Full Length Research Paper

Influences of rosuvastatin on the expression of matrix metallopeptidase 9 (MMP-9) through extracellular-signal-regulated kinases (ERK) pathway in mononuclear cell line (THP-1) derived macrophages induced by oxidized low density lipoprotein

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The current study aims to probe the changes in matrix metallopeptidase 9 (MMP-9) expression during differentiation as the mononuclear cell line THP-1 is converted into foam cells, as well as the underlying influence and mechanism of rosuvastatin. PMA was used to induce THP-1 cells transform into macrophages and treated with various concentrations, whereas rosuvastatin different concentrations were used for the intervention. Reverse transcription polymerase chain reaction and Western blot were adopted to detect the changes in MMP-9, extracellular-signal-regulated kinases (ERK) and pERK expressions in the cells of each group after the intervention of rosuvastatin. The expression of MMP-9 protein increased significantly in the conversion of THP-1 mononuclear macrophages into foam cells, and was negatively correlated with rosuvastatin in a dose dependent manner. The amount of MMP-9 and pERK decreased significantly by ERK pathway inhibitor PD98059, while ERK expression increased. Rosuvastatin intervention had no obvious effect on the expression levels of MMP-9, ERK and pERK protein. The expression of MMP-9 increased gradually in the conversion of THP-1 cells into foam cells. Rosuvastatin can reverse the expression of MMP-9 protein by suppressing ERK pathway.

Key words: Acute coronary syndrome (ACS), matrix metalloproteinase-9 (MMP-9), Rosuvastatin, oxidized low density lipoprotein (ox-LDL), extracellular-signal-regulated kinases (ERK), pERK.

INTRODUCTION

Angiocardiopathy, mainly in the form of acute coronary syndrome (ACS), is one of the diseases with the highest incidence and death rate (Al-Rasadi, 2011; Mahaffey et al., 2008). ACS pathogenic mechanism is very complicated, with combined action of many kinds of risk factors and pathogenic mechanisms involving rupture of plaque. Among them, oxidized low density lipoprotein (ox-LDL) is an important pathogenic factor of arteriosclerosis in pathological changes. Oxidized-LDL is mediated by

scavenger receptor and is endocytosed by mononuclear/macrophages and smooth muscle cells. In addition, it inhibits the discharge of cholesterin, resulting in large quantities of cholesterin accumulated in the cell to become foam cells (Kita et al., 2001). Research has proved that plenty of foam cells from macrophage apoptosis in plaque exist in most patients suffering from acute coronary artery syndrome. Foam cell apoptosis and lipid infiltration cause the occurrence of ACS, enlarge the

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(Littlewood and Bennett, 2003; Stoneman and extracellular lipid core and enhance plaque instability Bennett, 2004). In the development of atherosclerosis, the balance between the generation and degradation of extracellular matrix in the normal physiological state is disturbed (Nagase et al., 2006), the accumulation of monocyte-macrophages, increase in synthesized matrix metalloproteinases (MMPs), and decrease of inhibitors for endogenous MMPs are the prime reasons for reduction in the thickness of the fibrous cap of atheromatous plaques, strength of anti-injury and instability of the plaque (Newby, 2005). Previous studies show that the concentration of MMP-9 in the plasma of ACS patients increases obviously (Inokubo, 2001) and the excessive expression of MMP-9 can degrade the action of the extracellular matrix. Meanwhile, the concentration of MMP-9 can stimulate the generation of blood vessels, resulting in an unstable plaque, which causes plaque breakage and ACS generation (Heo, 2011). The drug used for inhibiting the expression and activity of MMP-9 has been proven to be helpful for the stability and even the long-term prognosis of ACS plaque, such as left ventricular remodeling and change of heart function (Amalinei, 2010; Nagase, 2006).

Among the drugs for ACS, statins (such as simvastatin, atorvastatin, pravastatin, and rosuvastatin) and drugs inhibiting platelet aggregation (such as aspirin, clopidogrel, and glycoprotein IIb/IIIa receptor antagonist) are used as basic drugs. Among these drugs, statins have the most numerous advantages, which include: improvement of endothelial function, having anti-inflammatory and immunoregulatory effect, oxidation resistance, inhibition of smooth muscle cell hyperplasia and immigration, inhibition of thrombosis, having plaque-stabilizing effect on arteriosclerosis, and other functions excluding lipid regulation (Kinlay et al., 2004; Crisby, 2001). Treatment has proved that statins can inhibit the development of subclinical atherosclerosis. Rosuvastatin, a kind of antioxidant that easily penetrates the endodermis after ingestion, has an obviously greater effect than other statins in improving the lipid level of patients suffering from hypercholesterolemia (Takayama et al., 2009). Previous large-scale clinical studies have proven that rosuvastatin can reduce plaque size, inhibit lipoprotein accumulation in macrophages, and decrease the proportion of low-density lipoprotein (LDL) plaque of the aortic arch while reducing LDL cholesterol (LDL-C). This leads to the promotion of the extinction of atherosclerosis plaque to a certain degree (Guo, 2009). Rosuvastatin can also reduce the incidence of acute coronary syndrome by lowering the hypersensitive C-reactive protein (Ridker et al., 2008; Ridker et al., 2009). However, the impact of rosuvastatin on MMP-9 and its mechanism of action remain unclear. Earlier studies have shown that CD147 can affect the expression of MMP-9 in the macrophages through ERK and NF-κB pathways (Kim et al., 2009). CD147 molecule, as we all know, is a highly glycosylated transmembrane protein; it belongs to an immunoglobulin super family, including two immunoglobulin domains (C2 and I). It can degrade the extracellular matrix by inducing the secretion of MMPs (Jouneau et al., 2011), and also simvastatin can inhibit the formation of abdominal aortic aneurysm in apolipoprotein E knockout mice induced by angiotensin II through ERKpathway (Zhang et al., 2009). Therefore, rosuvastatin is presumed to improve plaque stability by activating ERK pathway and reducing the expression of MMP-9.

To understand further the impact of rosuvastatin on MMP-9 and its mechanism, oxidized LDL is adopted to induce THP-1 monocyte-derived macrophages, which are converted into foam cells. Rosuvastatin in different contents and ERK pathway inhibitor PD98059 are used as interventions, respectively, to observe the effect of rosuvastatin on ERK pathway of the foam cells and the expression of MMP-9 protein. Based on the findings, the non-lipid mechanism of rosuvastatin on acute coronary syndrome can be investigated.

## MATERIALS AND METHODS

THP-1 cells culture

THP-1 monocytes (China Type Culture Collection, Shanghai, China) were maintained at 37°C in humidified 5% CO^2 atmosphere, in suspension with a cell density of 2.0×10^5 to 6.0×10^5/ml. They were placed in RPMI 1640 (GIBCO,UK) containing 10% fetal bovine serum (FBS)(GIBCO,UK), 2 mmol/L glutamine, 100 u/ml penicillin, 100 µg/ml streptomycin, and 0.05 mmol/L 2-mercaptoethanol (Sigma, USA). The cells were then cultured in phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) at a final concentration of 100 nmol/L for two days until they were completely differentiated into macrophage-like cells.

Grouping and drug intervention

All cells were divided into the following groups: (1) the blank control group (the PMA group); (2) the group with foam cells is the blank control group incubated with ox-LDL for 6 h; and (3) the group with foam cells and after that the group inducted with rosuvastatin calcium (0.01, 1, and 10 µmol/L). Equal amounts of ERK inhibitor (PD98059) were added to each group of cells.

Quantitation of immunoreactive MMP-9 by ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed on 96-well microtiter plates using standard protocols. Recombinant MMP-9 was used as the standard. For the detection of MMP-9, the total protein was measured. The microtiter plate was coated with monoclonal antibody (R&D, USA), and samples and standards were added. The bound proteins were detected with a secondary polyclonal antibody produced in human against MMP-9. A peroxidase-labeled antipeople-IgG (R&D, USA) was used for detection of the bound secondary antibody. Colour formation was measured at 450 nm (Anthos 2000 microplate reader; Lab, China) and calculations were done using a Multicalc program (Wallac,
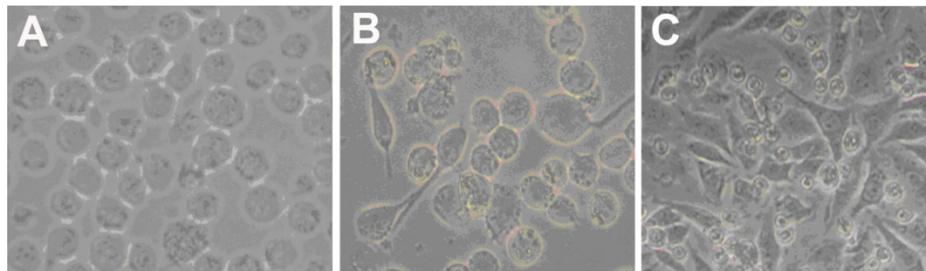
Figure 1. Transformation of PMA-induced THP-1 cells into macrophage. A) Uninduced THP-1 cells; B) THP-1 cells induced by PMA for 24 h; C) THP-1 cells induced by PMA for 48 h (magnification factor: 400×).

JIEDA) (Santhekadur et al., 2011). The monoclonal and polyclonal antibodies against MMP-9 were characterized to recognize both the pro- and active forms of MMP- 9.

RT-PCR

MMP-9 was: sense 5′-CCTGAAGAATGATGGGAGGCAAG-3′ Antisense 5′-GCTCTTGGCAAATCTGGCGTG-3′, Generating 652 bp product.

β-actin was: sense 5′-TCCATCGTGGGCCGCTCTAGGCACCAAGGT-3′, Antisense 5′-ATCTGGGTCATCTTTTCACGGTTGGC-3′, Generating 265 bp product.

Total cellular RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase treatment, as we reported previously (Santhekadu et al., 2011). cDNA was synthesized with Superscript II (RNase II) reverse transcriptase (Invitrogen, Carlsbad, CA) and 10 ng of extracted RNA. Then PCR was performed as described previously. We used the following PCR conditions: the initial denaturation step was at 94°C for 2 min, followed by 33 cycles of denaturation for 1 min at 94°C, annealing was 1 min at 58°C, and extension, 10 min at 72°C. As an internal control for RT-PCR analysis, β-actin transcripts were amplified from the same cDNA samples. Primer sequences are listed on the top. After amplification, PCR products were loaded onto 2% agarose gels, stained with ethidium bromide, electrophoresed, and visualized under ultraviolet illumination.

Western blot analysis

Samples (20 ug) of the cell lysate were subjected to 10% SDS-PAGE (Santhekadur et al., 2011), after which the resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% non-fat milk and 0.1% Tween 20 in TBS. They were probed with anti-ERK, pERK (Santa Cruz, Beverly, MA) and anti-MMP-9 mouse monoclonal antibodies (Abcam, Cambridge, United Kingdom), after which the blots were visualized using enhanced chemiluminescence.

Statistical analysis

All results in this study were expressed as the relative ratio over control (as 100%) unless otherwise indicated. Data were collected from at least three separate experiments and presented as means±SD. SPSS13.0 software was used to analyze difference between samples with one-way analysis of variance (ANOVA); S-N-

K post-test was used for difference between selected pairs of samples. $P<0.05$ was considered statistically significant.

RESULTS

Cellular morphology change of the THP-1 monocyte

THP-1 monocyte induced by PMA was transformed into macrophage after 48 h. Human monocytic line THP-1, which had a round mass, was suspended and cultivated in the culture medium. After PMA induction, the cellular morphology of this cell line became irregular, with enlarged cell body and vacuoles in the cytoplasm. Pseudopodia that stretched from the cell surface began to adhere gradually after 24 h; then became fully adherent, and transformed into macrophage after 48 h completely (Figure 1).

Effects of rosuvastatin on the MMP-9 expression in the transformation of THP-1 monocyte-derived macrophage into foam cells

Effects of rosuvastatin on the MMP-9 expression in the supernate of each group of cells

MMP-9 concentration in the supernate of each group of ox-LDL-induced cells was detected by ELISA. Compared with the uninduced mononuclear macrophage, the MMP-9 increased significantly in the supernate of THP-1 mononuclear macrophage induced by ox-LDL (486.8±38.6 vs 371.3±41.9, $P<0.05$). MMP-9 was negatively correlated with rosuvastatin concentration, like MMP-9 expression in the various groups of cells, where low, middle, or high rosuvastatin concentration was significantly decreased compared to that of the foam cells (299.1±34.3, 201.7±29.5, 115.3±36.1 vs 486.8±11.9, $P<0.01$) (Figure 2).

Effects of rosuvastatin on the MMP-9 gene expression in all groups of cells

RT-PCR was performed to detect the MMP-9 gene
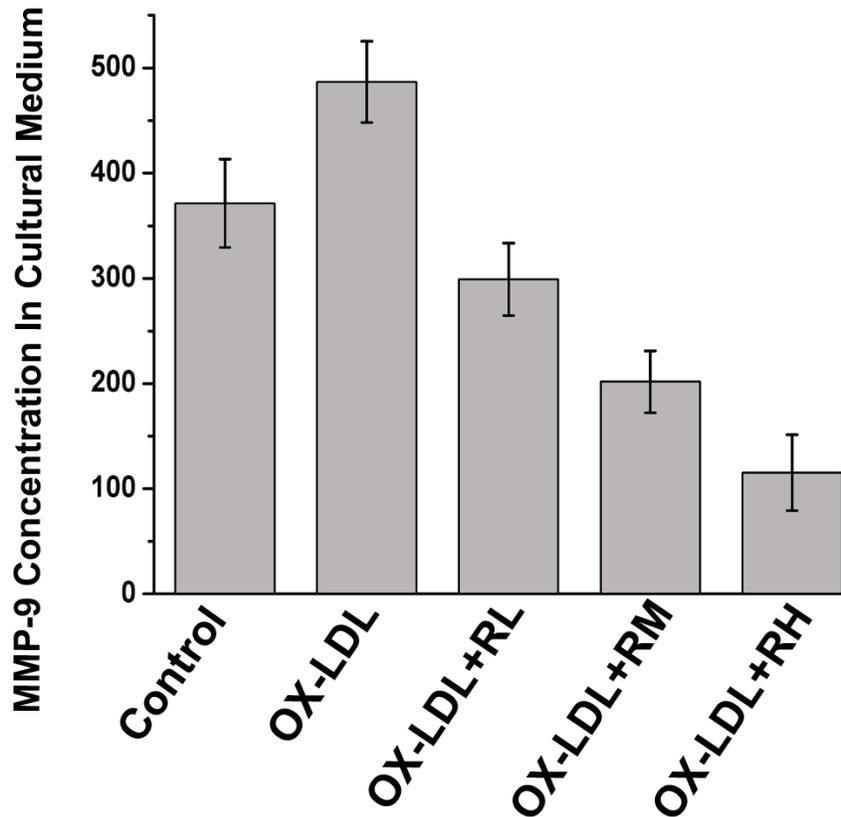
Figure 2. Concentration expression analysis of MMP-9 protein expression in the supernatant from each group of ox-LDL-induced cells by ELISA. This figure is a bar chart of MMP-9 expression in the supernate of each group of cells. In this chart, the control group is the PMA group, whereas the OX-LDL group is the OX-LDL group after induction for 6 h. The OX-LDL+RL group is the group of OX-LDL incubated with low-concentration rosuvastatin group for 6 h, whereas the OX-LDL+RM group is the group of OX-LDL incubated with middle-concentration rosuvastatin group for 6 h; and the OX-LDL+RH group is the group of OX-LDL incubated with high-concentration rosuvastatin group for 6 h.

expression in each group of ox-LDL-induced cells (Figure 3). MMP-9 gene expression in the THP-1 mononuclear macrophage induced by ox-LDL was remarkably higher than that of the uninduced mononuclear macrophage (0.261±0.031 vs 0.187±0.082), in agreement with the test results of the supernates. MMP-9 gene expression and the rosuvastatin concentration exhibited a dose-dependent decreasing trend compared to the foam cells (0.203±0.055, 0.175±0.016, 0.083±0.047 vs 0.261±0.031, respectively). However, there was significant difference in the MMP-9 gene expression compared with that in the group with foam cells at 10 μmol/L rosuvastatin (P<0.05).

Effects of rosuvastatin on MMP-9 protein expression in all groups of cells

MMP-9 protein expression in each group of ox-LDL-induced cells was detected by Western blot (Figure 4). MMP-9 in THP-1 mononuclear macrophage increased

significantly, which when compared with that in the uninduced mononuclear macrophage (0.398±0.041 vs 0.186±0.053, P<0.01), was consistent with those of the supernate and gene tests. MMP-9 protein expression and rosuvastatin concentration showed a dose-dependent decreasing trend compared with the foam cells (0.294±0.037, 0.164+0.032, 0.159±0.014 vs 0.398±0.041, respectively, P<0.01).

Effects of rosuvastatin on ERK and pERK protein expression in all groups of cells

ERK protein expression in each group of ox-LDL-induced cells was also detected by Western blot (Figure 5). ERK protein expression in THP-1 mononuclear macrophage induced by ox-LDL increased significantly compared with that in the uninduced mononuclear macrophage (0.575±0.091 vs 0.195±0.034, P<0.01), which had a remarkable decrease in pERK protein expression
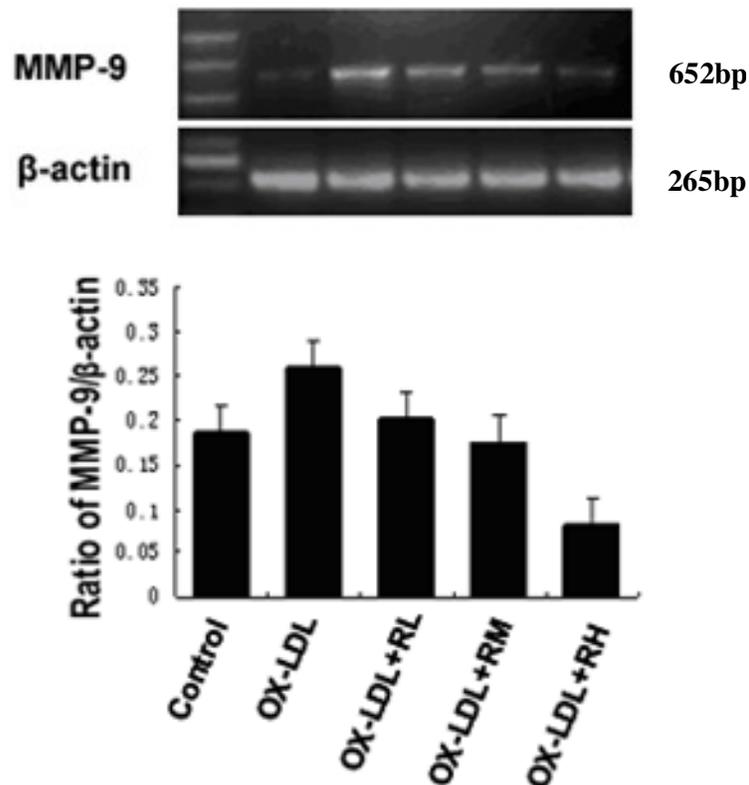
Figure 3. Gene expression analysis of total MMP-9 in each group of ox-LDL-induced cells by RT-PCR. A) Total RNA was extracted and analyzed for expression of MMP-9 RNA in each group, as described previously. β-actin was used as a loading control. B) Densitometric scanning of total MMP-9 RNA in each group (n=5). Quantitative analysis showed significant increase of MMP-9 RNA in OX+LDL group, P<0.05, then the MMP-9 gene exhibited a dose-dependent decreasing trend with rosuvastatin, P<0.05.

(0.188±0.038 vs 0.341±0.063, P<0.05). ERK protein expression and rosuvastatin concentration exhibited a dose-dependent decreasing trend compared with the foam cells (0.35±0.072, 0.248±0.049, 0.182±0.036 vs 0.575±0.091, P<0.01). pERK protein expression and rosuvastatin concentration showed a dose-dependent increasing trend compared with the same group (0.15±0.032, 0.272±0.064, 0.376±0.073 vs 0.188±0.038, P<0.01 at the last one).

**Effects of ERK pathway inhibitor PD98059 on MMP-9 protein expression in THP-1 monocyte-derived foam cell**

MMP-9 protein expression in all groups of cells intervened by the ERK pathway inhibitor (PD98059) was again detected by Western blot (Figure 6). After equal amounts of PD98059 were added into the four groups, THP-1 mononuclear macrophage induced by ox-LDL decreased significantly compared with the MMP-9 protein expression in the uninduced mononuclear macrophage (0.435±0.064 vs 0.589±0.072, P<0.05); while no evident change was noted in MMP-9 protein expression even after the addition of low, medium, or high-concentration rosuvastatin (0.322±0.058, 0.288±0.043 0.259±0.046 vs 0.435±0.064) compared with the foam cells.

DISCUSSION

The current study first proved via *in vitro* experiment that the increase in the activity of MARK/ERK pathway monocyte macrophages into foam cells. Rosuvastatin may reduce the expression of MMP-9 by inhibiting MARK/ERK pathway of macrophages, and it may be one of the mechanisms of action for ACS patients to stabilize atheromatous plaques.

The occurrence and development of lipid metabolism disturbances are closely related to coronary disease. The increase in total cholesterol (TC) concentration has proved to be an important factor or pathogenic risk factor of coronary disease (Uemura et al., 2001). Lipid modulation can stabilize and control the occurrence and development of plaque (Nicholls et al., 2011). However, ACS pathogenic mechanism is very complicated, with
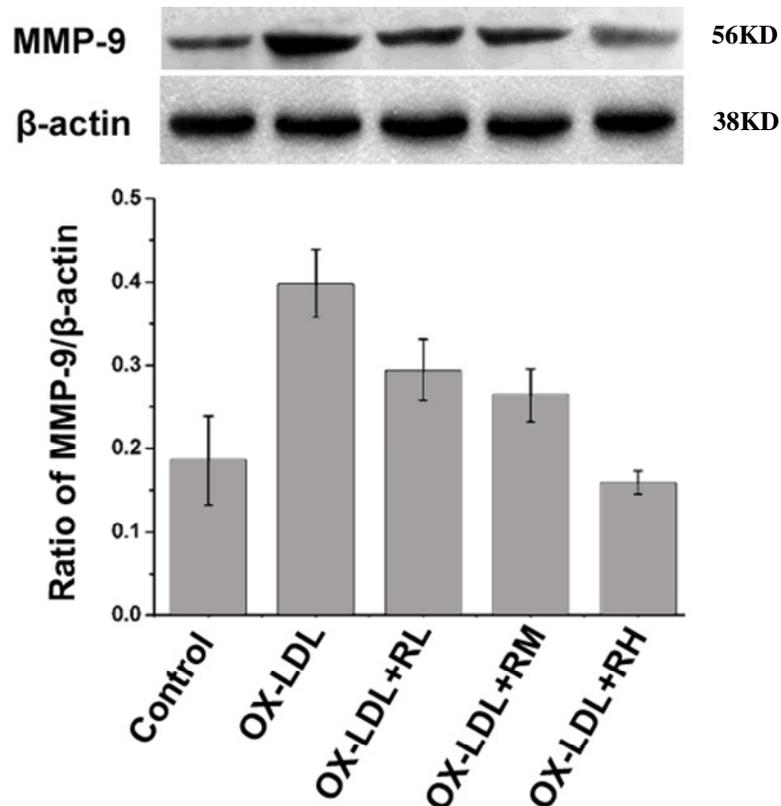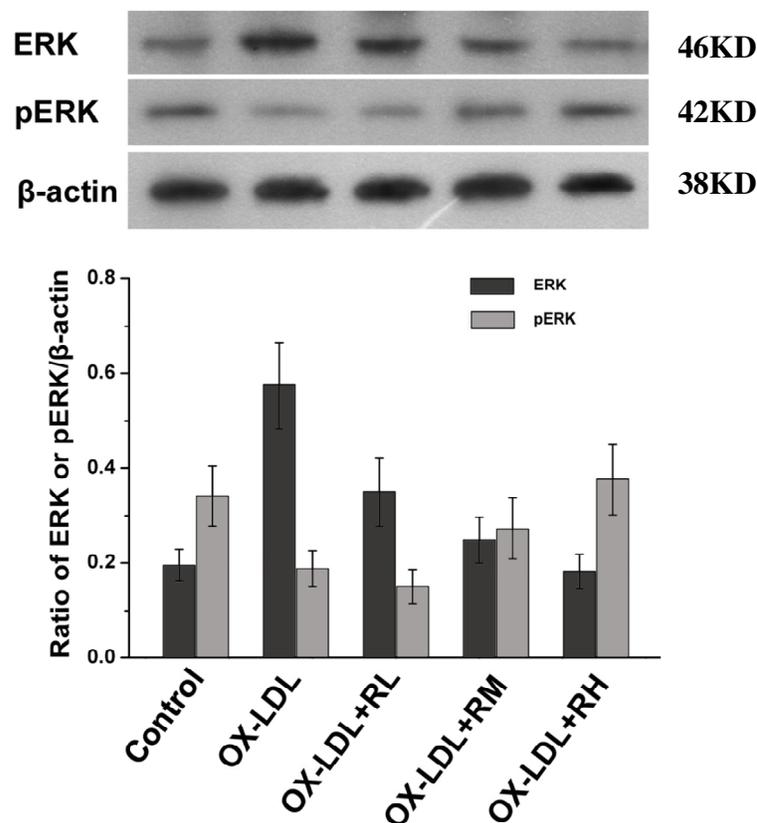
Figure 4. Immunoblotting analysis of total MMP-9 in in each group of ox-LDL-induced cells. A) Representative immunoblot of total MMP-9 protein in each group from the PMA group, as described previously. β-actin was used as a loading control. B) Densitometric scanning of MMP-9 protein in each group (n=5). Quantitative analysis showed significant increase of MMP-9 protein in OX+LDL group, P<0.05, then the MMP-9 protein exhibited a dose-dependent decreasing trend with rosuvastatin, P<0.05.

combined action of many kinds of risk factors and pathogenic mechanisms involving rupture of plaque. Among them, oxidized low density lipoprotein (ox-LDL) is an important pathogenic factor of arteriosclerosis in pathological changes. Ox-LDL can stimulate MMP-9 gene expression of the human mononuclear macrophage, increase the activity of the substrate metal protein, and can promote the degradation of the substrate in the arteriosclerosis plaque to induce the rapid wear of plaque (Xu et al., 1999). In addition, MMP-9 expression can be correlated positively with MMP-9 in the conversion of maintained at higher levels in the presence of ox-LDL. The over-expression of MMP-9 can inhibit the multiplication of smooth muscles, induce apoptosis, weaken plaque stability, accelerate plaque rupture, and promote pathological changes in arteriosclerosis and thrombus information. Therefore, the impact of ox-LDL on the expression of MMP-9 gene of human mononuclear macrophage is one of the key factors in the pathological changes of arteriosclerosis (Loftus et al., 2003; Wu and Li, 2006). In the present study, ox- LDL is used to induce the conversion of THP-1 macrophages into foam cells.

Rosuvastatin is used for this intervention, which proves that rosuvastatin can inhibit the expression of MMP-9 in the monocyte macrophages of THP-1 foam cells induced by ox-LDL.

MARK/ERK pathway is the representative and typical pathway of the MAPK signal pathway of human cells. ERK is a subfamily of MAPK, and is also one of the four big signal systems of the eukaryotic cell causing cell reaction through transduction of extracellular signals to the intracellular cells (Johnson and Lapadat, 2002). Protein phosphorylation and non-phosphorylation regulate almost all processes of life movement. Many kinds of extracellular signals can activate ERK. ERKs that are active show phosphorylation with Thr183/Tyr185, which can be represented in pERK. ERK activity could be induced by PMA, leading to increase in pERK expression; and PD98059 can significantly inhibit ERK activation, which causes down regulation of pERK (Aida, 2011). ERK pathway is Ras→Raf→MEK→ERK. ERK is a unique effector of MEK. Literature has shown that ERK can be activated by the activation of upstream PKC in most cases. PMA is a specific excitant of PKC, which can

Figure 5. Western blotting analysis of total ERK and pERK in in each group of ox-LDL-induced cells. A) Representative western blot of total ERK and pERK protein in each group, as described previously. β-actin was used as a loading control too. B) Densitometric scanning of total ERK and pERK in each group (n=5). Quantitative analysis showed significant increase of ERK ($P<0.05$), and significant decrease of pERK $P<0.01$) in the first two groups, then the ERK and pERK exhibited a dose-dependent decreasing trend with rosuvastatin in different dose ($P<0.01$).

inhibit the high quantity activity of PKCs. However, it can stimulate PKC to be low active content (Han, 2001). In the current study, PMA induces the THP-1 monocyte to change into adherent macrophages. Meanwhile, PMA activates ERK pathway and promotes the expression of MMP-9 as a specific protein kinase C excitant. After the addition of ERK pathway inhibitor PD98059, the result of the MMP-9 expression is the same as that of the phosphorylated extracellular signal-regulated kinases pERK). The result of the present study indicated that the activation of ERK pathway may be the key factor influencing the formation of foam cells and the expression of MMP-9.

Rosuvastatin, a lipid-regulating drug with the strongest effect on reduction of LDL cholesterol, can not only stop the development of atherosclerosis, but also reverse the accumulation of coronary atherosclerotic lipid plaques. In the present study, with the intervention of rosuvastatin in different concentrations for controlling the THP-1 genic monocyte macrophages, MMP-9 expression increased,

ERK protein expression decreased and pERK protein expression increased in the conversion of monocytes into foam cells. The inhibitory effect of rosuvastatin intervention on MMP-9 expression increased gradually with increase in its concentration. With the addition of ERK inhibitor PD98059, ERK protein increased obviously; whereas MMP-9 and pERK protein decreased significantly. These three kinds of proteins did not change obviously after the addition of rosuvastatin in different concentrations. The results of the current experiment show that ERK pathway induces the expression of MMP-9 protein, and the inhibitory effect of rosuvastatin on MMP-9 is achieved through this pathway.

Conclusion

The study shows that the increase in the activity of the MARK/ERK pathway correlates positively with the expression of MMP-9 in the ox-LDL-induced monocyte
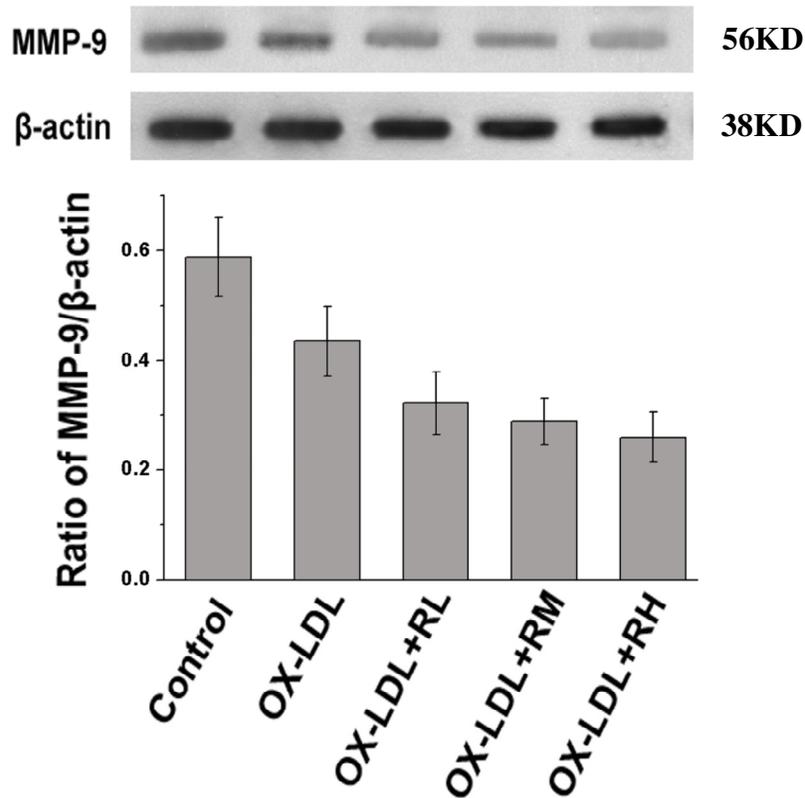
Figure 6. Western blotting analysis of total MMP-9 in each group incubated with PD98059. A) Representative western blot of total MMP-9 protein in each group, as described previously. After that it was incubated with PD98059. B) Densitometric scanning of total MMP-9 in each group (n=5). Quantitative analysis showed significant decrease of MMP-9 in the first two groups, $P<0.05$, and significant decrease in the OX-LDL+RL groups, $P<0.05$, in the OX-LDL+RM groups, $P<0.05$, in the OX-LDL+RH groups, $P<0.01$; however, these three groups had no evidence of change.

macrophages. Rosuvastatin could reduce the expression of MMP-9 by inhibiting the MARK/ERK pathway of macrophages, and it may be one of the mechanisms of action for ACS patients to stabilize atheromatous plaques.

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