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27-Hydroxycholesterol induces recruitment of monocytic cells by enhancing CCL2 production

Sun-Mi Kim [a], Sae-A Lee [a], Bo-Young Kim [a], Sun-Sik Bae [a], Seong-Kug Eo [b], Koanhoi Kim [a,*]

[a] Department of Pharmacology, Pusan National University – School of Medicine, Yangsan, Gyeongnam 626-870, Republic of Korea
[b] Laboratory of Microbiology, College of Veterinary Medicine and Bio-Safety Research Institute, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea

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ABSTRACT

Deposition of lipids in the intima is followed by infiltration of inflammatory cells, like monocytic cells and T lymphocytes, in atherosclerosis. However, roles of lipids in the infiltration of the inflammatory cells are not clearly defined. We investigated the possible involvement of cholesterol or its catabolites in recruitment of monocytic cells. Consumption of a high cholesterol-diet resulted in enhanced expression of CCL2 in arteries of ApoE$^{-/-}$ mice. 27-Hydroxycholesterol, the most abundant cholesterol oxide in atherosclerotic lesions, significantly induced the transcription of CCL2 and enhanced secretion of corresponding protein by THP-1 monocytic cells. However, cholesterol and 7-ketocholesterol did not influence expression of CCL2. Conditioned media containing CCL2 induced migration of monocytic cells, and migration was abrogated in the presence of CCL2-neutralizing antibody. TO-901317, a synthetic LXR agonist, inhibited both production of CCL2 and migration of monocytic cells induced by 27-hydroxycholesterol. Expression of CCL2 induced by 27-hydroxycholesterol was blocked when Akt inhibitor IV was added and when Akt1 was knocked down. We propose that 27-hydroxycholesterol will trigger a sequence of events leading to recruitment of monocytes into atherosclerotic lesions.

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1. Introduction

Leukocytes such as monocytes and lymphocytes are recruited to the intima where they participate in inflammatory response associated with initiation and development of atherosclerosis as well as complication of the disease [1]. Because leukocyte recruitment is directed by chemokines, expression of inflammatory chemokines affects severity of the disease. For instance, activity of chemokine (C–C motif) ligand 2 (CCL2), known as monocyte chemotactic protein-1 (MCP-1), is linked to atherosclerosis. Genetic deletion of the CCL2 gene or its receptor C–C chemokine receptor type 2 (CCR2) resulted in a significant reduction in development of atherosclerotic plaques in an animal model of atherosclerosis due to reduced monocyte/macrophage infiltration [2,3]. These findings indicate that CCL2 plays a critical role in recruitment of monocytic cells into atherosclerotic plaques. However, it is not known whether lipids detected from the lesions are involved in recruitment of monocytic cells in conjunction with CCL2.

Atherosclerosis is characterized by deposition and accumulation of extracellular cholesterol in the artery. The deposited cholesterol undergoes oxidative modification to cholesterol oxides (oxysterols). 27-Hydroxycholesterol (27OHChol) is the most abundant oxysterol, followed by 7-ketocholesterol, 7β-hydroxycholesterol (7βOHChol), and 7α-hydroxycholesterol (7αOHChol). These oxysterols comprised 75–85% of oxysterols detected in atherosclerotic plaques from different sites [4,5]. Oxysterols are believed to play active roles in plaque development because certain oxysterols perturb cellular cholesterol homeostasis, cause cytotoxicity, induce apoptosis, and enhance gene expression more potently than cholesterol by itself. For instance, 7K and 7βOHChol enhance expression of CXCL8 in human macrophages independent of the Toll-like receptors [6,7] and cause death of vascular cells by activation of intrinsic and/or receptor-mediated death pathways [7,8]. However, roles of oxysterols in terms of monocyte recruitment are unknown.

We attempted to determine whether cholesterol or the two most abundant oxysterols in the lesions influence recruitment of monocytic cells. We demonstrated the ability of 27OHChol to induce migration of monocytic cells by enhancement of CCL2 production. This is the first report on involvement of cholesterol oxides in migration of monocytic cells. We also sought to elucidate molecular mechanisms underlying enhanced production of CCL2 in the presence of 27OHChol.

2. Materials and methods

2.1. Cells and reagents

THP-1 monocytic cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as

* Corresponding author. Address: Department of Pharmacology, Pusan National University – School of Medicine, Busandaehak-ro 49, Mulgeum, Yangsan, Gyeongnam 626-870, Republic of Korea. Fax: +82 51 510 8068.
E-mail address: koanhoi@pusan.ac.kr (K. Kim).

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previously reported [9]. Cholesterol and 27OHChol were purchased from Research Plus, Inc. (Barnegat, NJ). 7K and TO-901317 were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against Akt1, Akt2, total Akt, and α-tubulin were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Reverse transcription (RT)-polymerase chain reaction (PCR)

Non-quantitative and quantitative polymerase chain reaction (PCR) was performed as described previously [9]. The primers for non-quantitative PCR were GAPDH: 5′-gagtcaacggatttggtcct-3′ (forward) and 5′-tgtggtcatgagtccttcca-3′ (reverse); CCL2: 5′-tctgtgc ctgctgctcatag-3′ (forward) and 5′-cagatctccttggccacaat-3′ (reverse). The primers for real-time PCR were GAPDH: 5′-atggggaaggt-gaaggtcg-3′ (forward) and 5′-ggggtcattgatggcaacaata-3′ (reverse); and CCL2: 5′-cagccagatgcaatcaatgcc-3′ (forward) and 5′-tggaatcct-gaacccacttct-3′ (reverse).

2.3. Enzyme-linked immunosorbent assay (ELISA)

The amounts of CCL2 released into the culture medium were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.4. Chemotaxis assay

Migration of THP-1 cells was measured using Transwell Permeable Supports (Costar, Cambridge, MA). THP-1 cells (5×10^5 cells in 100 μL of 0.1% BSA) were loaded into the top chamber of 5-μm-pore polycarbonate transwell inserts. Transwell chambers were inserted into wells filled with the supernatants containing CCL2 obtained after exposure of THP-1 cells to cholesterol or oxysterols. After incubation for 2 h at 37 °C, the number of cells that migrated to the bottom chamber was counted using a Vi-Cell cell counter (Beckman Coulter, Inc. Brea, CA).

2.5. Animal experiments

Animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines and protocols were approved by the Pusan National University Animal Care and Use Committee. Please see the Supplementary Material for additional details.

2.6. Akt knockdown

THP-1 cells were infected with lentiviruses expressing Akt1 shRNA or control lentiviruses, and infected cells were selected in the presence of puromycin as described previously [10].

2.7. Statistical analysis

Statistical analyses were performed as previously reported [9].

3. Results

3.1. Induction of CCL2 expression in macrophages in the presence of 27OHChol

We attempted to determine whether cholesterol and its oxides (oxysterols), which are detected in abundance in atherosclerotic lesions, could change expression of CCL2 by THP-1 cells. THP-1 cells were used because CCL2 is expressed by monocytes/macrophages in atherosclerotic lesions [11]. CCL2 transcripts were barely detected from THP-1 cells, whereas the levels of CCL2 transcripts were markedly elevated by 20-fold in the presence of 27OHChol (Fig. 1A and Supplementary Fig. 1A). However, cholesterol and 7K did not change transcription of CCL2. Time course and concentration experiments were performed using 27OHChol (Fig. 1B and C). Treatment with 27OHChol resulted in enhanced transcription of CCL2 at 48 h after treatment, which was sustained up to 72 h after treatment. Transcription of CCL2 was induced in the presence of 0.5 μg/mL or higher concentrations of 27OHChol.

We determined whether transcription of CCL2 necessitated protein synthesis using CHX, an inhibitor of protein synthesis (Fig. 1D). 27OHChol increased the levels of CCL2 transcripts, and the increase was significantly reduced in the presence of CHX, as determined by real-time PCR. The results indicate that 27OHChol induced expression of CCL2 via CHX-sensitive mechanisms. Polymyxin B was used to determine whether contaminating endotoxin in the reagents, if any, played a role in CCL2 expression (Fig. 1E). Polymyxin B inhibited the transcription of CCL2 induced by LPS whereas it did not affect the expression of CCL2 induced by 27OHChol.

3.2. Enhanced expression of CCL2 in ApoE$^{-/-}$ mice fed a high-cholesterol diet

Hypercholesterolemia leads to increased levels of oxysterols, including 27OHChol [4]. Therefore, we investigated the question of whether CCL2 expression was enhanced in the aortas and sera of ApoE$^{-/-}$ mice after a high-cholesterol diet. CCL2 transcripts were weakly detected in eight aortas out of eight wild-type mice, while the aortas of ApoE$^{-/-}$ mice showed increased transcription of CCL2 (Supplementary Fig. 1B). Expression of CCL2 transcripts was enhanced in six aortas out of seven ApoE$^{-/-}$ mice. When band intensity was normalized to its corresponding GAPDH band intensity, CCL2 intensity was significantly elevated in the aortas of ApoE$^{-/-}$ mice compared to that in wild-type mice (Fig. 1F). Sera isolated from ApoE$^{-/-}$ mice exhibited significantly elevated levels of CCL2, compared with wild-type mice (Fig. 2A). The mean level of CCL2 detected in sera of wild-type mice was 144.8 ± 14.6 pg/mL, and it increased to 465.8 ± 13.3 pg/mL, in sera from ApoE$^{-/-}$ mice. These results indicate an association of a high-cholesterol diet with enhanced production of CCL2 *in vivo*.

3.3. Enhanced secretion of CCL2 protein in the presence of 27OHChol and migration of monocytic cells in response to CCL2

The amount of CCL2 secreted from THP-1 cells was determined in order to determine whether monocytic cells secreted CCL2 proteins in response to 27OHChol (Fig. 2B). THP-1 cells a secreted low amount of CCL2 (19.7 ± 2.2 pg/mL) in the absence of stimulation, and showed an increase of approximately 8.6-fold in the presence of 27OHChol. Treatment with cholesterol and 7K did not resulted in enhanced secretion of CCL2, in agreement with real-time PCR results.

We performed chemotaxis assay in order to determine whether secreted CCL2 was functional. Migration of monocytic cells was significantly induced in response to the supernatant containing CCL2, which was isolated after stimulation of THP-1 cells with 27OHChol. However, the supernatants isolated from THP-1 cells treated with cholesterol and 7K, which contained basal amounts of CCL2, did not enhance migration of monocytic cells (Fig. 2C). A neutralizing antibody was used to determine whether CCL2 was critical for migration (Fig. 2D). Addition of anti-CCL2 neutralizing antibody resulted in complete inhibition of migration of monocytic cells. In contrast, addition of isotype IgG control antibody did not result in attenuated migration. These results indicate that CCL2

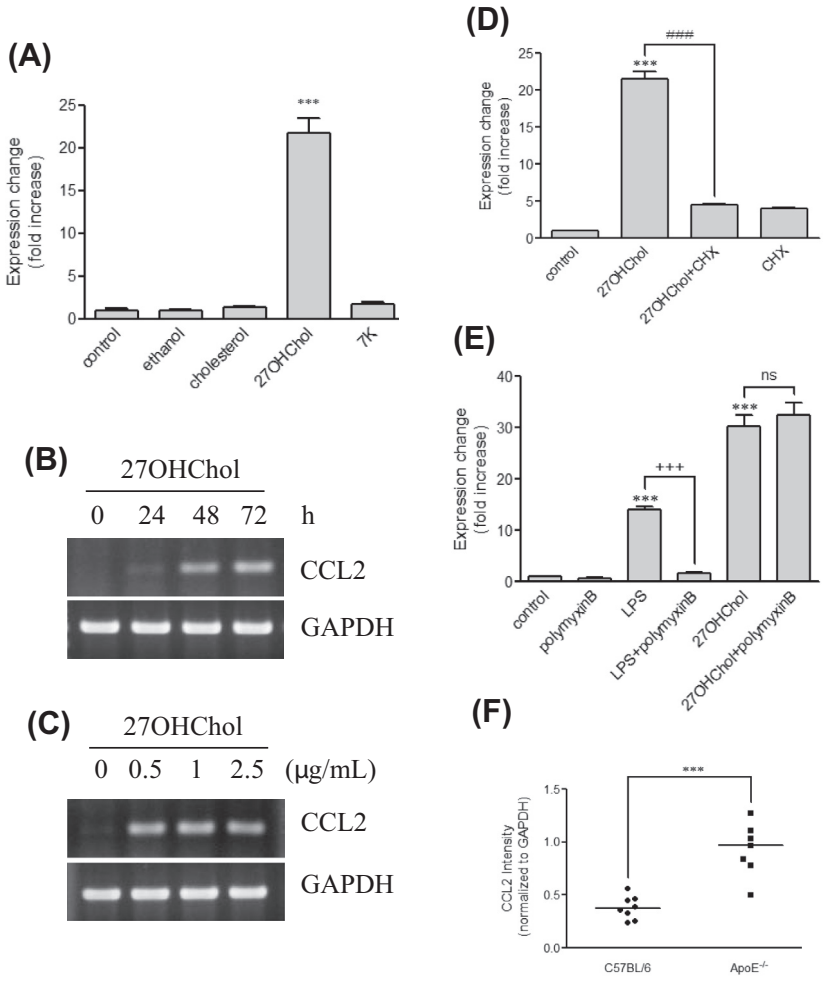

Fig. 1. Transcription of CCL2 in THP-1 cells in the presence of 27OHChol and in ApoE knockout mice after consumption of high-cholesterol-diet. (A) THP-1 cells were treated with cholesterol, 7K (5 μg/mL each), and 27OHChol (2.5 μg/mL) for 48 h. The relative expression ratio of CCL2 transcripts was determined by real-time PCR. The level of CCL2 transcripts was plotted as averages of fold changes in comparison with control. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control. (B, C) After treatment of THP-1 cells with 27OHChol (2.5 μg/mL) for the indicated time periods (B) or with the indicated amount of 27OHChol for 48 h (C), CCL2 transcripts were amplified by RT-PCR. (D) THP-1 cells were stimulated with LPS (100 ng/mL) and 27OHChol (2.5 μg/mL) in the absence or presence of polymyxin B (10 μg/mL). The relative expression ratio of CCL2 transcripts was determined. The level of CCL2 transcripts was plotted as averages of fold changes in comparison with control. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. 27OHChol; $^{+++}P < 0.001$ vs. LPS. (E) THP-1 cells were stimulated with 27OHChol (2.5 μg/mL) in the absence or presence of CHX (1 μg/mL). The relative expression ratio of CCL2 transcripts was determined. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. 27OHChol. (F) The intensity of CCL2 (Supplementary Fig. 1B) was normalized to GAPDH intensity. Each point represents normalized intensity of the CCL2 band amplified from the aorta of individual mice. (n = 8 wild-type mice and 7 ApoE$^{-/-}$ mice). $^{***}P < 0.001$ vs. C57BL/6 mice.

secreted from THP-1 cells in response to 27OHChol induced chemotaxis of monocytic cells.

3.4. Attenuation of 27OHChol-induced expression of CCL2 and cell migration in the presence of an LXR agonist

We attempted to investigate whether the LXR agonist regulated expression of CCL2. Transcription of CCL2 induced by 27OHChol was significantly attenuated in the presence of TO901317 (Fig. 3A). Treatment with TO901317 also resulted in a reduction in CCL2 production (Fig. 3B). We assessed the question of whether attenuated production of CCL2 affected migration of monocytic cells. Monocytic cells migrated in response to supernatants isolated from THP-1 cells treated with 27OHChol. However, the migration was significantly inhibited when monocytic cells were exposed to supernatants isolated from THP-1 cells treated with 27OHChol in combination with TO901317 (Fig. 3C). These results indicated that TO901317 attenuates CCL2 expression induced by 27OHChol, thereby reducing migration of monocytic cells.

3.5. Major role of Akt1 isoform in 27OHChol-induced expression of CCL2

We assessed roles of Akt kinases in 27OHChol-induced expression of CCL2 after pharmacological inhibition. Treatment with an Akt inhibitor resulted in blockade of CCL2 expression. Akt inhibitor IV almost completely inhibited transcription of the CCL2 gene and abrogated secretion of CCL2 protein by 27OHChol (Fig. 3D and Supplementary Fig. 2A). We attempted to investigate the role of Akt1 isoform in expression of CCL2. Akt1-knockdown cells were generated after infection of lentiviruses encoding *Akt1* shRNA, and knockdown efficiency was determined by western blot analysis. Cells infected with lentiviruses encoding *Akt1* shRNA showed markedly reduced level of Akt1 protein, however, the level of Akt2 protein remained unchanged (Fig. 3E). Treatment of THP-1 cells infected with control lentiviral vector with 27OHChol resulted in enhanced secretion of CCL2 protein and transcription of the CCL2 gene. However, such enhanced secretion and transcription did not occur in Akt1-knockdown cells (Fig. 3F and Supplementary

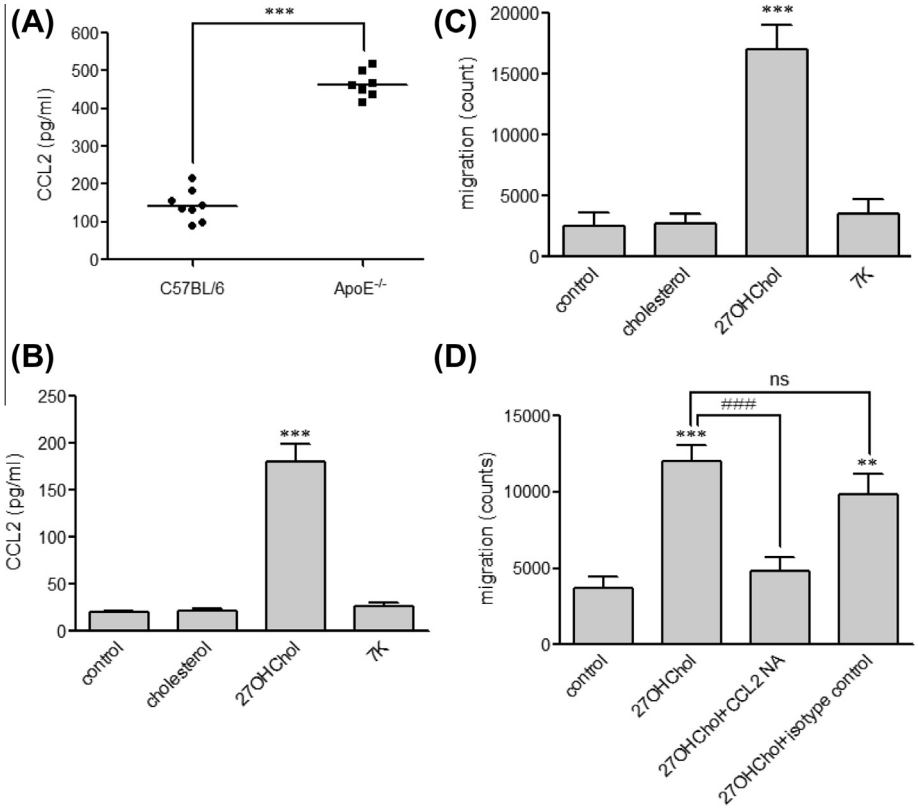

Fig. 2. Enhanced secretion of CCL2 in a cholesterol-rich milieu and activity of secreted CCL2 from THP-1 cells exposed to 27OHChol. (A) The levels of CCL2 in sera isolated from wild-type mice and ApoE$^{-/-}$ mice fed a high-cholesterol diet were measured by ELISA. Each point represents the blood level of CCL2 of individual mice (n = 8 wild-type mice and 7 ApoE$^{-/-}$ mice). $^{***}P < 0.001$ vs. C57BL/6 wild-type mice. (B) Conditioned media were harvested after treatment of THP-1 cells (2×10^5 cells/mL) with cholesterol, 7K, and 27OHChol for 48 h, as described above. The amount of CCL2 secreted into the media was measured by ELISA. Data are expressed as mean ± SD (n = 3 replicates/group). Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control. (C) Monocytic cells were exposed to the conditioned media isolated above and migration of monocytic cells was measured by chemotaxis assay. Data are expressed as mean ± SD (n = 3 replicates/group). $^{**}P < 0.01$ vs. control. (D) Monocytic cells were exposed to conditioned media isolated from THP-1 cells treated with 27OHChol after preincubation for 30 min with or without an anti-CCL2 neutralizing antibody (R&D Systems, clone # 24822) or isotype IgG control (10 μg/mL each). Migration of monocytic cells was determined by chemotaxis assays. Data are expressed as mean ± SD (n = 3 replicates/group). $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol.

Fig. 2B). These results indicated that inactivation of Akt1 was sufficient for regulation of 27OHChol-induced expression of CCL2 in THP-1 cells.

4. Discussion

27OHChol and 7K were chosen because they are the two most abundant cholesterol-derivatives, in addition to cholesterol, in atherosclerotic lesions [5]. 27OHChol, but not cholesterol and 7K, induced significant expression of CCL2 at the transcription and protein levels. These results are in agreement with those of a previous study reported by Leonarduzzi et al. in that certain types of oxysterols are active constituents involved in induction of chemokine expression. Leonarduzzi et al. reported upregulation of CCL2 by an oxysterol mixture and 7αOHChol in human promonocytic U937 cells; however, the study did not investigate the effects of the most abundant oxysterol in the lesions on CCL2 expression or examine possible contribution of endotoxin to CCL2 expression induced by oxysterol mixture and 7αOHChol [12]. In the current study that possibility was ruled out using polymyxin B, which binds to LPS and prevents its biological effects, as treatment with polymyxin B did not lead to attenuated expression of CCL2 induced by 27OHChol. These results indicate that 27OHChol is likely to be responsible for enhanced transcription of CCL2 in monocytic cells in the atherosclerotic artery.

CCL2 recruits monocytes to the site of inflammation, and the chemokine plays a critical role in pathogeneses of diseases characterized by monocytic infiltrates, such as psoriasis, rheumatoid arthritis and atherosclerosis [13]. We demonstrated enhanced migration of THP-1 monocytic cells in response to conditioned media containing high levels of CCL2 secreted in response to 27OHChol. Migration can be said to be specific due to the fact that it was completely inhibited by CCL2 neutralizing antibodies. CCL2 neutralizing antibodies have been employed in order to prove CCL2-mediated migration of mononuclear cells as well as cancer cells [14]. In addition, migration was significantly attenuated when secretion of CCL2 was impaired by an LXR agonist. These results indicate that CCL2 protein secreted from monocytic cells exposed to 27OHChol can induce migration of monocytic cells.

T-0901317, a synthetic LXR agonist, inhibited progression of atherosclerosis by reducing monocyte adhesion and lesional macrophage content in mice fed a high-cholesterol diet [15,16]. Therefore, LXR receptors have been proposed as targets for atherosclerosis therapy. However, mechanisms of action through which LXR agonists exert atheroprotective effects remain to be elucidated. Previously, we reported that T-0901317 inhibited expression of TNF-α induced by 27OHChol and 7αOHChol [9]. In the current study, we demonstrated a new role of the LXR agonist, inhibition of CCL2 production and monocytic cell migration induced by 27OHChol. We propose that regulatory effects of TO-901317 on expression of CCL2 as well as on migration of monocytic cells will contribute to anti-inflammatory and eventually atheroprotective effects of LXR agonists.

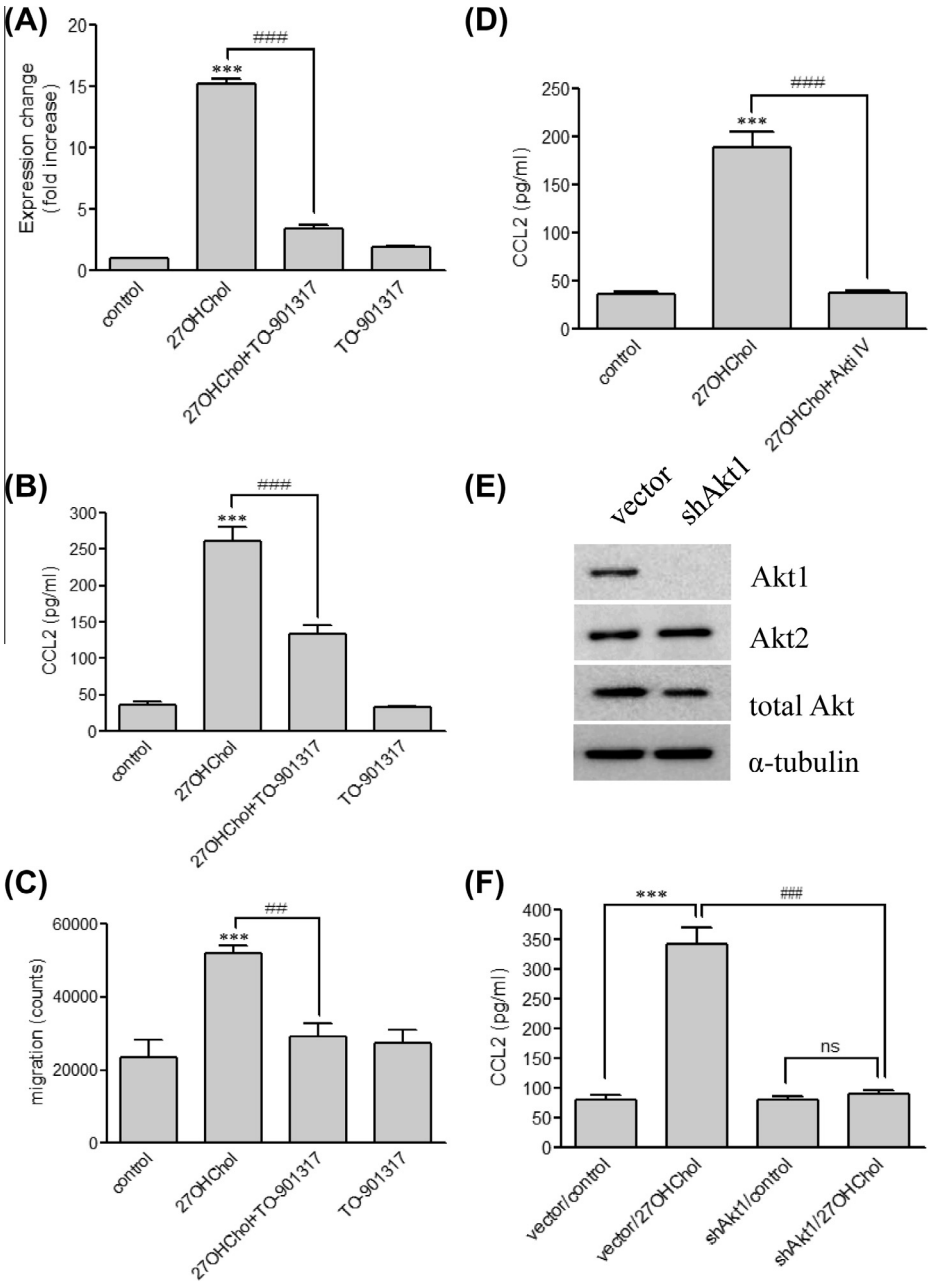

Fig. 3. Roles of LXR and Akt1 in 27OHChol-induced expression of CCL2. (A) THP-1 cells were stimulated for 48 h with 27OHChol in the absence or presence of TO-901317 (1 μM). The relative expression ratio of CCL2 transcripts was determined. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol. (B) The amount of CCL2 released into the media was measured. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol. (C) Conditioned media were isolated after stimulation of THP-1 cells with 27OHChol for 48 h in the absence or presence of TO-901317 (1 μM). Monocytic cells were exposed to the conditioned media, and migration of monocytic cells was assessed. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol. (D) After stimulation of THP-1 cells with 27OHChol for 48 h in the absence or presence of Akti IV, the amount of CCL2 secreted into the culture media was measured. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. 27OHChol. (E) THP-1 cells were infected with lentiviruses expressing Akt1 shRNA or control lentiviruses. After selection in the presence of puromycin, expression of Akt1 and Akt2 was examined by Western blot analysis. (F) THP-1 cells infected with lentiviruses expressing Akt1 shRNA or control lentiviruses were stimulated for 48 h with or without 27OHChol. The amount of CCL2 secreted from the cells into the culture media was measured. Data are expressed as mean ± SD (n = 3 replicates/group). $^{***}P < 0.001$ vs. vector/control; $^{\#\#\#}P < 0.001$ vs. shAkt1/control.

Production of CCL2 induced by 27OHChol was almost completely inhibited in cells that were selectively deficient in Akt1. The results are in agreement with those of a study by Di Lorenzo et al. which reported reduced inflammation as well as decreased leukocyte infiltration in Akt1-deficient mice [17]. Collectively, these results point to Akt1 as a critical factor determining inflammatory response. However, Fernandez-Hernando et al. reported that a global deficiency in Akt1 resulted in enhanced atherosclerotic lesion burden and promoted coronary atherosclerosis in a mouse model of atherosclerosis due to increased apoptosis in endothelial cells and macrophages and decreased bioavailability of nitric oxide [18]. Conduct of further study appears to be necessary in order to understand the roles of Akt1 in regulation of inflammatory responses using ApoE$^{-/-}$ or LDL receptor$^{-/-}$ mice that are deficient in Akt1 in monocytic lineage cells, as inflammatory response in atherosclerotic plaques are mediated primarily by monocytes/macrophages.

On the basis of our results and previous findings, we propose a model via which 27OHChol contributes to accumulation of monocytic cells in atherosclerotic lesions (Fig. 4). Cholesterol is

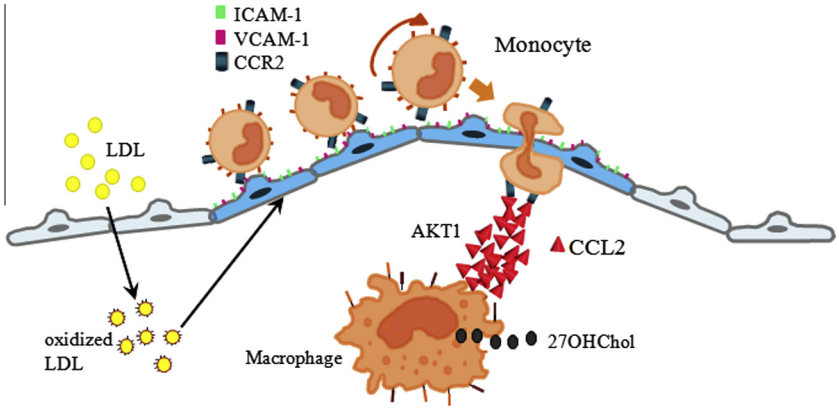

Fig. 4. A proposed model for monocyte recruitment in atherosclerosis. Macrophages secret CCL2 when they are exposed to 27OHChol via mechanisms that are mediated by Akt1. Monocytes express high levels of CCR2 on cell surface, the receptor for CCL2. Monocytes that are attached to endothelial cells of the atherosclerotic arteries migrate after recognition of the CCL2 that is produced in response 27OHChol.

deposited and undergoes oxidative modification to cholesterol oxides, including 27OHChol, in the intima of the arteries. Macrophages exposed to the oxysterol are activated and secrete CCL2 via mechanisms that are mediated by Akt1 and regulated by LXR. Monocytic cells that are attached to cell adhesion molecules expressed on activated endothelium recognize CCL2 and migrate following the signal of increased CCL2 concentration. Oxidized low-density lipoprotein (oxLDL) induces expression of cell adhesion molecules on endothelial cells and enhances attachment of monocytes to the endothelial cells [19]. Therefore, the presence of oxLDL will precipitate monocyte recruitment in response to 27OHChol.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.052.

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