PtdCho–induced migration was paralleled by an increase in the cellular level of GMP. Oxidized LDL– and lyso-PtdCho–induced migrations were inhibited by 8-bromo-cGMP. The results suggest that oxidized LDL and lyso-PtdCho stimulate the migration of human mesangial cells, at least in part, through a PKC-dependent process and that ANP and BNP inhibit this stimulated migration, probably through a cGMP-dependent process. (Hypertension. 2000;35:971-977.)

Key Words: lysophosphatidylcholines lipoproteins atrial natriuretic factors glomerular mesangium natriuretic peptide, brain

Hyperlipidemia is associated with a variety of renal diseases, and experimental evidence suggests a role for hyperlipidemia in the progression of glomerular diseases.1,2 Dietary cholesterol supplementation has been shown to accelerate the development of sclerotic glomerular lesions in experimental models of renal disease and in normal animals.1–4 LDL has been shown to be mitogenic for mesangial cells.5–9 However, oxidized LDL has been shown to possess more atherogenic properties than native LDL.10–13 Lysophosphatidylcholine (lyso-PtdCho) is a prominent phospholipid component of oxidized LDL.11 In oxidized LDL particles, the content of lyso-PtdCho is found to be dramatically increased.11,12 Furthermore, recent evidence indicates that lyso-PtdCho possesses mitogenic and chemotactic properties for smooth muscle cells (SMCs).10,13 monocytes, and lymphocytes.14,15 However, the migration of mesangial cells, which are modified SMCs, is believed to play an important role in the pathogenesis of certain glomerular diseases,16 and SMC migration may play a role in the pathogenesis of atherosclerosis. The present study was designed to determine whether oxidized LDL and lyso-PtdCho stimulate the migration of cultured human mesangial cells and, if so, to clarify the mechanism.

Natriuretic peptides are a family of hormones that are involved in the control of fluid balance. Atrial and brain natriuretic peptides (ANP and BNP) are 2 members of this family17–20 that are secreted through the coronary sinus from the heart.21,22 These hormones have peripheral effects on the vasculature and kidney, resulting in vasorelaxation, natriuresis, and diuresis.23–26 In addition to their vasorelaxant and natriuretic effects, these cardiac peptides have been shown to inhibit proliferation in mesangial cells as well as in vascular SMCs.27–29 Furthermore, we have recently shown that natriuretic peptides inhibit oxidized LDL–induced migration of human coronary artery SMCs. However, it is still uncertain whether these natriuretic peptides inhibit oxidized LDL– and lyso-PtdCho–induced migration of mesangial cells.

Accordingly, a study was designed to examine the possible effects of human ANP(1-28) and human BNP-32, the major circulating forms of ANP and BNP, on oxidized LDL– and lyso-PtdCho–induced migration of human mesangial cells.
Methods

Materials
Mesangial cell basal medium (MsGM) and human mesangial cells were purchased from Clonetics Corp. Synthetic human ANP(1-28), human BNP-32, and C-ANF (des-[Glu18,Ser19,Gly20,Leu21,Gly22]{ANP(4-23)}) were purchased from Peptide Institute. Lyso-PtdCho (palmitoyl), phosphatidylcholine (dipalmitoyl), lysophosphatidylinositol (palmitoyl), lysophosphatidylserine, human LDL, 3-isobutyl-1-methylxanthine, 8-bromo-cGMP, phorbol-12-myristate-13-acetate, and BSA were purchased from Sigma Chemical Co. Staurosporine was purchased from Calbiochem Corp. The protein kinase C (PKC) inhibitor H7 was purchased from Seikagaku Kogyo Co Ltd.

Mesangial Cell Cultures
Human mesangial cells were cultured in MsGM containing 5% FCS, 50 μg/mL gentamicin sulfate, and 50 μg/mL amphotericin B. The mesangial cells were identified according to the following criteria: (1) morphology, (2) typical microfilaments seen with transmission electron microscopy, (3) presence of receptors specific to angiotensin II (Ang II) and contraction in response to Ang II, and (4) absence of immunofluorescence with factor VIII antibody. Cells after passages 3 to 7 were used for the experiment.

Figure 1. Concentration-dependent effects of native and oxidized LDL on human mesangial cell migration. Migration activities were assayed in quadruplicate in 3 independent experiments, and values are expressed as mean±SD for number of cells observed in 4 HPF. *P<0.05 vs respective control.

Figure 2. Concentration- and time-dependent effects of lyso-PtdCho on human mesangial cell migration. Migration activities were assayed in quadruplicate in 3 independent experiments, and values are expressed as mean±SD for number of cells observed in 4 HPF. *P<0.05 vs control level of 5-hour incubation. †P<0.05 vs control level of 10-hour incubation.

Figure 3. Human mesangial cells after 5-hour exposure to various concentrations (b to f) of lyso-PtdCho (magnification ×100). a, Control. b, 10⁻⁷ mol/L. c, 10⁻⁶ mol/L. d, 10⁻⁵ mol/L. e, 10⁻⁴ mol/L. f, 5×10⁻⁴ mol/L.
Migration Assay

The migration of mesangial cells was assayed according to a modification of Boyden’s chamber method with the use of microchemotaxis chambers (Nucleopore Corp) and polycarbonate filters, as previously reported. In this experiment, polycarbonate filters with 12-μm-diameter pores were used. In all experiments, type IV collagen–coated filters were used. Migration activity is calculated as the mean number of migrated cells observed in 4 high-power fields (HPF) and is given as the mean value of 4 measurements.

Measurement of Lactate Dehydrogenase and Assessment of Cytotoxicity

The cytotoxic effects of high concentrations of lyso-PtdCho and oxidized LDL on human mesangial cells were assessed on the basis of lactate dehydrogenase (LDH) release according to a commercially available method (Sigma Chemical Co) with the procedure described by Wrobleski and LaDue. LDH was measured in cell culture supernatants after 5 hours of incubation at 37°C in MsGM with or without varying concentrations of lyso-PtdCho (10⁻², 10⁻⁴, and 10⁻⁶ mol/L) or oxidized LDL (1, 10, 50, 100, and 200 μg/mL). Cytotoxicity was expressed as percent LDH activity present in supernatants of cultured cells compared with total LDH activity present in 5×10⁵ cells/well incubated with 1 mL of 1% Triton X-100 for 30 minutes.

Calculations and Statistical Analysis

The statistical significance of differences in the results was evaluated with 1-way ANOVA, and probability values were obtained with Scheffe’s method. Values are expressed as mean±SD.

Results

Effects of Oxidized LDL and Lyso-PtdCho on Human Mesangial Cell Migration

Figure 1 shows the concentration-dependent effects of native and oxidized LDL on human mesangial cell migration. Oxidized LDL significantly induced migration in a concentration-dependent manner between 10⁻⁴ and 10⁻² mol/L. On the other hand, the migration-stimulatory effects of native LDL were modest.

Figure 2 shows the concentration- and time-dependent effects of lyso-PtdCho on human mesangial cell migration. Lyso-PtdCho significantly induced migration in a concentration- and time-dependent manner between 10⁻² and 10⁻⁵ mol/L. In contrast, the migration-stimulatory effects of 10⁻⁴ and 5×10⁻⁴ mol/L lyso-PtdCho were nonexistent. Lyso-PtdCho at 10⁻² and 5×10⁻⁴ mol/L appeared to be cytotoxic for human mesangial cells, because these concentrations of lyso-PtdCho clearly caused morphological injury or cell lysis (Figure 3) and a marked increase in LDH release from the cells (Figure 4, top). On the other hand, oxidized LDL did not cause clear morphological injury or cell lysis, at least not at the concentration used in the current experiment, and did not cause a significant increase in LDH release even at the concentration of 200 μg/mL (Figure 4, bottom).

Table 1 shows the effects on mesangial cell migration of other lysophospholipids that are structurally similar to lyso-PtdCho. When these phospholipids were tested at 2 comparable concentrations, phosphatidylcholine had no significant activity, and lysophosphatidylinositol and lysophosphatidylserine appeared to be less effective than lyso-PtdCho.

Effects of the PKC Inhibitors on Lyso-PtdCho– and Oxidized LDL–Induced Human Mesangial Cell Migration

The stimulatory effect of lyso-PtdCho (10⁻⁵ mol/L) on migration was significantly inhibited by the PKC inhibitors H 7 and staurosporine (Figures 5A and 5B). These inhibitory effects of H 7 and staurosporine were relatively concentration dependent. On the other hand, the PKC inhibitors did not affect the basal migration.

The migration-stimulatory effect of oxidized LDL (50 μg/mL) was also significantly inhibited by H 7 (10⁻⁶ and 10⁻⁵ mol/L).
mol/L) (oxidized LDL alone, 38.2±5.7 cells/4 HPF; oxidized LDL+H 7 at 10⁻⁵ mol/L, 28.7±3.2 cells/4 HPF, P<0.05; oxidized LDL+H 7 at 10⁻⁴ mol/L, 23.1±2.5 cells/4 HPF, P<0.05) and staurosporine (10⁻⁵ and 10⁻⁷ mol/L) (oxidized LDL alone, 37.7±4.9 cells/4 HPF; oxidized LDL+staurosporine at 10⁻⁴ mol/L, 26.2±2.6 cells/4 HPF, P<0.05; oxidized LDL+staurosporine at 10⁻⁷ mol/L, 20.7±2.0 cells/4 HPF, P<0.05).

To confirm the importance of the PKC-dependent mechanism in the stimulation by lyso-PtdCho and oxidized LDL of migration activity, PKC depletion was generated through preincubation with a high dose of phorbol-12-myristate-13-acetate (10⁻⁷ mol/L) for 24 hours. In PKC-depleted mesangial cells, 10⁻⁵ mol/L lyso-PtdCho and 50 μg/mL oxidized LDL failed to stimulate migration (Table 2).

Effects of ANP and BNP on Lyso-PtdCho- and Oxidized LDL-Induced Human Mesangial Cell Migration
To clarify whether the alteration in cell number by oxidized LDL, lyso-PtdCho, ANP, or BNP modifies the result of migration response, we examined the effect of these substances on mesangial cell number for 5- and 10-hour incubations. However, during the 5- and 10-hour incubations, lyso-PtdCho and oxidized LDL, as well as ANP(1-28) and BNP-32, did not affect mesangial cell number under our experimental conditions (data not shown). Therefore, in our experiments with 5- and 10-hour incubations, proliferation could not have affected the result of the migration assay.

Figures 6A and 6B show the effects of varying concentrations (10⁻⁹, 10⁻⁸, and 10⁻⁷ mol/L) of human ANP(1-28) and BNP-32 on 10⁻⁵ mol/L lyso-PtdCho- or 50 μg/mL oxidized LDL–induced mesangial cell migration. Both human ANP(1-28) and human BNP-32 significantly inhibited lyso-PtdCho– and oxidized LDL–induced migration at concentrations of 10⁻⁹ and 10⁻⁷ mol/L. On the other hand, 10⁻⁷ mol/L ANP and BNP had no significant effect on nonstimulated mesangial cell migration (baseline, 10.3±2.3 cells/4 HPF; ANP 10⁻⁷ mol/L, 9.3±1.9 cells/4 HPF; BNP 10⁻⁷ mol/L, 8.5±1.3 cells/4 HPF). ANP and BNP increased cellular cGMP in a concentration-dependent manner (control, 3.3±0.5 pmol/5×10⁵ cells; ANP 10⁻⁷ mol/L, 7.3±1.3 pmol/5×10⁵ cells; BNP 10⁻⁷ mol/L, 7.0±1.4 pmol/5×10⁵ cells; ANP 10⁻⁸ mol/L, 15.5±2.1 pmol/5×10⁵ cells; BNP 10⁻⁸ mol/L, 14.8±1.7 pmol/5×10⁵ cells; ANP 10⁻⁹ mol/L, 34.8±3.6 pmol/5×10⁵ cells; BNP 10⁻⁹ mol/L, 30.8±2.2 pmol/5×10⁵ cells), and these effects paralleled the inhibition of migration.

To clarify the role of biological receptors on the migration-inhibitory effects of natriuretic peptide, we examined the effects of C-ANF, a specific ligand for ANP-clearance receptors, on lyso-PtdCho– and oxidized LDL–induced migration of human mesangial cells. As shown in Table 3,
C-ANF could not inhibit lyso-PtdCho– and oxidized LDL–induced migration between $10^{-5}$ and $10^{-4}$ mol/L.

Effects of 8-Bromo-cGMP on Lyso-PtdCho– and Oxidized LDL–Induced Mesangial Cell Migration

To elucidate whether the inhibitory effects of ANP and BNP on lyso-PtdCho– and oxidized LDL–induced mesangial cell migration are causally linked to the increase in cellular cGMP, we examined the effect of 8-bromo-cGMP on lyso-PtdCho (10$^{-5}$ mol/L)– and oxidized LDL (50 µg/mL)–induced mesangial cell migration. The inhibition of lyso-PtdCho– and oxidized LDL–induced mesangial cell migration by ANP and BNP could be reproduced by this analog at concentrations of $10^{-6}$ and $10^{-5}$ mol/L (Table 4).

Discussion

In the present study, we have shown that lyso-PtdCho at concentrations of $<10^{-5}$ mol/L stimulates the migration of cultured human mesangial cells. In contrast, the migration-stimulatory effect of $10^{-4}$ and $5 \times 10^{-4}$ mol/L was nonexistent.

TABLE 4. Effects of 8-Bromo-cGMP on Lyso-PtdCho– and Oxidized LDL–Induced Human Mesangial Cell Migration

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Migration Activity, cells/4 HPF/5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>9.0±1.4</td>
</tr>
<tr>
<td>Lyso-PtdCho 10$^{-5}$ mol/L</td>
<td>27.3±1.9</td>
</tr>
<tr>
<td>Lyso-PtdCho 10$^{-5}$ mol/L+8-bromo-cGMP 10$^{-5}$ mol/L</td>
<td>3.2*</td>
</tr>
<tr>
<td>Lyso-PtdCho 10$^{-5}$ mol/L+8-bromo-cGMP 10$^{-5}$ mol/L</td>
<td>2.4*</td>
</tr>
<tr>
<td>Oxidized LDL 50 µg/mL</td>
<td>37.5±6.4†</td>
</tr>
<tr>
<td>Oxidized LDL 50 µg/mL+8-bromo-cGMP 10$^{-6}$ mol/L</td>
<td>27.4±2.7†</td>
</tr>
<tr>
<td>Oxidized LDL 50 µg/mL+8-bromo-cGMP 10$^{-6}$ mol/L</td>
<td>23.6±4.1†</td>
</tr>
<tr>
<td>Oxidized LDL 50 µg/mL+8-bromo-cGMP 10$^{-6}$ mol/L</td>
<td>17.8±3.2‡</td>
</tr>
</tbody>
</table>

Values are mean±SD of 4 measurements. Migration activities are expressed as number of cells per 4 high-power fields (HPF).

*P<0.05 vs baseline.
†P<0.05 vs lyso-PtdCho alone.
‡P<0.05 vs oxidized LDL alone.

Furthermore, $10^{-4}$ and $5 \times 10^{-4}$ mol/L lyso-PtdCho clearly caused morphological cell injury or cell lysis for these cells and a marked increase of LDH release from the cells. These results suggest that $10^{-4}$ and $5 \times 10^{-4}$ mol/L lyso-PtdCho might be cytotoxic for human mesangial cells and that lyso-PtdCho at concentrations within this approximate range, as expected to be present in the levels of oxidized LDL, can stimulate migration. On the other hand, phosphatidylcholine had no significant effect. In addition, we have shown that oxidized LDL stimulates human mesangial cell migration and that this effect of native LDL was modest. Furthermore, both oxidized LDL and lyso-PtdCho have been shown to possess mitogenic properties. Therefore, these observations raise the hypothesis that the conversion of phosphatidylcholine to lyso-PtdCho may render it chemotactic for mesangial cells, thus contributing to hypercholesterolemia-aggravated glomerulosclerosis. However, there may be intrinsic differences between the effects of exogenously applied lyso-PtdCho and oxidized LDL in the glomerular lesions. In the former case, lyso-PtdCho at concentrations of $<10^{-5}$ mol/L was immediately incorporated from the medium into cellular membranes. During the oxidation of LDL, however, various possible degradation products other than lyso-PtdCho, such as other lysolipids, lipid peroxides, and oxygenated sterols, are generated and might exert effects on mesangial cells.

In the present study, we have shown that lyso-PtdCho in solution and lyso-PtdCho in oxidized LDL. During the oxidation of LDL, loss of esterified cholesterol, with a relative and absolute increase in free cholesterol content, is observed. Changes in the free cholesterol content can alter membrane physicochemical properties, the function of membrane-bound enzymes, and ion transport. Furthermore, free cholesterol is shown to impart resistance to oxidative modification. Therefore, caution should be exercised in extrapolation of the present in vitro data to in vivo conditions.

The specific mechanisms of action of oxidized LDL and lyso-PtdCho as chemotactic factors for human mesangial cells at least allow us to conceptualize the possibilities. The PKC inhibitors H 7 and staurosporine inhibited the oxidized LDL– and lyso-PtdCho–stimulated mesangial cell migration. The stimulation of migration by oxidized LDL and lyso-PtdCho is also abolished in the PKC-depleted cells. These results suggest that oxidized LDL and lyso-PtdCho stimulate human mesangial cell migration, at least in part, via a mechanism that probably involves the activation of PKC. On the other hand, lysophosphatidylinositol and lysophosphatidylserine were less effective than lyso-PtdCho. However, the precise structural specificity of lyso-PtdCho that has such an effect on human mesangial cell migration remains to be clarified at this time.

Next, we showed that human ANP(1-28) and BNP-32 strongly inhibited oxidized LDL– and lyso-PtdCho–induced migration of human mesangial cells in a concentration-dependent manner. In fact, 50 µg/mL oxidized LDL– and $10^{-5}$ mol/L lyso-PtdCho–stimulated mesangial cell migration was significantly inhibited by ANP and BNP at concentrations of $10^{-8}$ to $10^{-7}$ mol/L. Although human ANP(1-28) and human BNP-32 are the major circulating forms of ANP and BNP, respectively, the normal plasma concentrations ($\approx 10^{-11}$ to $10^{-10}$ mol/L).
mol(L) are much lower than those of synthetic natriuretic peptides that inhibited mesangial cell migration in the present study. However, plasma ANP and BNP concentrations were found to be high in patients with various renal diseases or severe hypertension.21,22 Furthermore, ≈10% of cardiac output delivered to 1 kidney and a high density of ANP-A receptors are expressed in glomerular mesangial cells.23 These observations may suggest the possibility that elevated plasma ANP and BNP concentrations in patients with renal diseases or severe hypertension may represent a compensatory mechanism that tends to offset further progression of glomerulosclerosis through the inhibition of mesangial cell migration. However, it remains to be clarified whether ANP and BNP have physiological roles as modulators of mesangial cell migration, because high concentrations of ANP and BNP are required to inhibit the lyso-PtdCho and oxidized LDL effects on mesangial cell migration.

In the present study, ANP and BNP did not inhibit the basal migration activity of nonstimulated human mesangial cells. Furthermore, in a trypan blue exclusion test, dead cells stained with trypan blue were not found 5 hours after treatment with 10⁻⁷ mol/L ANP or BNP. Therefore, it is likely that the observed migration-inhibitory effect of natriuretic peptides was not a result of cytotoxicity or diminished cell viability.

We have obtained some evidence for a causal link between cGMP production and the inhibition of mesangial cell migration after stimulation with oxidized LDL and lyso-PtdCho. First, ANP and BNP increased cGMP levels, and these effects paralleled the inhibition of migration. Second, a cGMP analog, 8-bromo-cGMP, significantly inhibited oxidized LDL- and lyso-PtdCho-stimulated migration. Third, C-ANF, a specific ligand for ANP clearance receptors,40 could not significantly inhibit oxidized LDL- and lyso-PtdCho-induced migration. These results suggest that cardiac natriuretic peptides inhibit oxidized LDL- and lyso-PtdCho-induced migration, at least in part, through a cGMP-dependent process and that the biological rather than the clearance ANP receptor may be involved in this effect. Recently, nitric oxide is shown to inhibit Ang II–induced migration of rat aortic SMCs in part via a cGMP-dependent mechanism.41 This finding may support our hypothesis. However, further studies are necessary to elucidate the involvement of cGMP and its related systems in the inhibition by natriuretic peptides of oxidized LDL– or lyso-PtdCho–induced migration of human mesangial cells.

In summary, our present findings suggest that oxidized LDL and its major phospholipid component, lyso-PtdCho, stimulate human mesangial cell migration, at least in part, via a PKC-dependent process and that ANP and BNP can inhibit this stimulated migration, probably through a cGMP-dependent process. Taken together with an antimitogenic effect29 of ANP and BNP on glomerular mesangial cells, migration inhibition by these natriuretic peptides may exert an important beneficial effect that tends to lessen the severity of hypercholesterolemia-induced glomerular damage that involves mainly the mesangium.

Acknowledgments

This work was supported by a grant-in-aid-for Scientific Research from the Ministry of Education, Science and Culture, Japan. The authors gratefully acknowledge the technical assistance of Asami Ohnishi, Yuka Inoshita, and Kazuko Shimagawa (Division of Hypertension and Atherosclerosis, The First Department of Internal Medicine, Osaka City University Medical School).

References

Effects of Cardiac Natriuretic Peptides on Oxidized Low-Density Lipoprotein– and Lysophosphatidylcholine-Induced Human Mesangial Cell Migration
Masakazu Kohno, Kenichi Yasunari, Kensaku Maeda, Hiroaki Kano, Mieko Minami, Takao Hanehira and Junichi Yoshikawa

Hypertension, 2000;35:971-977
doi: 10.1161/01.HYP.35.4.971

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/35/4/971

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/