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Regular Article

Chemokine CX3CL1 and its receptor CX3CR1 are associated with human atherosclerotic lesion volnerability

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ABSTRACT

Background: CX3CL1 and its receptor CX3CR1 have been emphasized in atherosclerosis recently. In this study we investigated the role of the chemokines CX3CL1 and their receptor CX3CR1 in atherogenesis and identified whether the genetic variations in CX3CL1 and CX3CR1 impacted the atherosclerosis process in coronary artery disease (CAD) or not.

Methods: CX3CL1/CX3CR1 expression in coronary and carotid artery specimens were analysed by immunohistochemistry. CX3CR1 expression on $CD4^+$ $CD28^-$ T cells was analysed by flow cytometry. We also screened for CX3CL1/CX3CR1 sequence variations selected from the hapmap database and examined the association between CX3CL1/CX3CR1 and CAD in the Chinese Han population.

Results: Immunohistochemical staining of tissue from CAD patients showed increased CX3CL1/CX3CR1 expression in atherosclerotic coronary and carotid artery plaques compared with normal arteries. CX3CL1/CX3CR1 expression was correlated with the severity of the atherosclerosis lesion. Patients with CAD also showed an increased number of $CX3CR1^+$ $CD4^+$ $CD28^-$ T cells. Compared with their corresponding wild-type genotypes, CX3CL1 rs170364 and CX3CR1 rs17793056 were associated with increased susceptibility to CAD.

Conclusions: CX3CL1 and CX3CR1 may contribute to the formation of coronary atherosclerotic plaque in CAD.CX3CL1 rs170364 and CX3CR1 rs17793056 polymorphisms may be independent genetic risk factors for CAD.

Introduction

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality globally. However the challenge remains to elucidate the pathogenesis of CAD and develop ways to accurately determine a patients existing disease risk [1,2].

An increasing number of studies indicate that the inflammatory response is an important factor in the progression of atherosclerosis in CAD; with immune impairment being involved in disease initiation and progression [1,2]. Increasing evidence suggests that inflammatory immune cells are the most important effector cells in the atherosclerotic response and that chemokines are involved in atherosclerosis progression by activating and directing the inflammatory leukocytes to atherosclerotic lesions [3–5].

Fractalkine (CX3CL1), a member of the CX3C chemokine subfamily, exists as both membrane-anchored and soluble forms, thus mediating its different biological functions [6–8]. The membrane-anchored form serves as an adhesion protein, promoting the retention of monocytes and T cells. The soluble form acts as a chemokine and strongly induces chemotaxis [5,9]. The adhesive and chemotactic properties of CX3CL1 and CX3CR1 play important roles in the progression of various inflammatory disorders [10,11]. A direct role of CX3CL1 and CX3CR1 in atherogenesis had been implicated from murine models. More studies suggest that deletion of the CX3CL1 and CX3CR1 gene in mice inhibit atherosclerosis progression. CX3CR1 is expressed on several cell types such as smooth muscle cells, monocytes, endothelial cell and T-lymphocytes [12,13]. Immobilized to the membrane it mediates the capture and firm adhesion of CX3CR1-expressing leukocytes while the soluble form acts as leukocyte chemo attractant at CX3CR1 via the G-coupled signalling pathway. $CX3CR^{-/-}/ApoE^{-/-}$ knockout mouse model shows that the CX3CL1 and CX3CR1 axis recruits leukocytes to the subendothelial layer in atherosclerosis procession [14,15]. At present it is crucial to clarify the roles of CX3CL1 and CX3CR1 in human atherosclerotic lesions to gain better understanding the disease.

Several studies report the relationship between CX3CL1/CX3CR1 gene variants and CAD, but there is no consistent results. Since the important role of CX3CL1 and CX3CR1 gene variants for the atherosclerotic cardiovascular disease, the aim of our study systematically is to screen for sequence variations in human CX3CL1 and CX3CR1 gene and examine the association between these variations and susceptibility to CAD.

Our studies demonstrate the increased expression of CX3CL1 and CX3CR1 in the human atherosclerotic coronary lesions and provide

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the genetic data about the association between the CX3CL1 and CX3CR1 variants and CAD in Chinese Han population.

Materials and Methods

Coronary and Carotid Artery Tissue

A total of 10 human left anterior descending (LAD) coronary arteries with normal regions and atherosclerotic plaque were obtained from 8 donors at autopsy, and from 2 patients undergoing heart transplant surgery. We get the atherosclerotic coronary and carotid arteries from 4 individuals (with history of CAD and possessing carotid atherosclerosis), and normal coronary and carotid arteries from 4 normal controls (individuals who died in traffic accidents, with no history of CAD) were obtained at autopsy. All specimens were collected in accordance with the Declaration of Helsinki that the locally appointed ethics committee had approved the research protocol and that informed consent had been obtained from the subjects and the relatives.

Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded left anterior descending (LAD) coronary and carotid arteries were serially sectioned (4 μm thick). Immunostaining of serial sections was performed using primary antibodies against CX3CL1 (Abcam, Cambridge, UK), CX3CR1 (Abcam), CD3 (Dako, Glostrup, Denmark) and CD68 (Dako). Secondary antibodies were biotinylated goat anti-rabbit or anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA and Chemicon, Merck Millipore, Billerica, MA, USA). Immunoreactivity was detected with DAB staining and sections counterstained with hematoxylin. Immunofluorescence was conducted on OCT-embedded sections of frozen samples. Briefly, 6-μm-thick cryostat sections were fixed in 99% acetone for 10 minutes then rehydrated 3 times for 5 minutes each time in phosphate-buffered saline (PBS).In immunofluorescence staining, the secondary antibodies were goat anti-rabbit Alexa Fluor 488 or donkey anti-mouse Alexa Fluor 555 (Invitrogen, Life Technologies, Carlsbad, CA, USA). Slides were observed and photographed under a light microscope (Leica Microsystems, Wetzlar, Germany) or a confocal laser scanning microscope (Olympus FV500, Tokyo, Japan).

Determination of Plasma CX3CL1 Levels

Blood samples for the determination of serum CX3CL1 levels were collected on ice in tubes containing EDTA and aprotinin and centrifuged at 3,000 g for 15 min at 4 °C to isolate plasma. Plasma samples were then frozen at -70 °C until use.

A 100 μl sample diluent, standards and test samples were each added to 3 plates and incubated for 2 hours at room temperature. After 3 washes, conjugate was added to the plates and incubated for 2 hours at room temperature. After 3 washes, the plates were incubated for 30 min in the dark before the reaction was stopped with 50 μl stop solution. In order to investigate whether the CX3CL1 rs170364 G/T variant influenced the expression of CX3CL1, the plasma concentration of CX3CL1 was determined in 90 CAD and 90 control subjects. For the determination of plasma CX3CL1 levels, CX3CL1 45 GG homozygotes, 30 GT heterozygotes and 15 TT homozygotes were selected from the control group, 45 GG homozygotes, 30 GT heterozygotes and 15 TT homozygotes were selected from CAD patients.

Flow Cytometry

Flow cytometric analysis of whole blood was performed with FITC-conjugated anti-CD4, PE-conjugated anti-CD28 and APC-conjugated anti-CX3CR1 (BD, Franklin Lakes, NJ, USA). 100 μl heparinized whole blood was incubated with the conjugated monoclonal antibodies for 30 min. Next 2 ml of 1× FACS lysis solution was added to each tube and incubated in the dark for 10 min then centrifuged for 5 min and the supernatant discarded. Each tube was then washed in FACS buffer. Cells were resuspended in 0.5 ml PBS with 1% formaldehyde. Cell populations were analysed with a FACS Calibur instrument and CellQuest software (BD).

Genotype Study Participants

Between January 2007 and February 2012, a total of 1922 subjects were enrolled in the current study. Among them, 1043 CAD patients were recruited from the Northern Chinese population (ShenYang Military General Hospital, China). Patients were considered eligible regardless of the clinical symptoms, including unstable angina, ST-elevated myocardial infarction and non-ST-elevated myocardial infarction at

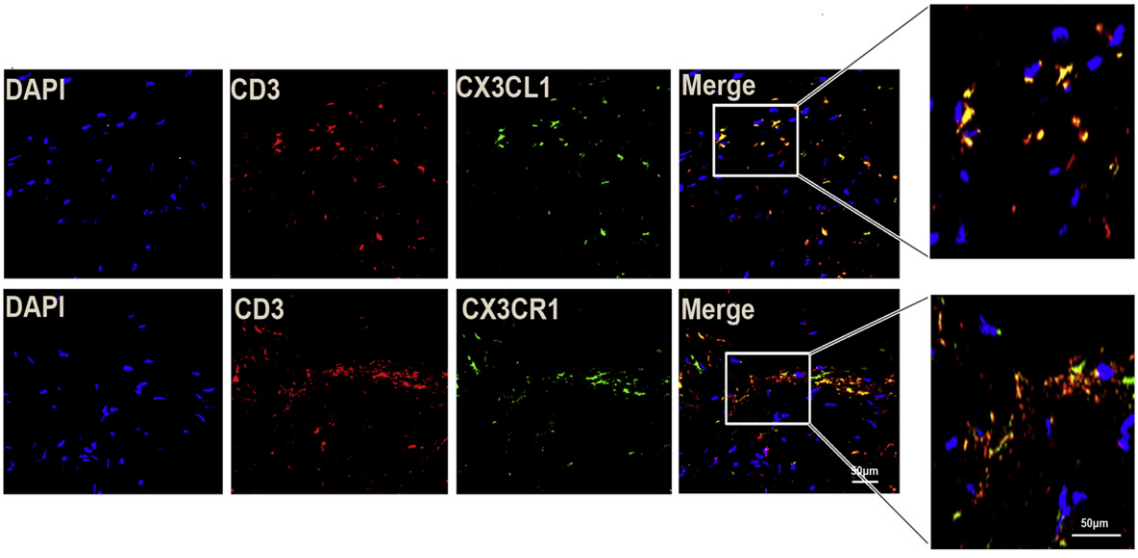

Fig. 1. Immunofluorescence staining of CX3CL1 and CX3CR1 in human coronary artery atherosclerosis plaques. Representative immunohistochemical staining for CD3, CX3CL1 and CX3CR1 in human coronary arteries. CX3CL1 and CX3CR1 were co-localised in T cells in human coronary arteries. T cells were identified by staining for CD3 (red). Immunofluorescence staining showed CD3 (red) and CX3CL1 and CX3CR1 (green), with a merged image (×20). Co-localisation of $CD3^+$ T cells and CX3CL1 and CX3CR1 was seen in the lesion region in human coronary arteries.