

Elevated Cholesterol Levels in the Plasma Membranes of Macrophages Inhibit Migration by Disrupting RhoA Regulation

Tomokazu Nagao, Chunbo Qin, Inna Grosheva, Frederick R. Maxfield, Lynda M. Pierini

Objective—Atherogenesis begins as small subendothelial accumulations of foam cells that develop through unregulated uptake of modified and aggregated low-density lipoprotein (LDL). The reason why foam cells remain in the atherosclerotic plaque rather than migrating out of the area is unclear. We tested the hypothesis that elevated membrane cholesterol levels, which may result from interactions with aggregated LDL, affect macrophage migration.

Methods and Results—Cholesterol loading by incubation with cholesterol-chelated methyl-β-cyclodextrin decreased migration of J774A.1 macrophages toward complement 5a (C5a) in transwell migration assays, even though cholesterol-loaded macrophages responded to a bath application of C5a. In a micropipette polarization assay, cholesterol-loaded cells polarized toward a C5a gradient. In a transwell migration assay, cholesterol-loaded cells extended lamellae through the filter pores but were unable to translocate their cell bodies. Cholesterol loading decreased both the cellular levels of GTP-bound active RhoA and the phosphorylation of myosin light chain. Expression of constitutively active RhoA largely prevented the inhibition of cell migration by cholesterol loading.

Conclusions—These results suggest that increases in plasma membrane cholesterol content alter RhoA activation, resulting in inhibition of cell migration. These findings provide one possible explanation for the retention of foam cells in atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2007;27:1596-1602.)

Keywords: foam cells ■ atherosclerosis ■ actin ■ motility ■ cyclodextrin

Atherosclerosis is a complex process that is thought to begin when hypercholesterolemia leads to deposition of low-density lipoprotein (LDL) into the subendothelial space.1–3 Subendothelial LDL becomes oxidatively modified, aggregated, and then attached to the surrounding matrix by arterial wall cell enzymes.4,5 Macrophages take up cholesterol from the matrix-bound LDL aggregates and become lipid-filled foam cells.6 Gradual thickening of the arterial wall caused by the accumulation of foam cells leads to the development of an atherosclerotic plaque. It has been proposed that plaque progression may result in part from long-term retention of foam cells within the plaque,7 suggesting that promoting emigration of foam cells from atherosclerotic lesions might lead to plaque regression.

See cover

It is unclear why macrophage foam cells remain within the atherosclerotic lesion. A possible explanation is that the lesion environment, and specifically interaction with lipoproteins, changes the migratory behavior of macrophages. Several studies have investigated the possibility that macrophage motility is altered by interactions with modified LDLs. Oxidatively modified LDL strongly inhibited the chemotactic responses of mouse resident peritoneal macrophages,8,9 and oxidized or aggregated LDL changed the organization of the F-actin cytoskeleton and decreased the ability of macrophages to generate locomotor forces.10 However, the molecular mechanism(s) by which lipoprotein interactions caused these changes in macrophages is not understood.

The interactions of macrophages with aggregated and matrix-bound LDL have some unique features that are not apparent when macrophages bind soluble LDL. When macrophages begin to engulf aggregated matrix-bound LDL particles, they maintain prolonged contact with the LDL aggregates.11 During this prolonged contact there can be rapid transfer of cholesterol from the LDL aggregates to the macrophage. As a result, transient increases in the cholesterol content of macrophage plasma membranes may result. These findings suggest 2 possible ways that lipoprotein interactions could lead to changes in macrophage F-actin organization and migration: (1) The binding of modified lipoproteins to their receptors could initiate signals that negatively regulate the cytoskeletal changes required for migration; or (2) Cholesterol transfer from lipoproteins could modulate macrophage...
plasma membrane cholesterol levels, and this change in membrane cholesterol could alter signal transduction mechanisms in the macrophage. To directly test the latter possibility, in a previous study we raised macrophage membrane cholesterol levels acutely by treating cells with cholesterol-chelated methyl-β-cyclodextrin (chole-MβCD). We found that elevation of plasma membrane cholesterol levels with chole-MβCD changed F-actin organization and decreased macrophage motility, and these changes mimicked those obtained when cells were treated with soluble lipoproteins and an ACAT inhibitor, which cause cellular cholesterol levels to rise.12 In this study, we sought to identify the step at which the migration of cholesterol-loaded cells was inhibited and to investigate the molecular mechanisms of this inhibition.

The steps that make up the cell migration process can be summarized as follows: sensing the chemoattractant signal, lamellipodium protrusion toward the signal (ie, polarization), translocation forward (which requires contractile forces), and tail detachment and retraction. We tested the effects of cholesterol loading on each stage of migration and found that the translocation step (ie, contractility) was affected in cholesterol-loaded macrophages that had been stimulated to migrate through the filters of a transwell migration chamber. Arrest at this stage of migration appears to result from inhibition of the RhoA/Rho kinase signaling pathway, which regulates cell contraction.

**Methods**

**Plasmids and Transfection**

Plasmid DNA encoding EGFP-tagged wild-type RhoA (WT-RhoA, Addgene plasmid 12965) and EGFP-tagged constitutively active (Q63L) RhoA (CA-RhoA, Addgene plasmid 12968) were obtained from Addgene (Cambridge, Mass).13 Transient transfection of RAW 264.7 cells (RAW cells) was performed using FuGENE HD transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions.

**Transwell Migration Assay**

J774A.1 macrophage-like cells (J774 cells) or transfected RAW cells were plated into transwell migration filter inserts seated in 24-well dishes (Corning). The cells were incubated with 5 mmol/L cholesteryl chloroform and placed in wells containing FBS and media but no C5a. After 3 hours incubation at 37°C in a CO2 incubator, the samples were processed for fluorescence microscopy imaging and analysis as described (please see supplemental materials, available online at http://atvb.ahajournals.org).

**Micropipette Protrusion Assay**

J774 cells were plated into Poly-d-lysine-coated glass coverslip-bottom dishes (coverslip dishes) and incubated overnight. The cells were then treated with or without 5 mmol/L cholesteryl chloroform for 15 minutes in 20 mmol/L HEPES-buffered DMEM, washed, and mounted onto the stage of a Leica DMIRB inverted microscope equipped with a micromanipulation system (Narishige). A micropipette filled with 1 μmol/L C5a was positioned close to the cells by using a micromanipulator, and phase-contrast time-lapse images of the cells within the field were recorded at 30-second intervals using MetaMorph image acquisition software (Molecular Devices).

**RhoA Activation Assay**

J774 cells, RAW cells, or human monocyte-derived macrophages (hMDM) treated with or without chole-MβCD were stimulated with 0.5 mmol/L C5a or left unstimulated, then lysed with lysis buffer (50 mmol/L Tris, 10 mmol/L MgCl2, 150 mmol/L NaCl, 1% Triton X-100, pH 7.5) on ice and clarified by centrifugation; supernatants were used for the RhoA activation assay. The amount of GTP-bound RhoA was determined using the RhoA activation assay kit from Cytoskeleton, Inc.

**MLC Phosphorylation**

J774 cells were plated onto 35-mm tissue culture dishes (BD Falcon) and incubated overnight. The cells were treated with or without 5 mmol/L cholesteryl chloroform or 10 μmol/L Y-27632 (Sigma), washed, and lysed with Laemmli buffer. After boiling, the samples were subjected to SDS-PAGE followed by immunoblotting with primary anti-myosin light chain (MLC) or anti-phospho MLC (Thr18/Ser19) antibodies (Cell Signaling Technology) and secondary HRP-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology).

**Statistical Analysis**

All data are expressed as mean±SD and are representative of 3 independent experiments, unless otherwise noted. For individual pair-wise comparisons, Student t test was used.

For detailed Materials and Methods, please see http://atvb.ahajournals.org.

**Results**

**Increasing Macrophage Membrane Cholesterol Levels Impaired Chemotaxis Toward C5a**

To examine the effects of cholesterol loading in the absence of any contributions from lipoprotein receptor-mediated signaling, the cholesterol content of macrophages was acutely raised or lowered by treating cells with chole-MβCD or MβCD, respectively. Supplemental Figure I (available online at http://atvb.ahajournals.org) recapitulates and extends our earlier findings12 on the effects of these treatments on cellular cholesterol content and cell migration for J774 cells and human monocyte-derived macrophages. Briefly, cholesterol loading causes a 51% decrease in the migration of J774 cells, and this decrease is largely reversible when cholesterol levels are returned to normal.

**Cholesterol-Loaded J774 Cells Retained the Ability to Respond to C5a and Polarize in the Direction of the Source of Chemoattractant**

Control and cholesterol-loaded cells were stimulated with a bath application of C5a, and the resulting reorganization of F-actin was evaluated by confocal microscopy. Figure 1A shows that raising membrane cholesterol led to an increase in membrane ruffling and cell spreading even in the absence of exogenous stimulation, consistent with our previous findings.12 In response to C5a, control cells extend F-actin-rich lamellae. Because the concentration of C5a in this assay was equivalent in all directions, the cells often extended several lamellae, and the cells that polarized did so in random directions. When C5a was added to cholesterol-loaded cells, the cells spread more than untreated C5a-stimulated cells, and they put out many more F-actin–containing lamellae. Under
these conditions, almost no polarized cells were found. Therefore, the impaired chemotaxis of cholesterol-loaded cells was not caused by an inability to respond to C5a.

To test the ability of the cells to sense a gradient of chemoattractant and extend a protrusion toward the chemoattractant source (ie, polarize), a micropipette polarization
assay was used. A micropipette was filled with C5a and then placed in the proximity of cells within the field of view of the microscope. Time-lapse images of the field of cells were recorded. As shown in Figure 1B, control cells extended protrusions toward the micropipette tip emitting C5a. When the position of the micropipette was changed, the cells rapidly responded by changing the direction of their lamellae (see supplemental Movie I). Similarly, although cholesterol-loaded J774 cells generally had larger lamellipodia than untreated cells, the cholesterol-loaded cells still showed polarization toward the micropipette (Figure 1B) and responded to changes in the position of the chemoattractant source (see supplemental Movie II).

**J774 Cells With Increased Membrane Cholesterol Levels Showed an Impaired Ability to Undergo Translocation**

After extending a lamella toward the source of chemoattractant, cells complete migration by translocating their bulk forward and subsequently retracting their uropods or tails. The effect of cholesterol modulation on translocation and tail retraction was evaluated in the transwell migration model described for supplemental Figure IB. The positions and morphologies of C5a-stimulated J774 cells were evaluated by confocal imaging of fluorescently-labeled cell nuclei and F-actin cytoskeletons. As indicated by the F-actin staining, the number of protrusions extended by the cells was not altered by cholesterol modulation (Figure 1D, i, ii, v, vi). Quantification of these images (Figure 1E) revealed that 21% (s.d. 5%, n=3) of untreated cells had protrusions in the absence of C5a stimulation, and for cholesterol loaded cells the number of spontaneous protrusions was about the same (18%, s.d. 5%, n=3). In the presence of C5a, the fraction of both untreated and cholesterol-loaded cells with protrusions was increased to nearly identical levels (untreated: 82%, s.d. 9%; cholesterol-loaded: 81%, s.d. 5%). The lamellae extended by untreated and cholesterol-loaded cells in response to C5a were phenotypically distinct: cholesterol-loaded cells could extend very large pseudopodia through the pores without translocating their cell bodies (note absence of nuclei; arrows in Figure 1D, panel vi), but this was rarely seen for untreated cells. These results suggest that the defect in migration of cholesterol-loaded cells was attributable to a defect in the translocation step of migration.

**Rho/Rho Kinase Signaling Pathway Was Inhibited by Cholesterol Loading**

It has been shown that Rho family small GTPases are involved in migration, mainly through regulation of F-actin organization and actin-myosin contraction. Rac, Cdc42, and RhoA are well-characterized members of the Rho GTPase family, and it was shown that for migrating fibroblasts RhoA is necessary for cell translocation and tail retraction (see supplemental Figure II). We investigated the activation state of RhoA in J774 cells before and after cholesterol loading using a RhoA pull-down assay. As shown in Figure 2.
2A, the amount of active (ie, GTP-bound) RhoA in resting J774 cells was decreased in cells with elevated membrane cholesterol. After 3 minutes of activation with a bath application of C5a, untreated cells showed approximately a 25% decrease in active RhoA, which returned close to the level of resting cells by 30 minutes after C5a addition. On the other hand, in cholesterol-loaded cells, the amount of active RhoA remained well below that of untreated cells even after C5a treatment, which caused a slight increase in active RhoA levels. Lowering the cellular cholesterol to control levels with MβCD reversed the inactivation of RhoA seen in cholesterol-loaded cells (supplemental Figure IIIA). The reduction in RhoA activity by cholesterol loading was also found in hMMDM and RAW cells (supplemental Figure IIIB).

**Inhibition of Rho Kinase Largely Recapitulates the Phenotype of Cholesterol-Loaded Cells**

GTP-bound RhoA subsequently activates the serine/threonine kinase Rho kinase (supplemental Figure II),19–21 so inhibition of Rho kinase might mimic the phenotype elicited by RhoA inactivation through cholesterol loading. J774 cells were pretreated with Rho kinase inhibitor Y-27632, and F-actin organization of the cells before and after C5a stimulation was examined (Figure 2B). Y-27632 treatment on its own induced cells to form single or multiple F-actin–rich lamellipodia (Figure 2B, ii). C5a stimulation of the Y-27632–treated cells caused the cells to spread, and this spreading was greater than that of untreated C5a-stimulated cells (Figure 2B, iii, iv). Quantification of cell area revealed that C5a-stimulated Y-27632–treated cells spread to a similar extent as C5a-stimulated cholesterol-loaded cells (Figure 2C).

Transwell migration of Y-27632–treated J774 cells was decreased as compared with untreated cells (supplemental Figure IVA). Y-27632–treated cells had a number of large lamellipodia extending through the filter pores, but they showed reduced translocation across the pore because many of the nuclei remained above the filter (supplemental Figure IVB). However, the fraction of cells with protrusions was unchanged by Y-27632 treatment (supplemental Figure IVC). Similar phenotypes were observed in J774 cells in which RhoA was inhibited by C3 transferase treatment (supplemental Figure V).

**Phosphorylation of MLC Was Decreased by Cholesterol Loading**

Rho kinase phosphorylates MLC phosphatase and inhibits its activity, which results in an increase in MLC phosphorylation, and consequently an increase in actomyosin contractility.22 Because cholesterol-loaded cells exhibited a phenotype similar to cells in which Rho kinase was inhibited (ie, Y-27632–treated cells), we probed the phosphorylation status of MLC by Western blot. The phosphorylation of MLC was slightly but significantly decreased by cholesterol loading as shown in Figure 3. As a control, the phosphorylation of MLC in Y-27632–treated cells was measured and found to be decreased a similar amount as in cholesterol-loaded cells.

**Expression of Constitutively Active RhoA Protects Macrophage Migration From Inhibition by Cholesterol Loading**

RAW cells were used to test the effects of the expression of constitutively active RhoA (CA-RhoA) on migration of cholesterol-loaded cells. Like J774 cells, RAW cells showed inactivation of RhoA after cholesterol loading (supplemental Figure IIIB). As shown in Figure 4, cholesterol loading greatly inhibited the migration of untransfected RAW cells (light gray bars, 41% migration compared with untreated cells, s.d. 17%), but cholesterol loading was less effective at inhibiting the migration of cells expressing CA-RhoA (dark gray bar on right, 84% migration compared with untreated cells, s.d. 20%). Expression of WT-RhoA did not affect the inhibition of migration by cholesterol loading (Figure 4, dark gray bar on left), and neither transfection of WT-RhoA nor CA-RhoA affected the number of migrated cells with normal cholesterol levels (data not shown).

**Discussion**

Previously, we showed that raising the cholesterol content of macrophage membranes either by chol-MβCD or by modified LDL (and ACAT inhibition) resulted in Rac-mediated membrane ruffling and increased macropinocytic activity in the absence of cell stimulation by chemoattractants.12 We also showed that chemoattractant-stimulated migration was decreased in cells with elevated cholesterol levels.12 In the present study, we have investigated the mechanism by which cholesterol loading decreases the C5a-stimulated migration of J774 macrophages. We show here that cholesterol loading inactivates RhoA in macrophages, thereby inhibiting migration of the cells attributable to impaired translocation/contraction.

RhoA regulates contraction of migrating cells by activating Rho kinase, which in turn promotes MLC phosphorylation by blocking the activity of MLC phosphatase. The phosphorylation of MLC creates tension on actin filaments, which results in contractile force generation. Inhibition of myosin light chain kinase caused lamellar extension in all directions in neutrophils.23 This is similar to what is seen in macrophages with either cholesterol loading or Rho kinase inhibition, both of which caused a decrease in the levels of phosphorylated myosin light chain. We have demonstrated that cholesterol-loaded cells are phenotypically similar to...
Rho kinase inhibitor–treated cells; both cell populations exhibit increased cell spreading and a block in cell body translocation during transwell migration. In addition, we have shown that cholesterol loading leads to RhoA inactivation and dephosphorylation of MLC. Furthermore, the expression of constitutively active RhoA largely protected cells from the effects of cholesterol loading on migration. Based on these findings, we propose that impaired chemotaxis in cholesterol-loaded macrophages is, at least in part, a result of deficient RhoA/Rho kinase signaling. Because tail retraction is also an important step for cell migration and is regulated by the RhoA/Rho kinase signaling pathway, impaired tail retraction may also contribute to the inhibition of chemotaxis by cholesterol loading. Because the morphological changes in cholesterol-loaded cells were not identical to those in Y-27632–treated cells, it is likely that cholesterol loading may affect other regulators of cell migration, such as Rac. Another possible reason for reduced migration after cholesterol loading is an increase or decrease in cell adhesiveness. We found that cell adhesiveness, as measured by vigorous rinsing of cells, was neither increased nor decreased by cholesterol loading (supplemental Figure VI).

Lamellar extension is one of the first morphological changes induced by chemotactic stimulation, and it is thought to be positively regulated by the Rho-GTPases Rac and Cdc42, but negatively regulated by RhoA. Inactivation of the RhoA/Rho kinase signaling pathway caused THP-1 monocytes and human primary monocytes to extend protrusions, whereas unnecessary protrusions were prevented by active RhoA in unstimulated cells. C5a stimulation leads to a modest inhibition of RhoA activity (Figure 2A) yet a strong chemotaxis response (supplemental Figure 1B). The current model for RhoA activation in a migrating cell calls for a local inhibition of RhoA activity within protruding lamellae but continued RhoA activation in the cell body and uropod. So, the apparently modest decrease in RhoA activation after C5a treatment may represent a large local (ie, leading edge) decrease in a subset of migrating cells. The morphological features of cholesterol-loaded cells can be explained by changes in the activation states of the Rho GTPases. In our previous report we showed that cholesterol loading increased the levels of GTP-bound active Rac and spontaneous membrane extensions were promoted. Here we found that a bath application of C5a led to enhanced spreading in cholesterol-loaded cells. Observed changes in morphology and Rho GTPase activation are summarized in Figure 4B. In the context of Rho GTPase regulation, membrane protrusion leading to increased spreading may be increased in these cells by both activation of Rac and inactivation of RhoA. When a gradient of chemoattractant is present, cholesterol-loaded cells could extend membrane protrusions toward the source in both 2-D and 3-D settings. The formation of especially large lamellipodia in response to chemotactic stimulation supports the idea that Rho may be misregulated in cholesterol-loaded cells. There is a negative interaction between Rac and Rho activation in neutrophils, and this might be related to our results in which these 2 GTPases are regulated in opposite directions by cholesterol.

Figure 4. Expression of constitutively active RhoA protected cell migration from the effects of cholesterol loading. A, RAW cells, transfected with EGFP–tagged wild-type (WT-RhoA) or constitutively active RhoA (CA-RhoA), were used for transwell migration assays. Numbers of transfected (DAPI+, EGFP+) and untransfected (DAPI+, EGFP−) cells on the bottom of the same transwell filter were counted and normalized to that of cells without cholesterol loading. B, Summary of observed morphological changes in response to cholesterol modulation or stimulation with C5a. Cholesterol loading of macrophages induced ruffling and cell spreading in the absence of stimulation with chemotactant (i, ii). In an isotropic bath of chemoattractant (ie, C5a), cholesterol-loaded cells (iv) showed increased spreading with many lamellar extensions and ruffling as compared with untreated cells (iii). Both untreated (v) and cholesterol-loaded cells (vi) showed polarization toward a chemotactic source, though cholesterol-loaded cells had larger lamellipodia than untreated cells. Red lines and blue background represent lamellipodia extended and C5a distribution, respectively.
In summary, our findings demonstrate that increases in plasma membrane cholesterol content interfere with RhoA activation and consequently inhibit macrophage migration attributable to reduced contractile force generation. These findings provide a possible explanation for the retention of foam cells in atherosclerotic lesions. Besides its role in the regulation of cell motility, RhoA can also regulate the cell cycle and apoptosis. It will be interesting to investigate the effects of cholesterol-induced Rho inactivation on these aspects of foam cell biology.

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Disclosures

None.

References

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Materials and Methods

Cell culture

J774 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were routinely grown at 37 °C in a humidified incubator (5% CO₂) in DMEM supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. Cell cultures were grown to confluence and then used for experiments. Human monocyte-derived macrophages (hMDMs) were obtained from human peripheral blood mononuclear cells isolated by density gradient sedimentation in Ficoll (Amersham Biosciences, Uppsala, Sweden) as described previously.¹

Quantification of free cholesterol content

Measurement of free cholesterol was based on filipin staining and image analysis. Cells were fixed with 3.3% paraformaldehyde in phosphate buffered saline (PBS; Mediatech) for 15 minutes, washed and incubated in 50 μg/ml filipin (Sigma) for 90 min at room temperature. Fluorescence images were obtained with a Leica DMIRB inverted microscope using a 25x objective (NA=0.40). The integrated fluorescence intensity per cell was measured by image analysis as previously described.¹ The accuracy of this method for quantification of cellular cholesterol was demonstrated previously by comparing quantitative image analysis results with those obtained using gas chromatography.¹
Transwell migration assay

To quantify migration, cells in transwell migration chambers were fixed with 0.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 5 min. Cells remaining on the upper surface of the filters were removed by scraping, and the filters were stained with 1 μM of the nuclear stain TO-PRO-3 iodide (Molecular Probes, Eugene, OR). The cells that had migrated through the filter were observed by confocal microscopy (LSM510, Carl Zeiss, Thornwood, NY) using a 10x objective. The number of cells on the underside of the filter was counted in at least 5 random fields per sample. For RAW cells, the samples were fixed with 3.3% paraformaldehyde and stained with DAPI. After scraping the cells on the upper surface of the filters, fluorescence images for both EGFP and DAPI were obtained with a Leica DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a 25x oil immersion objective (NA=0.75). For evaluating the step at which migration was arrested, the samples were fixed with 3.3% paraformaldehyde, and the cells on the upper surface of the filter were left intact. The cells were double-stained with Alexa Fluor 488 phalloidin (Molecular Probes) and TO-PRO-3 after fixation to allow observation of F-actin organization and the position of nuclei. Confocal z-stack images were acquired from below the cells on the lower surface of the filters to above the cells on the upper surface of the filters using a 40x objective (see Fig. 2A). The image stacks were analyzed with Zeiss Image Browser Ver.3.5 and used to generate depth-coded images. Nuclei of cells that had migrated were imaged in a single plane underneath the porous filter.
**Cell spreading assay**

J774 cells were plated on coverslip dishes and incubated overnight. The cells were treated with or without 5 mM chol-MβCD or 10 μM Y-27632 for 15 min in 20 mM HEPES-buffered DMEM and then stimulated with 0.5 nM C5a for 3 min. After fixation with 0.5% glutaraldehyde, cellular F-actin organization was visualized by staining with Alexa Fluor 488 phalloidin and then imaging by confocal microscopy. For quantification of cell area, the lower adherent surface of the samples was imaged by wide-field fluorescence microscope using a 25x objective. The fluorescence images were thresholded to exclude cell-free regions, and the number of pixels above the fixed threshold was quantified using MetaMorph Imaging System. The average pixel area per cell was obtained by dividing total thresholded area by the number of the cells in the field.

**Measurement of cell adhesiveness**

Attached J774 cells were labeled with 2 μM calcein-AM (Molecular Probes) for 1 h, then harvested and suspended in 20 mM HEPES-buffered DMEM. The cell suspension was divided into two tubes and treated with or without 5 mM chol-MβCD for 15 min at 37 °C. The cells were washed, resuspended in DMEM containing 0.5% FBS, and then plated into two 96-well plates. After 3 min incubation, one of the plates was washed with gentle pipetting to remove nonadherent cells, while the other plate was washed vigorously by vortexing to remove nonadherent and lightly attached cells. The remaining attached cells in each well were lysed with 50 μl of 0.1% Triton X-100 in PBS and calcein fluorescence emission at 530 nm with excitation at 485 nm was measured with a microplate spectrofluorometer (Molecular Devices).
Figure legends

Figure I. Transwell migration of macrophages was decreased by cholesterol loading. (A) Quantification of cellular cholesterol levels. J774 cells were plated on 35 mm coverslip dishes. After overnight incubation, the cells were incubated for 15 min in the absence (Untreated) or presence of 5 mM chol-MβCD (Chol-MβCD), fixed and stained with filipin. To test reversibility of cholesterol loading, cholesterol-loaded J774 cells were subsequently incubated with 5 mM MβCD for 10 min (Chol-MβCD→MβCD). The relative free cholesterol levels were determined by quantification of filipin fluorescence intensity per cell. Data (Mean ± SD) from one representative experiment out of 3 with similar results are shown. (B) Transwell migration of cholesterol modulated cells. The cells on transwell migration filters were treated with Chol-MβCD as above, and then tested for their chemotactic response to 10 nM C5a. The numbers of migrated cells were normalized to that of untreated cells stimulated with C5a. Only a small percentage of untreated or cholesterol-modulated cells migrated through the filter when the lower well contained media alone. When C5a was present in the lower well, 22.9% (s.d. 5.6%, n=3) of control cells migrated through the transwell filter over 3 hrs. After increasing cellular cholesterol levels by treatment with chol-MβCD, only 11.2% (s.d. 2.9%, n=3) of the cells crossed the filter, a 51% decrease in the migration. When the cholesterol-loaded cells were subsequently treated with MβCD, which lowered their cellular cholesterol content to that of control cells (supplemental Fig. IA), the chemotactic response of the cells returned to levels comparable to control cells. Data (Mean ± SD) from one representative experiment out of 3 with similar results are shown. (C) Transwell migration through filters with 8 μm pores. The transwell migration assay was carried out following the same
procedure as described in part B, except for the use of 8 μm pore transwell migration filters. (D) Transwell migration of untreated and cholesterol-loaded human monocyte-differentiated macrophages (hMDMs). The migration protocol was exactly the same as for J774 cells. Filters with 8 μm pores were used. Data (mean ± SD) shown are from one experiment out of two. In the other independent experiment, the number of migrated macrophages in response to C5a stimulation decreased by 73% (± 7%) after cholesterol loading.

**Figure II.** Schematic illustrating the role of Rho GTPase in regulating cell migration. Both Rac and Cdc42 are required at the leading edge of migrating cells. Rac promotes actin polymerization at the lamellipodia and Cdc42 determines the direction of migration. RhoA activity is associated with cell contractility and is responsible for cell body contraction and tail retraction. GTP-bound active RhoA activates the serine/threonine kinase Rho kinase which interacts with and phosphorylates myosin light chain (MLC) phosphatase and thereby inactivates it. This increases MLC phosphorylation, leading to cross-linking of actin filaments and generation of contractile force. In unstimulated cells, unnecessary lamellipodial protrusions are prevented by active RhoA.

**Figure III.** (A) Lowering cellular cholesterol content to normal levels reversed RhoA inactivation that resulted from cholesterol loading. J774 cells were grown overnight on tissue culture dishes and incubated for 15 min in the absence or presence of 5 mM chol-MβCD. The cells were further incubated with 5 mM MβCD for 5 or 10 min. GTP-bound
active RhoA and total RhoA levels were analyzed using Rhotekin pull-down assay as described in Materials and Methods. (B) Cholesterol loading caused RhoA inactivation in hMDMs and RAW cells. Cells were loaded with cholesterol by incubation with 5 mM chol-MβCD for 15 min, and then lysed. Active and total RhoA levels were analyzed in lysates.

**Figure IV.** Migration, but not protrusive activity, was inhibited by Rho kinase inhibitor. (A) Transwell migration was decreased by inhibition of Rho Kinase. The cells on transwell migration filters were treated with or without 10 μM Y-27632 for 15 min and then tested for their chemotactic response to 10 nM C5a. The numbers of migrated cells were normalized to that of untreated control cells. (B) Morphology of untreated and Y-27632-treated J774 cells migrating through transwell filters was examined by the same method described for Fig. 1D. (C) Y-27632-treated J774 cells were impaired in cell body translocation. Quantification of the percentage of cells with protrusions was performed as described in Fig. 1E.

**Figure V.** RhoA inhibitor, C3 transferase, has similar effects as cholesterol loading. (A) Transwell migration of J774 cells was decreased by C3 transferase. J774 cells were treated for 24 h with or without C3 transferase in the presence of 1% FBS. The cells were then tested for chemotactic response to 10 nM C5a as described in Materials and Methods. (B) C3-treated J774 cells showed a similar protrusive activity as control cells. The morphologies of untreated and C3-treated J774 cells moving through transwell filters was examined by the same method described in Fig. 2 and representative images are shown.
**Figure VI.** Cell adhesiveness was unchanged by cholesterol loading. After cholesterol loading in suspension, J774 cells were plated into the wells of a 96-well plate and incubated for 3 min in a CO$_2$ incubator. Nonadherent cells were removed by gentle pipetting (Gentle wash), or both weakly adherent and nonadherent cells were detached by mechanical stress produced by vortexing the plate (Vortex). The number of cells remaining attached was measured as described in Materials and Methods. Data were normalized to untreated gently washed control cells.
References


Figure II

Lamellipod extension → Rac / Cdc42

Rac / Cdc42 → RhoA

Contractile Force

Myosin activation

Rho kinase inhibitor (Y-27632)

Rho kinase

MLC

MLC phosphatase

MLC → P
### Figure IIIA

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</thead>
<tbody>
<tr>
<td>Chol-MβCD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Active RhoA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total RhoA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure IVA

![Graph showing migration normalized to control between Untreated and Y-27632](image-url)
Figure IVB

Depth coding (F-actin)

Untreated

Y-27632

Migrated cells (Nuclei)

Overlay

ii

iv

vi

iii

iv

vi
Figure VB

- **Depth coding (F-actin)**
  - Untreated
  - C3 transferase

- **Migrated cells (Nuclei)**
  - iii
  - iv

- **Overlay**
  - v
  - vi
Figure VI

![Bar graph showing attached cells normalized to control for untreated and cholesterol-loaded conditions with gentle wash and vortex methods.](image)