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Increased Expression and Activity of MMP-9 in C-reactive Protein- induced Human THP-1 Mononuclear Cells Is Related to Activation of Nuclear Factor Kappa-B

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Summary：The relation between the expression and activity of MMP-9 in C-reactive protein (CRP)-induced human THP-1 mononuclear cells and the activation of nuclear factor kappa-B (NF-κB) was studied to investigate the possible role of CRP in plaque destabilization. Human THP-1 cells were incubated in the presence of CRP at 0 (control group), 25, 50 and 100 μg/mL (CRP groups) for 24 h. In PDTC (a specific NF-κB inhibitor) group, the cells were pre-treated with PDTC at 10 μmol/L and then with 100 μg/mL CRP. The conditioned media (CM) and human THP-1 cells in different groups were harvested. MMP-9 expression in CM and human THP-1 cells was measured by ELISA and Western blotting. MMP-9 activity was assessed by fluorogenic substrates. The expression of NF-κB inhibitor α (IκB-α) and NF-κB P^{65} was detected by Western blotting and ELISA respectively. The results showed that CRP increased the expression and activity of MMP-9 in a dose-dependent manner in the human THP-1 cells. Western blotting revealed that IκB-α expression was decreased in the cells with the concentrations of CRP and ELISA demonstrated that NF-κB P^{65} expression in the CRP-induced cells was increased. After pre-treatment of the cells with PDTC at 10 μmol/L, the decrease in IκB-α expression and the increase in NF-κB P^{65} expression in the CRP-induced cells were inhibited, and the expression and activity of MMP-9 were lowered too. It is concluded that increased expression and activity of MMP-9 in CRP-induced human THP-1 cells may be associated with activation of NF-κB. Down-regulation of the expression and activity of MMP-9 may be a new treatment alternative for plaque stabilization by inhibiting the NF-κB activation.
Key words：C-reactive protein; human THP-1 mononuclear cell; matrix metalloproteinase-9; nuclear factor kappa-B

Vascular inflammation plays a key role in all stages of atherosclerosis, ranging from the initiation and progression of the atherosclerotic lesion to plaque rupture, and eventually to the occurrence of cardiovascular events[1]. C-reactive protein (CRP) is not only a biomarker of inflammation but also a potent vasoactive mediator that can promote atherogenesis. Clinical trails have shown the association of plasma CRP levels with acute coronary syndromes[2–5] and the possible role of CRP in plaque vulnerability. A recent histopathological study showed that plasma CRP level in patients who died of severe coronary artery disease was correlated with the immunoreactive CRP in atherosclerotic lesions and the number of thin cap atheroma[6]. Another study using angioscopical examination reported an association between plasma CRP level and the intensity of yellow color in plaques, which is a sign of plaque vulnerability[7]. In addition, CRP could induce apoptosis of human endothelial cells[8] and coronary vascular smooth muscle cells[9], and it could stimulate matrix metalloproteinases (MMPs) expression in cells of which the atherosclerotic plaque is composed. All these studies indicated the possible roles of CRP in plaque destabilization.

MMPs can degrade extracellular matrix of fibrous cap, which plays an important role in plaque destabilization[10]. MMP-9 is a member of MMPs and its expression is believed to be regulated by nuclear factor kappa-B (NF-κB)[11]. CRP can activate NF-κB in smooth muscle cells and endothelial cells[12]. A recent study demonstrated that CRP could activate NF-κB in peripheral monocytes in patients with unstable angina[13]. However, the relation between MMP-9 expression and NF-κB activation in monocytes induced by CRP is not well established. The study aims to elucidate the relation between MMP-9 expression and NF-κB activation in CRP-induced monocytes and further investigate the underlying mechanism by which CRP causes plaque destabilization.

1 MATERIALS AND METHODS

1.1 Cell Culture

The human THP-1 monocyte cell line was grown in RPMI-1640 medium supplemented with 2% glutamine, 2% penicillin/streptomycin, and 10% FBS in a 5% CO_2 humidified atmosphere at 37°C. Human THP-1 cells were maintained in logarithmic growth phase (1×10^5 cells/mL) by passage every 3–4 days. When reaching

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80% confluence, the cells were used for following experiments.

1.2 Study Protocols

Human THP-1 cells were randomly divided into five groups with each group containing 1×10^6 human THP-1 cells. The cells were incubated in RPMI-1640 medium in the presence of CRP at 0 (control group), 25, 50 and 100 μg/mL (CRP groups) for 24 h. In PDTC group, the cells were pre-treated with a specific NF-κB inhibitor PDTC at 10 μmol/L for 30 min and then induced with CRP at 100 μg/mL for 24 h. The cell viability was assayed by Trypan blue exclusion method and was found to be greater than 90% in the presence of PDTC.

1.3 Western Blotting

After treatment, human THP-1 cells were collected. Protein extraction kit (Beyotime, China) was used to extract nuclear and cytoplasmic protein from 5×10^5 human THP-1 cells in accordance with the manufacturer's instructions. BCA method was used for protein quantification, and then the total protein was boiled for 5 min and stored for preparation. Cytoplasmic protein (50 μg/lane) was subjected to 10% SDS-PAGE. The fractionated proteins were transferred to nitrocellulose membranes (Amersham, UK), which was followed by blockage with 5% non-fat dry milk for 2 h at room temperature. After washing in PBS, the membranes were incubated overnight at 4°C with the following PBS-diluted antibodies respectively: anti-human MMP-9 (1:2000, Boster, China), anti-human IκB-α (1:1000, Boster, China) and anti-human GAPDH (1:5000, Kangchen Biological Co., China), and were further made to react with HRP-conjugated secondary antibodies (1:5000, Boster, China) in 5% milk-PBS for 1 h at room temperature. Immunoreactive products were visualized using enhanced chemiluminescence (ECL) system (Amersham, UK). All the protein bands were densitometrically quantified.

1.4 ELISA for Active NF-κB P^{65}

The amount of activated NF-κB P^{65} subunit in nuclear extract (5 μg), which was prepared from 5×10^5 human THP-1 cells (as described in preceding text), was assessed by a sensitive ELISA assay for active NF-κB. NF-κB P^{65} subunit in nuclear extract was measured by using NF-κB ELISA kit (Kangchen Biological Co., China) according to the manufacturer's instructions.

1.5 MMP-9 Activity Assay

After treatment, the conditioned medium (CM) was harvested and concentrated 10 times by using Centricon centrifugal filter devices (YM-30) through a filtering unit with a 30 kDa cut-off (Millipore, USA). Proteins in the concentrated media were quantified by BCA method. MMP-9 activity was measured as described previously[14].

1.6 ELISA for Measurement of MMP-9 in Supernatants

Following treatment, the supernatants of human THP-1 cells were collected and used for MMP-9 measurements with an ELISA kit (Boster, China).

1.7 Statistical Analysis

All the experiments were performed at least three times, with each group having 3 wells. Data were expressed as $\bar{x}\pm s$ and analyzed by ANOVA and tested for differences by using SNK-q test for multiple comparisons. A statistical probability of $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 IκB-α Expression in Human THP-1 Cells

IκB-α expression was the highest in the control group. CRP could obviously inhibit the IκB-α expression in human THP-1 cells in a dose-dependent manner. Pre-treatment of human THP-1 cells with the NF-κB inhibitor PDTC at 10 μmol/L could inhibit the decrease in IκB-α expression ($P<0.05$, fig. 1).

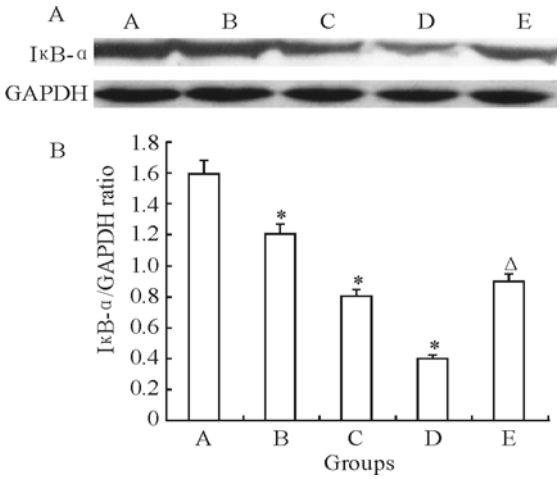

Fig. 1 IκB-α expression in human THP-1 cells

IκB-α protein in cytoplasmic extract of human THP-1 cells was detected by Western blotting (A). All bands were quantified by densitometry (B). IκB-α protein was expressed relative to GAPDH ($\bar{x}\pm s$).

$^*P<0.05$ as compared with A; $^\Delta P<0.05$ as compared with D

A: control group; B: 25 μg/mL CRP group; C: 50 μg/mL CRP group; D: 100 μg/mL CRP group; E: 100 μg/mL CRP +10 μmol/L PDTC group

2.2 NF-κB P^{65} Expression in Human THP-1 Cells

When compared with other groups, NF-κB P^{65} expression in human THP-1 cells was the lowest in control group. Treatment of human THP-1 cells with CRP at 25, 50 and 100 μg/mL could markedly increase NF-κB P^{65} expression in a dose-dependent manner. Pre-treatment of human THP-1 cells with PDTC could inhibit the increase in NF-κB P^{65} expression (fig. 2).

2.3 MMP-9 Expression in Human THP-1 Cells and Supernatants

MMP-9 expression in cytoplasmic extract and supernatants of human THP-1 cells was the lowest in control group as compared with other groups. CRP could dose-dependently increase MMP-9 expression. Pre-treatment of human THP-1 cells with PDTC could inhibit the increase in MMP-9 expression (fig. 3 and 4).

2.4 MMP-9 Activity in Human THP-1 CM

MMP-9 activity in human THP-1 CM was the lowest in the control group than in other groups. After treatment of human THP-1 cells with CRP at 25, 50 and 100 μg/mL, MMP-9 activity was markedly increased in human THP-1 CM and the effect was in a dose-dependent

fashion. PDTC pre-treatment could limit the increase in MMP-9 activity in human THP-1 CM (fig. 5).

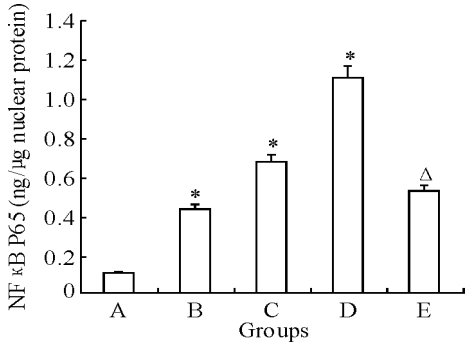

Fig. 2 NF-κB P^{65} expression in human THP-1 cells
NF-κB P^{65} in nuclear extract of human THP-1 cells was measured by ELISA. $^{*}P<0.05$ as compared with A; $^{\Delta}P<0.05$ as compared with D
Group designation is same as in fig. 1

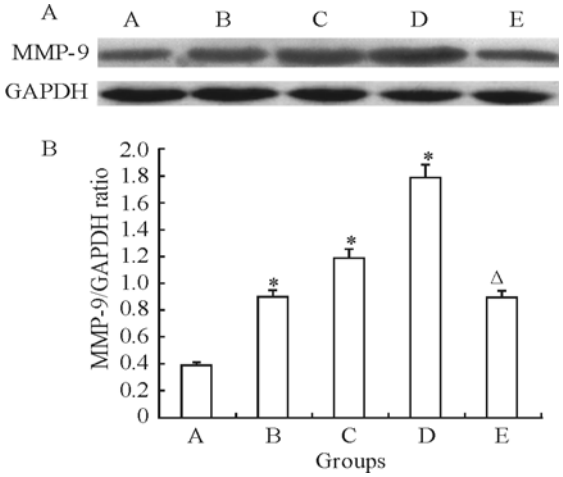

Fig. 3 MMP-9 expression in human THP-1 cells
MMP-9 protein in cytoplasmic extract of human THP-1 cells was detected by Western blotting (A). All bands were quantified by densitometry (B). MMP-9 protein was expressed relative to GAPDH ($\bar{x}\pm s$).
$^{*}P<0.05$ as compared with A; $^{\Delta}P<0.05$ as compared with D
Group designation is same as in fig. 1

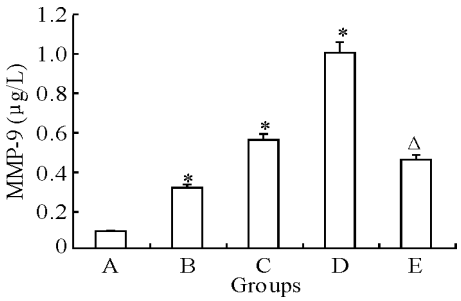

Fig. 4 MMP-9 expression in supernatants
MMP-9 expression in supernatants of human THP-1 cells was measured by ELISA. $^{*}P<0.05$ as compared with A; $^{\Delta}P<0.05$ as compared with D
Group designation is same as in fig. 1

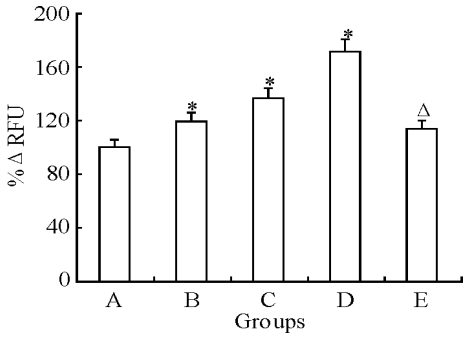

Fig. 5 MMP-9 activity in human THP-1 CM
MMP-9 activity in human THP-1 CM was assessed by fluorogenic substrates. $^{*}P<0.05$ as compared with A; $^{\Delta}P<0.05$ as compared with D
RFU: relative fluorescent unit
Group designation is same as in fig. 1

3 DISCUSSION

This study showed that CRP could increase MMP-9 expression and activity in human THP-1 cells in a dose-dependent manner. CRP could decrease IκB-α expression in cytoplasmic extracts as well as increase NF-κB P^{65} expression in nuclear extracts of human THP-1 cells. NF-κB inhibitor PDTC could inhibit the decrease in IκB-α expression and the increase in NF-κB P^{65} expression in the human THP-1 cells induced by CRP, and PDTC could also reduce the MMP-9 expression and activity in CRP-induced human THP-1 cells.

Pathological studies have shown that atherosclerotic plaques prone to rupture contain numerous macrophages that produce MMPs capable of degrading extracellular matrix molecules. MMP-9 is a member of MMPs and increased expression and activity of MMP-9 in human atherosclerotic plaques contribute to plaque destabilization[15]. Macrophages in atherosclerotic plaques are the major source of MMP-9[16]. Moreover, precursor monocytes recruited from the circulation provide a renewable source of macrophages as needed in tissues. So stimuli that can up-regulate MMP-9 expression and activity of monocytes/macrophages will lead to plaque destabilization.

Studies have also shown that CRP may play a role in plaque destabilization. A recent histopathological study demonstrated that serum CRP level in patients who died of severe coronary artery disease was correlated with the level of immunoreactive CRP in atherosclerotic lesions and the number of thin cap atheroma[6]. Another study using angioscopical examination reported an association between plasma CRP level and the intensity of yellow color in plaques, which is a sign of plaque vulnerability[7]. In addition, CRP could induce apoptosis in human endothelial cells[8] and coronary vascular smooth muscle cells[9]. All these studies supported that CRP might be involved in plaque destabilization, but the precise role of CRP in plaque destabilization was not fully understood. In the present study, we used precursor human THP-1 cells, a renewable source of macrophages, as a material, and found that CRP could augment MMP-9 expression and activity in human THP-1 cells, which might be interpreted as a potential role of CRP in plaque

vulnerability. Our findings were consistent with previous studies which revealed that CRP could increase MMP-9 expression and activity in macrophages[17] and monocytes[8].

Activation of NF-κB is reported to be important in the induction of MMP-9 gene expression in human THP-1 cells[18]. In support of this observation, there is a consensus NF-κB binding site in the promoter region of the MMP-9 gene[19]. CRP can activate NF-κB in smooth muscle cells and endothelial cells[12]. A recent study demonstrated that CRP could activate NF-κB in peripheral monocytes in patients with unstable angina[13]. However, the relation between NF-κB activation and MMP-9 expression in human THP-1 cells induced by CRP remain veiled. In this study, we presented several lines of evidence which showed that the CRP-induced increase in MMP-9 expression and activity in human THP-1 cells was dependent, at least in part, on NF-κB activation. Firstly, according to the classic pathway of NF-κB activation in stimulated cells, the IκB-α unit of NF-κB is phosphorylated, ubiquitinated, and degraded, which allows free NF-κB to be transferred to the nucleus, where it activates target genes by binding to cognate DNA regulatory elements. Our results showed that CRP could dose-dependently inhibit IκB-α expression in cytoplastic extract of human THP-1 cells and it could increase NF-κB P^{65} expression in nuclear extract of human THP-1 cells in the same manner. We inferred that the NF-κB activation in CRP-induced human THP-1 cells led to increased MMP-9 expression and activity. Secondly, the role of NF-κB activation in increased MMP-9 expression was further confirmed by pre-treatment of CRP-induced human THP-1 cells with PDTC, a chemical that stabilizes the NF-κB /IκB-α complex[20] and inhibits the nuclear translocation of activated NF-κB. Because the effect of CRP was in a dose-dependent manner, we investigated the role of PDTC in human THP-1 cells treated with CRP at 100 μg/mL and found that pre-incubation of human THP-1 cells with PDTC inhibited the decrease in IκB-α expression and the increase in NF-κB P^{65} expression. Moreover, PDTC could decrease MMP-9 expression and activity of CRP-induced human THP-1 cells. These data clearly suggested the importance of NF-κB activation in CRP-induced increase in MMP-9 expression and activity in human THP-1 cells. However, the involvement of other transcription factors could not be ruled out.

In conclusion, CRP could increase MMP-9 expression and activity in human THP-1 cells, which might be associated with NF-κB activation. And these effects could be reversed by NF-κB inhibitor PDTC. Increased expression and activity of MMP-9 in CRP-induced monocytes through activation of NF-κB might be a possible mechanism by which CRP plays a role in plaque destabilization. Decreasing MMP expression and activity by inhibiting NF-κB may furnish a new therapeutic choice for plaque stabilization.

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