Inflammation plays a pivotal role in the formation of atherosclerosis. In addition to being a risk marker for cardiovascular diseases, the role of C-reactive protein (CRP) in atherogenesis has been supported by more recent data. CD40–CD40L system is proven to be an important mediator of several auto-immune and chronic inflammation diseases. Interruption of CD40–CD40L signaling pathway not only reduces the initiation and progression of atherosclerotic lesions, but also modulates plaque architecture. By using a flow cytometry and western blotting, we found that incubation of human umbilical vein endothelial cells (HUVECs) with CRP resulted in a time- and dose-dependent increase in the cell-surface expression of CD40 and CD40L. In addition, CRP (25 μg/ml) increased gelatinolytic activities of MMP-2 and MMP-9. Anti-CR40 antibody significantly reversed the upregulated activities of MMP-2 and MMP-9 induced by CRP with gelatin zymography. Furthermore, lovastatin (10⁻⁷, 10⁻⁶, 10⁻⁵ mol/l) and fenofibrate (5×10⁻⁵, 10⁻⁴, 2×10⁻⁴ mol/l) significantly diminished the expression of CD40, CD40L and gelatinase activities (MMP-2, MMP-9) induced by CRP in HUVECs. In conclusion, our data provide evidence to support the direct pro-inflammatory effects of CRP via CD40–CD40L signaling pathway involved in the pathogenesis of atherosclerosis, and lovastatin and fenofibrate possess anti-inflammatory effects independent of their lipid-lowering action.

Key words C-reactive protein (CRP); CD40; CD40L; MMP-9; lovastatin; fenofibrate

MATERIALS AND METHODS

Reagents Dulbecco’s Modified Eagle Medium Dulbecco (DMEM) and fetal bovine serum (FBS) were produced by Gibco RBL (Grand Island, NY, U.S.A.). lovastatin, fenofibrate and endothelial cell growth supplement were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Membrane protein extraction reagent kit (containing reagents A, B, and C) was purchased from Pierce Biotechnology Co. (U.S.A.). Mouse anti-human CD40 and CD40L, goat anti-mouse FITC-conjugated IgG and alkaline phosphatase conjugated goat anti-mouse antibody immunoglobulin IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Penicillin and streptomycin were from Medical Faculty of Beijing (China). Recombinant human C-reactive protein (CRP) was purchased from Biodesig (U.S.A.). CRP was checked by SDS-PAGE, yielding a single band when 1 μg was loaded on the gel. Endotoxin was removed from CRP with Detoxigel column and found to be <0.125 EU/ml by limulus assay. All media were tested for endotoxin and found to be <0.125 EU/ml.

Cell Culture Human umbilical vein endothelial cells
(HUVECs) were isolated from fresh umbilical cords obtained at normal deliveries, which was in accordance with the ethical standards formulated in the Helsinki Declaration. The umbilical vein was cannulated with 50 ml of PBS to remove any blood. After that the vein was filled with 20 ml of 0.1% collagenase dissolved in PBS and incubated for 15 min at 37 °C. The collagenase solution was drained from the cord and collected, and the cord was gently flushed with 20 ml of PBS, which was added to the collagenase solution. The cells in these pooled solutions were recovered by centrifugation at 1000 rpm for 5 min and transferred to dishes in DMEM containing 20% FBS, penicillin (100 U/ml), streptomycin (100 mg/l), and 25 µg/ml endothelial cell growth supplement at 37 °C in 5% CO2. The cultured cell monolayer was identified with phase-contrast microscopy. At confluency, cells were trypsinized, counted and seeded in 4 or 24-well flat-bottomed plates with a density of 1×10^5 or 2×10^4 cells/cm².

After the cells grew for 24 h, culture fluid was changed and HUVECs were washed by D-Hank’s solution (NaCl 8 g, KCl 0.4 g, Na2HPO4 0.06 g, KH2PO4 0.06 g, NaHCO3 0.35 g in 1 l) for three times and then was further cultured in DMEM with free FBS for the experimental treatment. In all the experiments, HUVECs were used at second or third passage.

**Flow Cytometry** After the cells grew for 24 h in free FBS, (1) Different concentrations (0, 5, 10, 25, 50, 100 µg/ml) of CRP were added to the wells, the plates were incubated for another 24 h. The medium of cells was collected for flow cytometry; (2) The 25 µg/ml CRP was added to the wells, the plates were incubated for another 3, 6, 9, 12, 24 and 48 h, the medium of cells was collected for flow cytometry; (3) Different concentrations of lovastatin (0, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/l) and fenofibrate (0, 5×10⁻⁵, 10⁻⁴, 2×10⁻⁴ mol/l) in DMEM were added to the wells. The plates were incubated for 24 h; cells were then incubated with CRP (25 µg/ml) for another 24 h. In control group, DMEM with free FBS was used instead of CRP. Expression of CD40 and only a little CD40L. However, treatment of HUVECs, a dose–response experiment on cell-surface protein expression of these molecules in cultured HUVECs was performed.

**Western Blotting** Membrane protein extractions were separated by SDS-PAGE under reducing conditions and blotted to polyvinylidene difluoride membranes (Bio-Rad) using a semidry blotting apparatus. After 24 h, blots were washed 3 times with PBS and then, alkaline phosphatase conjugated goat anti-mouse antibody immunoglobulin IgG was used as the secondary antibody (1 : 500) and immunoreactive bands were revealed by NBT/BCIP colorimetry.

**Gelatin Zymography** Gelatinolytic activities of MMP-2 and MMP-9 were analyzed using gelatin zymography. Medium samples containing DMEM with CRP for another 24 h after exposed to lovastatin (0, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/l) and fenofibrate (0, 5×10⁻⁵, 10⁻⁴, 2×10⁻⁴ mol/l) for 24 h were collected and stored at -20 °C and used for measurements of MMP-2 and MMP-9 activities by zymography. Samples were fractionated in 8% polyacrylamide gel (SDS-PAGE) containing gelatin (1 mg/ml) by electrophoresis at 100 V for 90 min at 4 °C. Molecular weight standard proteins (BioRad) were run simultaneously. The gels were soaked in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS, and incubated in a digestion buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 2 µM ZnSO₄, and 0.01% Triton X-100) at 37 °C overnight to allow protease digestion of its substrate. Gels were rinsed again in distilled water, stained with 0.25% Coomassie brilliant blue R-250 in methanol : acetic acid : water (30 : 10 : 60, v/v/v) for 2 h, and destained with methanol : acetic acid : water (10 : 10 : 80, v/v/v). Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

**Statistical Analysis** Data were presented as mean±standard deviation (S.D.), and groups were compared using ANOVA. A p value of ≤0.05 was considered significant.

**RESULTS**

**Dose-Dependent Manner of CD40 and CD40L Cell-Surface Expression Induced by CRP in HUVECs** To investigate the cell-surface expression of both CD40 and CD40L induced by CRP involved in atherosclerosis in HUVECs, a dose–response experiment on cell-surface protein expression of these molecules in cultured HUVECs was performed. Unstimulated HUVECs expressed lower level of CD40 and only a little CD40L. However, treatment of HUVECs with CRP (5, 10, 25, 50, 100 µg/ml) for 24 h significantly increased the cell-surface expression of basal CD40 and CD40L protein in a dose-dependent manner. These effects of CRP were already present at concentration of 10 µg/ml (p<0.01), were a significant increase at 25 µg/ml and were maximal at 50—100 µg/ml. An increase in CRP concentration up to 100 µg/ml resulted in only a further modest increase in the cell-surface expression of CD40 cells that were incubated on ice for 30 min with overwetting every 5 min, and then centrifuged at 10000 rpm for 2 min at 4 °C. The supernatant was transferred to new tubes and centrifuged at 10000 rpm for 2 min to isolate the hydrophobic fraction containing membrane protein. The membrane protein was measured with BradFord.

**HUVEC Membrane Protein Extraction** Different concentrations ofLovastatin (0, 10⁻⁷, 10⁻⁶, 10⁻⁵ mol/l) and fenofibrate (0, 5×10⁻⁵, 10⁻⁴, 2×10⁻⁴ mol/l) in DMEM were added to the wells. The plates were incubated for 24 h; cells were then incubated with CRP (25 µg/ml) for another 24 h. HUVECs (1×10⁶/ml) in the above condition were harvested by trypsinization and were washed three times with PBS. 150 µl solutions of reagent A was added to the cell pellet and pipetted up and down to obtain a homogenous cell suspension, and then incubated for 10 min at room temperature with occasional overwetting. Diluted reagent C (diluted by B) of 450 µl was added to each tube containing 10 µl of the lysed
and CD40L (Fig. 1A), suggesting that the cell-surface expression of CD40 and CD40L depended on CRP concentration.

**Time-Dependent Manner of CD40 and CD40L Cell-Surface Expression Induced by CRP in HUVECs** The treatment of HUVEC with 25 μg/ml of CRP time-dependently increased the expression of basal CD40 and CD40L protein after 6 h \((p<0.01)\) between 6 h and 24 h. The maximal cell-surface expression of CD40 and CD40L occurred after 24 h, maintaining a plateau at least 24 h. In other words, CRP did not further significantly increase the cell-surface expression of CD40 and CD40L beyond 24 h (Fig. 1B). It indicated that CRP induced the cell-surface expression of CD40 and CD40L in a time-dependent manner in the limited concentrations.

**Lovastatin and Fenofibrate Reduced the Expression of CD40 and CD40L Induced by CRP in HUVECs** To examine whether lovastatin and fenofibrate influenced the expression of CD40 and CD40L induced by CRP in HUVEC involved in the pathogenesis of atherosclerosis, we explored the expression of CD40 and CD40L induced by CRP with flow cytometry and western blotting. After the preincubation of cells with lovastatin \((0, 10^{-7}, 10^{-6}, 10^{-5}\ \text{mol/l})\) for 24 h and then using CRP 25 μg/ml as a stimulus for another 24 h, results from western blotting revealed that the unstimulated cells showed a little expression of CD40 and CD40L protein, and CRP-stimulated cells showed more expression. Treatment with lovastatin reduced CD40 and CD40L expression induced by CRP in a dose-dependent manner. At concentration of \(10^{-6}\ \text{mol/l}\), lovastatin reduced the expression of both CD40 and CD40L \((p<0.01)\); maximal inhibition was achieved at more than \(10^{-5}\ \text{mol/l}\). To determine whether the modulation of the expression of CD40 and CD40L extended to fenofibrate, the paralleled expression was observed in fenofibrate \((0, 5\times10^{-5}, 10^{-4}, 2\times10^{-4}\ \text{mol/l})\). Fenofibrate at low concentration similarly reduced CD40 and CD40L expression induced by CRP (Figs. 2A, B). In accordance with the findings for whole-cell lysates, results from flow cytometry showed that lovastatin and fenofibrate concentration-dependently diminished the cell-surface expression of CD40 and CD40L induced by CRP (Fig. 3). Notably, concentrations of lovastatin and fenofibrate required to inhibit the expression of CD40 and CD40L resembled each other in the western blotting and flow cytometry. Neither cell synthesis nor protein synthesis was affected at these concentrations (data not shown).

Combined, studies with flow cytometry and western blotting suggested that lovastatin and fenofibrate reduced expression of CD40 and CD40L induced by CRP in a dose-dependent way.

**Effects of Lovastatin and Fenofibrate on Gelatinolytic Activities of MMP-2 and MMP-9 Induced by CRP in HUVECs** To evaluate the effect of CRP (25 μg/ml) on the regulation of MMP-2 and MMP-9 gelatinolytic activities and action of lovastatin and fenofibrate on the gelatinolytic activities induced by CRP, the relative enzyme activities of MMP-2 and MMP-9 present in the above conditioned media were determined with gelatin zymograph (Fig. 4). All conditioned media showed a major gelatinase band of 92 kD, representing pro-MMP-9. Under the unstimulated conditions, there was not the 72 kD pro-MMP-2 and the activated MMP-2 at 66 kD. Zymograph analysis showed that culture supernatants from the CRP-induced cells had a significant increase of gelatinase activities when compared with those from the unstimulated cells. This increase was located at molecular weight of 92 kD corresponding to the molecular weight of the precursors of pro-MMP-9 and two bands at 72 kDa and 66 kD were also seen corresponding to the molecular weight of pro-MMP-2 and the activated MMP-2. Addition of an anti-CD40 MAb blocked the gelatinolytic activities of MMP-2 and MMP-9 induced by CRP. Lovastatin and fenofibrate markedly inhibited MMP-2 and MMP-9 activities induced by CRP in a dose-dependent way. The inhibitory effects occurred at concentration of lovastatin \(10^{-6}\ \text{mol/l}\) \((p<0.01)\) and fenofibrate \(10^{-4}\ \text{mol/l}\) \((p<0.01)\). These results indicated regulation of MMP-2 and MMP-9 gelatinase activities via CD40-CR40L signaling pathway induced by CRP and lovastatin and fenofibrin significantly inhibited the stimulatory effect of CRP on the MMP-2 and MMP-9 activities in a dose-dependent way in HUVECs.
DISCUSSION

Atherosclerotic cardiovascular disease represents the most common cause of death, and results from an intricate interplay between diverse factors such as lipid metabolism, blood coagulation elements, cytokines, hemodynamic stress and behavioral risk factors. For some years, endothelial cells, smooth muscle cells, and macrophages have been accorded crucial roles in the process of atherosclerosis. The mechanisms by which these cells contribute to atherosclerosis include the augmented expressions of adhesion molecules and matrix metalloproteinases, the increased secretion of proinflammatory cytokines in human and experimental atheroma.\textsuperscript{17} Our knowledge of the pathogenesis and treatment of atherosclerosis has progressed exponentially and however, less than 50% of coronary artery disease can be ascribed to traditional risk factors. It is now well accepted that inflammation plays a central role in the development of atherosclerosis and its complication.\textsuperscript{18,19} Chronic inflammation results in endothelial dysfunction and facilitates the interactions between the modified lipoproteins, monocyte-de-
rived macrophages, T cells and normal cellular elements of the arterial wall. Indeed, inflammation processes have been implicated in each facet of atherogenesis described above. Attention has thus focused on identifying the novel markers and mechanisms of atherosclerosis, of which CRP has emerged as one of the most important.29,40

Accumulating evidences suggest that the elevated levels of CRP have emerged as one of the most powerful independent predictors of myocardial infarction, stroke and vascular death, with prognostic value exceeding that of LDL cholesterol.21 In addition to being a powerful risk marker, recent evidences suggest that CRP not only participates in lesion formation, but also alters plaque architecture in the favor of rupture.22,23 Human recombinant CRP, at concentrations known to predict vascular diseases, elicits a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype. For example, CRP potently downregulates eNOS transcript and destabilizes eNOS mRNA, with resultant decreases in both basal and stimulated NO release.8 In a synchronous fashion, CRP has been shown to stimulate endothelin-1 and interleukin-6 and -8 releases, to upregulate adhesion molecules, complement-inhibitory factors and to stimulate monocyte chemotactic protein-1, while facilitating macrophage LDL uptake.24—27 More recently, the proatherosclerotic effects of CRP also seem to be modified by risk factors and treatment strategies. For example, hyperglycemia potentiates the effects of CRP on endothelial cell activation and pharmacological interventions with statins, PPAR gamma agonist (rosiglitazone), and bisentan attenuate these processes.28 Thus, CRP is not only an inflammatory marker of atherosclerosis/coronary events, but also a mediator of the diseases because it contributes to the substrate underlying lesion formation, plaque rupture and coronary thrombosis by interacting with and altering the endothelial cell phenotype.

Despite the increasing appreciation that atherogenesis involves participation of CRP within cellular interactions, mediators of local communication between the major cell types within atherosclerotic plaques remain incompletely defined. Recent studies have demonstrated that CD40–CD40L inflammatory signaling pathway is a potent activator of endothelial cells and promoter of atherosclerosis.29,30 Increasing evidences support the central role of the CD40–CD40L signaling pathway in atherosclerosis. Our present studies were to assess the possible CRP-induced expression of CD40—CD40L signaling pathway in HUVECs involved in atherosclerosis. Activation of vascular cells via CD40—CD40L signaling pathway interactions has shown to induce inflammatory responses with expression of adhesion molecules, secretion of pro-inflammatory cytokines, matrix metalloproteinases, tissue factor and chemokines,30 molecules considered as crucial players in atherogenesis. CD40—CD40L blockade has shown to prevent atherosclerotic plaque progression, to promote plaque stability and to prevent transplant associated vasculopathy, an accelerated form of atherosclerosis.31,32 Our studies found that CRP, at concentration known to predict adverse cardiovascular events, caused a marked and dose-dependent increase in the cell-surface expression of CD40 and CD40L protein in HUVECs. Unstimulated HUVECs with CRP expressed lower level of CD40 and only a little CD40L, which was in accordance with previous report.33 But our results showed that CRP markedly increased the cell-surface expression of CD40 and CD40L, with maximal effect observed at 50—100 μg/ml. Increases in CRP concentrations beyond 50 μg/ml did not further significantly increase CD40 and CD40L expression, indicating the potent effect at low concentrations of CRP. The effect of CRP on increase of CD40 and CD40L cell-surface expression was maximal at 24 h. Data from the present study suggest that CRP increases CD40 and CD40L expression, which can further increase expression of adhesion molecules, secretion of pro-inflammatory cytokines and MMP to augment inflammatory response in atherosclerosis. Although some cytokines like IL-6 are potent hepatic stimuli for CRP,7 maybe it represents a positive feedback mechanism for signaling pathway of CRP-induced CD40—CD40L expression, which markedly enhances inflammatory response in atherosclerosis.

We also found that culture supernatants from CRP-induced cells had a significant increase in gelatinase activities (MMP-2, MMP-9) when compared with those from the unstimulated cells. The MMP family of enzymes probably plays a crucial role in undermining the integrity of the tissue in an atherosclerotic lesion, favoring plaque rupture and precipitation of the unstable coronary syndromes.34 Several members of the MMP family contribute to collagen degrada-
tion: interstitial collagenase (MMP-1) initiates degradation of collagen types I, II and III with a limited cleavage, followed by further breakdown performed by gelatinase A (MMP-2, 72 kD gelatinase), stromelysin (MMP-3) and gelatinase B (MMP-9, 92 kD gelatinase). These enzymes also degrade elastin, collagen type IV, gelatin and fibronectin as well as other components of the extracellular matrix.\textsuperscript{35} Initially synthesized as inactive zymogens, biological activity of the MMPs requires processing of the precursor.\textsuperscript{36} Human vascular cells \textit{in vitro} express MMP-1, gelatinases A and B (MMP-2, MMP-9) and MMP-3 upon stimulation with soluble mediators such as IL-1 and tumor necrosis factor-\textalpha. We recently found that CRP increased gelatinase activities (MMP-2, MMP-9) in HUVECs, but preincubation with anti-CD40 antibody significantly reversed the upregulation activities of MMP-2 and MMP-9 induced by CRP. It is reported that preincubation of endothelial cells with macrophages for 18 h increases MMP-2 and MMP-9 expression, and anti-CD40 antibody block the expression of MMP-2 and MMP-9.\textsuperscript{37} Therefore, increase in MMP-2 and MMP-9 gelatinase activities induced by CRP depends on CD40–CD40L signaling pathway in HUVECs. It is reported that MMP-2 and MMP-9 expression is regulated \textit{via} CD40–CD40L signaling pathway in human vascular smooth muscle cells by T lymphocyte.\textsuperscript{38} So far, there is no report for CRP to activate MMP by another pathway except CD40–CD40L signaling pathway in HUVECs. However, CRP stimulates MMP expression in U937 histiocytes through Fc[gamma]RII and extracellular signal-regulated kinase pathway.\textsuperscript{39} The intracellular signaling mechanisms on an increase in CD40–CD40L expression and MMP activities induced by CRP are under study. In a word, CRP influences not only inflammatory signal, but also plaque stability in atherosclerosis.

From above we reported a new finding to support the involvement of inflammatory response in atherosclerosis. In order to provide further evidence, we studied whether lipid-lowering agents, lovastatin and fenofibrate, possess anti-inflammatory properties independent of their lipid-lowering action \textit{via} CRP-induced CD40–CD40L way and effects on MMP-2 and MMP-9 in treatment of atherosclerosis. \textit{In vitro} and \textit{in vivo} findings have indicated that statins have anti-inflammatory actions, such as the diminished expression of chemokines, major histocompatibility complex II molecules, matrix degrading enzymes, and the procoagulant tissue factor as well as the augmented expression of nitric oxide.\textsuperscript{40,41} Furthermore, statins may reduce serum CRP levels and simvastatin partially inhibits the induction of MCP-1 by CRP on endothelial cells. Recently, experimental studies have suggested that statins reduce the basic, LDL- and IFN-\gamma-induced expressions of CD40 and CD40L in human vascular cells.\textsuperscript{42,43} Furthermore, Statins produce protective action on vascular endothelial cells through stimulation of production of nitric oxide and inhibit the production and development of atherosclerosis through stimulation of interleukin-6 release and reduction of the expression of adhesion molecules in monocytes.\textsuperscript{44,45} Fenofibrate is the peroxisome proliferators-activated receptor activators (PPAR), which may reduce the proinflammatory effects of cytokines on vascular cells and may have beneficial effects on the progression of atherosclerosis in animal models.\textsuperscript{46} It also completely blocks the induction of MCP-1 by CRP. In the present study, lovastatin and fenofibrate significantly diminished the expression of CD40 and CD40L as well as gelatinase activities (MMP-2, MMP-9) induced by CRP in HUVECs. Because CRP can increase CD40 and CD40L expression as well as regulation of MMP-2 and MMP-9 gelatinase activities \textit{via} CD40–CD40L signal pathway, our studies suggested that lovastatin and fenofibrate exert anti-inflammatory effects \textit{via} CD40–CD40L signaling pathway induced by CRP. By inhibiting inflammatory signaling pathway of CD40–CD40L expression, they reduce inflammatory response and thus stabilize atherosclerotic plaques, features believed to account for the beneficial effects of lovastatin and fenofibrate on cardiovascular morbidity and mortality. Notably, concentration of fenofibrate beyond $10^{-4}$ mol/l did not further decrease the expression of CD40 and CD40L induced by CRP. The specific mechanism needs to be found out. Our findings not only provide an additional explanation for previously unknown non-lipid effects of lovastatin and fenofibrate in treatment of atherogenesis, but also suggest their further application in immuno-inflammatory diseases in which CD40–CD40L is involved. In addition, cerivastatin decreases MMP-2 secretion in endothelial cells by Ras inhibition.\textsuperscript{47} In the study, we only showed that lovastatin and fenofibrate diminished gelatinase activities (MMP-2, MMP-9) induced by CRP. This is possibly due to direct inhibition of gelatinase activities or indirect inhibition of gelatinase activities by depression of gelatinase expression. The further work is to be done.

In summary, our studies demonstrate that CRP is able to induce the expression of CD40 and CD40L and to regulate of MMP-2 and MMP-9 gelatinase activities \textit{via} CD40–CD40L signal pathway in HUVECs. These effects can be modulated by pharmacological interventions, particularly lovastatin and fenofibrate. Taken together, our data provide evidences to support direct pro-inflammatory effects of CRP \textit{via} CD40–CD40L signaling pathway involved in the pathogenesis of atherosclerosis, and lovastatin and fenofibrate possess anti-inflammatory effects independent of their lipid-lowering action. It opens a way to new pharmacological strategies for treatment of atherosclerosis.

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REFERENCES