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The expression of CD40-CD40L and activities of matrix metalloproteinases in atherosclerotic rats

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Abstract

This study investigated the expression of CD40, CD40 ligand (CD40L) and matrix metalloproteinases (MMPs) in dietary-induced atherosclerosis in rats. Wister rats were fed with high cholesterol diet (As group, $n = 6$) or with normal diet (N group, $n = 6$). Blood cells that express CD40 and CD40L were sorted by flow cytometry, the MMP-2 and MMP-9 were measured by zymography method. The morphological locations of MMP-2 and MMP-9 in the aorta were studied with immunohistochemistry and by microscopy. The results showed that the expression of CD40, CD40L and matrix metalloproteinase were higher in As group than those in control group. The MMP-2 and MMP-9 were positive in As group but negative in control group by immunohistochemistry study. Our results suggest that the expression of CD40 and CD40L in the blood cells and the activities of MMP-2 and MMP-9 in plasma were higher in As group than those in Normal group, indicating that they may contribute to the formation of atherosclerosis. (Mol Cell Biochem **282:** 141–146, 2006)

Key words: adhesion factor, atherosclerosis, CD40, CD40L, matrix metalloproteinase

Abbreviations: CD40L, CD40 ligand; DAB, diaminobenzidine; FACS, fluorescence-activated cell sorter; FITC, fluorescence isothiocyanate; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; TF, tissue factor; TNF, tumor necrosis factor

Introduction

Atherosclerosis is one of the most prevalent diseases in the developed world. Recently the intricate interplay between diverse factors such as lipid metabolism, blood coagulation elements [1], cytokines [2], hemodynamic stress [3], and behavioral risk factors [4] have been shown to be major risk factors for the development of atherosclerosis. Atherosclerosis has also been known to involve the immune system and chronic inflammation [5]. The accumulation of extracellular matrix proteins in the endothelium and media of the artery may lead to the rupture of lipid-rich coronary plaques and the release of materials from the clot is an important mechanism underlying the sudden onset of acute coronary syndromes and stroke. The degradation of the connective tissue matrix protein by activated matrix metalloproteinases (MMPs) within the atherosclerotic plaque may play a major role in this process [6]. The immune-mediator CD40 ligand (CD40L), a member of the tumor necrosis factor family, and its receptor CD40 that belongs to the nerve growth factor/tumor necrosis factor receptor family, were originally considered to be restricted to T and B cells [7]. The activated CD40-CD40L in blood cell results in the secretion of some chemical factors such as MMPs.

MMPs are members of a family of Zn^{2+}- and Ca^{2+}-dependent endopeptidases, which are essential for cellular migration and tissue remodeling in both physiological and pathological conditions [8]. Since MMPs appear to be

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involved in monocyte invasion and vascular smooth muscle cell migration, derangement of MMPs regulation is considered to be a critical factor in the development of vascular lesions [9]. Hence it is tempting for us to hypothesize that CD40–CD40L system and MMPs may contribute to the development of atherosclerosis. To further elucidate the possible pathogenic role of the CD40, CD40L and MMPs in blood and plasma in the development of atherosclerosis, we investigated these factors in a rat atherosclerotic model fed with high cholesterol diet, and compared them with results obtained from the control group.

Materials and methods

Animals

Wister rats were obtained from the laboratory center of the Third Military Medical University. All animal experiments were conducted in accordance with the established protect of the Shantou University. At the age of 8–10 weeks, 12 rats were divided into two groups. One group was fed with normal diet ($n = 6$) and the other group was fed with high cholesterol diet ($n = 6$) (4% cholesterol, 0.5% cholate). After 12 weeks, the rats were sacrificed using sodium pentobarbital (50 mg/kg, i.p.), blood was collected by cardiac puncture and the aortic arch was dissected. Plasma was obtained by centrifugation, and analyzed as described below.

Materials

Wister rats were obtained from the laboratory center of the Third Military Medical University. Anti-rat CD40, CD40L, MMP-2 and MMP-9 antibodies were provided by Santa Cruz Biotechnology, Inc. Gelatin and anti-rat MMP-9 antibody were from Sigma.

Hematoxylin and eosin staining

Paraffin sections (4 μm) from normal and As rat aortic arch were used for routine staining of aortic sections to show the atherosclerotic plaque by hematoxylin and eosin staining kit (Santa Cruz Biotechnology, Inc.). Slides were de-paraffinized and rinsed in hematoxylin for 5 min and washed in tap water for 10 min. Then the slides were rinsed in eosin for 10 s and mounted with a resinous medium. Images were taken using a Zeiss Axioscop microscope using Axiovision software.

Flow cytometry

Harvested rat blood was incubated with the CD40 and CD40L antibodies (Santa Cruz Biotechnology, Inc.) at a final concentration of 10 μl per 100 μl of blood for 30 min on ice, respectively, washed twice before and after lysis of erythrocytes by adding 2 mL of ice-cold erythrocyte-lysing solution (NH_4Cl 2.08 g, Na_2EDTA 0.0108 g, $NaHCO_3$ 0.21 g in 250 mL H_2O) and then incubated with FITC-conjugated second antibody at room temperature for 30 min and analyzed by FACScan flow cytometer. Data were analyzed using CELLQUEST software (Becton Dickinson). For each treatment the mean fluorescence intensity (MFI) value for the control stained population was subtracted from the MFI value of the positive-stained sample. MFIs are the geometric mean intensities and refer to values normalized to the log scale [10, 11].

Western blotting

Proteins in the plasma were separated by SDS-PAGE and blotted onto polyvinydene difluoride membranes (Bio-Rad) with the use of a semidry blotting apparatus. Blocking of nonspecific binding and dilution of the primary (1:100 anti-rat MMP-2, Santa Cruz Biotechnology, Inc.) and secondary (1:10,000) antibody was done by using 5% fat-free milk/PBS/0.1% Tween 20. Proteins in the blot were visualized by incubation of the blot in a buffer solution containing 1 mg/mL diaminobenzidine (DAB) in a buffer containing 17 mmol/L acetic acid/65 mmol/L Na_2HPO_4/0.01% thimerosal/ 0.1% H_2O_2.

Zymography

The zymography method for measuring MMP-2 and MMP-9 has been described as previously reported [12]. Plasma obtained from centrifuged blood was employed for zymography in SDS gels with gelatin. Electrophoresis of the gel was run at 50 V for 3.5 h. The gels were incubated in 2.5% Triton x-100 for 30 min to remove the SDS, briefly washed in 1 μM $ZnCl_2$/5 mM $CaCl_2$/10 mM HCl, pH 7.5 and incubated at 37 °C in this buffer for 24 h. After incubation, the gels were stained with Coommassie G-250 dye. Zymography was scanned with Bio-Rad ScanJet, and black-and white images were analyzed using an image analysis software program, running on a Bio-Rad PC.

Immunhistochemistry

Paraffin sections (4 μm) from normal and As rat aortic arch were used for immunohischemistry using the high-SABC Kit (Santa Cruz Biotechnology, Inc.). Aortic arch sections were incubated with sheep anti-rat MMP-2 (1:100, Santa Cruz Biotechnology, Inc.), or sheep anti-rat MMP-9 (1:100, Sigma) for 1 h at room temperature, followed by incubation

with biotinylated anti-sheep secondary antibody for 1 h at room temperature. The sections were stained with DAB (Diaminobenzidine, Sigma) and nuclei were counter stained with haematoxylin QS (Vector Laboratories Inc.). Images were taken using a Zeiss Axioscop microscope using Axiovision software.

Statistical analysis

Results obtained for each group of animals were presented as the mean ± standard error (S.E.). Results were compared by SPSS10.0 software. Data were analyzed using the *t*-test. $P < 0.05$ was considered statistically significant.

Results

The cholesterol levels in plasma

In the study, blood was collected at the end of the experiment in each group. The plasma cholesterol level in each group was detected by automated biochemical machine (Beckman counter LX20). As shown in Table 1, the levels of rats in As group were higher then those in Normal group. The endothelium of the aorta in Normal group was more smooth than that in As group and there were foam cells in As group as shown in Figs. 1A and B.

CD40 and CD40L expressions

In this study, blood samples were collected from both animal groups. As depicted in Table 2, the expression of CD40 and CD40L by blood cells of the rats in As group were significantly higher than those observed in the control group.

MMP-2 and MMP-9 expression

To test if there were any changes in the proteases that would degrade the extracellular matrix, we measured the activity of MMP-2 and MMP-9 in plasmas from normal and As rats using gelatine zymography. As shown in Fig. 2, there was a significant increase in MMP-2 and MMP-9 activity in the plasma of As group when compared with those in Normal group. Western blot analysis of the total protein in the plasma for MMP-2 also showed similar results as the activity one, as showed in Fig. 3.

Table 1. Plasma cholesterol level (mmol/l)

Normal group	As group
3.12 ± 0.18	8.04 ± 0.40*

*$P < 0.05$ *versus* N group.
Samples were collected and plasma analyzed for the cholesterol level at the end of experiments in both Normal and As group. The level of cholesterol in the As group was significantly higher than that in the Normal group.

Table 2. CD40 and CD40L levels

	Normal group	As group
CD40 (MIF)	11.90 ± 0.41	17.76 ± 0.38*
CD40L (MIF)	11.72 ± 0.26	20.10 ± 0.41*

*$P < 0.05$ *versus* N group.
Samples were collected as described in methods section and were analyzed for CD40 and CD40L expression by flow cytometry. The expression of CD40 and CD40L on peripheral blood cells of the As group were significantly higher than those observed in the Nomal group.

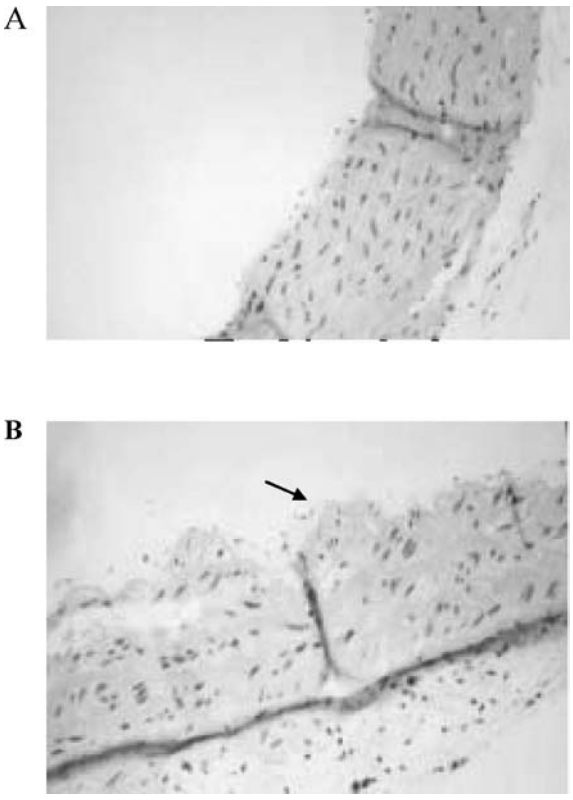

Fig. 1. Hematoxylin and eosin staining showed the structure of normal (A) and As rat aortic arch (B). The endothelium of the aorta in normal group was more smooth than that in As group and there were foam cells in As group as shown by the arrow in (B).

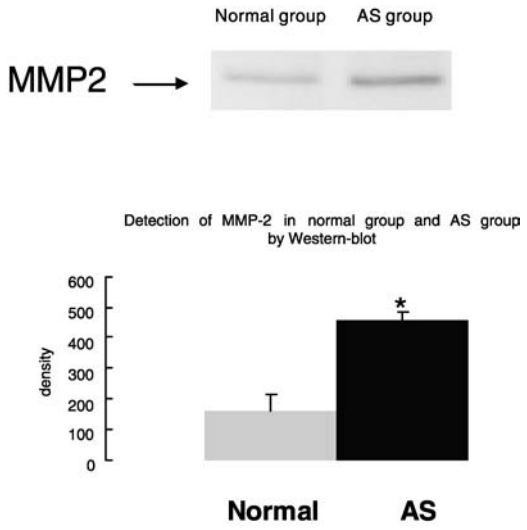

Fig. 2. Western blot analysis of MMP-2 protein level in plasma of the As and normal groups. Samples were collected as described in methods section. Thirty microgram protein from the plasma was separated on SDS-PAGE. Blots were probed with antibodies to MMP-2. Bands corresponding to MMP-2 were quantified by densitometry using the QuantityOne Program. Bar graph shows the mean ± S.E. of the MMP protein expression from three independent experiments. $^{*}P < 0.01$ significantly different compared to the normal group.

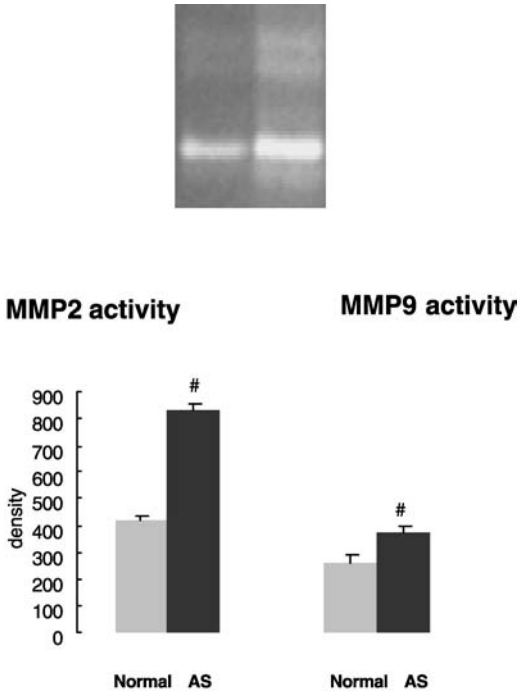

Fig. 3. Analysis of MMP-2 and MMP-9 activities in normal group and As group using gelatin zymography. Zymography was carried out as described in methods section. The *upper panel* shows a representative zymography of the plasma from normal and AS groups. The *lower panel* shows densitometric quantification of the bands corresponding to MMP-2 and MMP-9 activities. Bar graphs are the mean ± standard error (S.E.) of density of MMP-2 or MMP-9 from four independent experiments. $\#P < 0.01$ significantly different compared to the normal group.

Immunhistochemistry of MMPs

To determine if the changes in the MMP-2 and -9 proteins in plasma was a reflection of the *in vivo* situation, we used the tissue for immunohistochemical staining with MMP-2 and nine specific antibodies to localize these proteins in tissue from normal and As rat aorta arches. As shown in Fig. 4 (A, B, C, D). MMP-2 and MMP-9 expression are higher in the As rat aorta arch as compared to the normal tissue.

Discussion

Immune mediator CD40 and CD40L have recently been found to localize in a variety of cell types involved in the immune system (B cells, T cells), monocytes and dendritic cells, fibroblasts, endothelial cells and vascular smooth muscle cells [10]. The interaction of CD40 and CD40L can activate T and B lymphocytes and up-regulate some of the cytokines and adhesion factors, such as IL-1, IL-12, TNF-α (tumor necrosis factor-α) [13]. Therefore, the CD40-CD40L signal system not only acts as a regulator of humoral and cellular immunity, but also plays an important role in the inflammation process mediated by T lymphocyte.

MMP-2 and MMP-9 are synthesized and secreted by endothelial cells, fibroblasts, neoplastic cells, inflammatory cells, myocardiocytes [14]. MMP-2 and MMP-9 could be activated by some inflammatory factors such as TNF-α, MCP-1. They also can be activated by CD40-CD40L interaction [15]. MMP-2 and MMP-9 are increased in the process of serious myocardial pathology, e.g. myocardial infarction, ischemic reperfusion injuries [16].

CD40–CD40L interaction and MMPS play important roles in the process of atherosclerosis [15]. The activated CD40–CD40L in blood cell results in the secretion of some chemical factors such as IL-8, MCP-1 [17], cytokines (IL-6, TNF-α), tissue factor [17], adhesion factors [18] and matrix metalloproteinase [15]. Schonbeck *et al.* [15] revealed that CD40L was expressed by CD40 on activated T lymphocytes and induced the expression and release of MMP-1, MMP-3, MMP-9 and it also activated MMP-2 in human vascular smooth muscle cells. It could also activate blood coagulation, plaque rupture, procoagulant activity potential and collagen formation [15]. In our results, CD40 and CD40L levels were in peripheries blood cells significantly higher in As group than those in Normal group. They suggests that CD40–CD40L interaction may be involved in the formation of As. Recently, it has been reported that CD40–CD40L system plays a role in

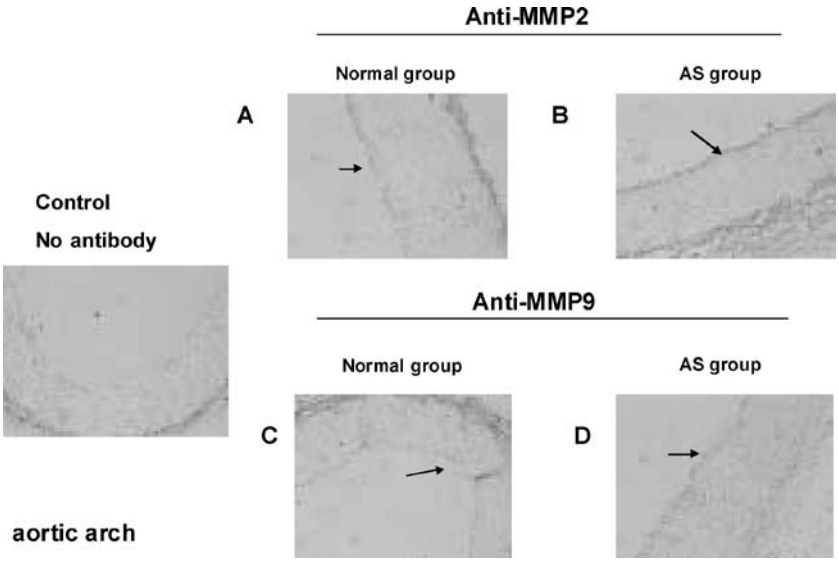

Fig. 4. Immunohistochemical staining of the aortic arch from normal or the As group using antibody for MMP-2 (A, B) and MMP-9 (C, D). Sections (4 μm) of the rat aortic arch were incubated with the polyclonal antibody to MMP-2 (A, B) or MMP-9 (C, D), followed by biotinylated labelled secondary antibody and DAB. Cell nuclei were counter stained with haematoxylin.

the location of foam cells and leading to the development of atherosclerosis [19].

Our data showed that MMP-2 and MMP-9 in plasma were significantly higher in As group than those in Normal control diet group. Again it suggests that MMP-2 and MMP-9 may contribute to the formation of As. It was reported that MMPs in coronary lesion was involved with plaque rupture [20]. Indeed, Zaltman [21] reported that cholesterol feeding significantly increased secretion of MMP-2 and MMP-9 in vessels, and the activation of MMP-9 in atherosclerotic plaque.

Our study shows that the expression of CD40, CD40L in blood cells and the activities of MMPs were higher in As group than those in normal group. The results lend further support to the involvement the development of CD40, CD40L and MMP-2 and MMP-9 in atherosclerosis. Schonbeck *et al.* [22] also demonstrated that CD40 and matrix metalloproteinase participate in the unstability of the plaque *in vivo* and *in vitro*.

The "CD40–CD40L hypothesis," however, does not depend on the "infection hypothesis," and several other factors may well lead to T-cell activation in atherosclerosis patients. For example, enhanced oxidative stress and oxidized-LDL might also lead to increased CD40L levels and T-cell activation through monocyte activation or direct effects on T cells [23]. Therefore down-regulating CD40–CD40L interaction may represent a new therapeutic approach in atherosclerosis.

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