Original Paper

Daintain/AIF-1 Plays Roles in Coronary Heart Disease via Affecting the Blood Composition and Promoting Macrophage Uptake and Foam Cell Formation

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Key Words

Inflammatory factor daintain/AIF-1 • Superoxide dismutase (SOD) • Oxidized low density lipoprotein (ox-LDL) • Scavenger receptor A (SRA) • Atherosclerosis

Abstract

Background: Daintain/AIF-1 is an inflammatory polypeptide factor/allograft inflammatory factor 1 derived from macrophages. It is characterized in APOE^{-/-} mice as a novel inflammatory factor associated with atherosclerosis. The purpose of this study was to characterize its function in human atherosclerosis. **Methods:** Immunohistochemistry was used to identify the expression of daintain/AIF-1 in vessel segments within and far from atherosclerotic plaques; High-performance liquid chromatography (HPLC) was used to display the effects of daintain/AIF-1 on C-reactive protein (CRP), oxidative capacity and superoxide dismutase (SOD) *in vivo*; Oil Red O Staining was used to show the effects of daintain/AIF-1 on uptake of oxidized low density lipoprotein (ox-LDL) into U937 cells, a macrophage line; Western Blot was used to test scavenger receptor A (SRA) expression. **Results:** A high density of daintain/AIF-1 was observed in the tunica intima and media of coronary artery with atherosclerotic plaque, and fewer daintain/AIF-1 in the vessels without atherosclerotic plaque; Daintain/AIF-1 injected intravenously into BALB/c mice boosted oxidative capacity, significantly impaired SOD activities and augmented the CRP level in blood. According to the oil red O test, daintain/AIF-1 profoundly facilitated the uptake of ox-LDL in U937 macrophages and formation of foam cells in the endothelium. We also found that the molecular mechanisms are effective by promoting overexpression of SRA on macrophages. **Conclusion:** These findings implicate that the inflammatory factor daintain/AIF-1 is closely associated with atherogenesis, and could be further characterized as a novel risk factor for atherosclerosis.

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Introduction

Recent advances in medical science have established a fundamental role for inflammation in mediating all stages of atherosclerosis leading to the thrombotic complications of atherosclerosis [1-3]. These new findings have renovated and expanded the knowledge of the LDL metabolism pathway and low density lipoprotein receptor (LDLR) pathway in the formation of atherosclerosis, which were mainly discovered and elucidated by Brown and Goldstein [4, 5]. Although atherosclerosis has been recognized as a chronic inflammatory condition, the links between the risk factors and molecular mechanisms of pathogenesis have yet to be fully understood, and more novel risk factors may be found and identified. In the mid-1990s, daintain/AIF-1 was cloned out from rat with cardiac allografts chronic rejection [6], isolated and chemically characterized from porcine intestines [7]. According to the later investigations on this peptide and its synonyms Iba1 (ionized Ca^{2+} -binding adapter) [8], MRF-1 (microglia response factor-1) [9], this polypeptide takes important roles in other inflammatory diseases including autoimmune neuritis, systemic sclerosis, type 1 diabetes and breast cancer [10, 11]. In this study, we further, from newly visual angle, investigated daintain/AIF-1 is involved in atherogenesis via facilitating uptake of oxidized low density lipoprotein (ox-LDL) and foam cell formation in the endothelium.

Materials and Methods

Immunohistochemistry

The artery vessel specimens were collected from the CHD patients during surgical treatment in Wuhan Asia Heart Hospital. The tissues were fixed with 4% formalin for overnight and embedded in paraffin. The procedures for the collection of these samples were carried out in accordance with guidelines from the Ministry of Health of The People's Republic of China, and approved by the Local Hospital Ethics Committee.

The daintain/AIF-1 used in this study was prepared as reference [7]. The monoclonal antibodies against daintain/AIF-1 were prepared [12] and diluted 1:50. The formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections. Routine immunohistochemical methods were employed.

Assays of C-reactive protein and activity of superoxide dismutase in the blood

BALB/c mice (18-20 g, from the standard animal center of the China Medical College) in the experimental group ($n=10$) were intravenously injected with daintain/AIF-1 in saline at a dose of 5 $\mu\text{g/g}$ body weight, and the mice in control group were injected with saline alone. The administration was repeated at 24 h after the first injection. At 30 h, the mice were bled into evacuated tubes containing trisodium citrate as anticoagulant. The plasma was separated in a refrigerated centrifuge at 4 °C. C-reactive protein was analyzed with a Clinical Chemistry Analyzer (CL-8000S, HIMADZU, Japan). SOD activity was measured with the Sigma-Aldrich 19160 superoxide dismutase assay kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's protocol.

We took 20 μL of plasma from the centrifuged blood of each mouse and made a plasma mixture. A total of 200 μL mixture was obtained from the experiment and control groups. After addition of dithiothreitol (DTT) 528 μg into the plasma mixture, the two kinds of plasma were stirred at 4 °C for 30 min. For denaturation and sedimentation of proteins, we added 40 μL of 5% concentration of trichloroacetic acid into the two mixtures respectively and shook for 30 min at 4 °C. The two samples of plasma were centrifuged at 10,000 rpm for 10 min at 4 °C. 80 μL supernatants of each group were used for lyophilization. The dried materials were then dissolved in 120 μL of 0.1% trifluoroacetic acid respectively. After centrifugation, the supernatants were applied in aliquots to HPLC on column Pharmacia-LKB TSK ODS 120-T (10 μm , 300 $\times$ 7.8 mm). For elution, we used a linear gradient of 0-30% acetonitrile in 0.1% trifluoroacetic acid (60 min, 1.5 mL/min).

Effect of daintain/AIF-1 on U937 macrophages uptaking ox-LDL

U937 cells were obtained from The American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and cultured in Dulbecco's modified eagle medium supplemented with 10% heat-inactivated fetal calf

Fig. 1. Location of daintain/AIF-1 in CHD artery with Atherosclerotic plaque (antibody 1:50 dilution). A. daintain/AIF-1 in the intima of CHD artery, B. daintain/AIF-1 in the media of CHD artery, C. the section stained with the neutralized antibody by antigen daintain/AIF-1, D. fewer daintain/AIF-1 in the vessel section (control group) far from Atherosclerotic plaque. (magnification $\times 100$).

plasma, 100 U/ml penicillin and 100 μ g/ml streptomycin. Human native LDL was purchased from Sigma-Aldrich Corp. (St. Louis, Missouri, USA); ox-LDL was prepared as described by Hone et al. [13]. The PMA-derived U937 cells were prepared [14] and incubated in the presence of daintain/AIF-1 at 0, 1, 10, and 100 ng/mL for 24 h in Series II water Jacketed CO₂ Incubator.

The cultured monolayer cells were washed and exposed to ox-LDL (20 μ g/ml) for 24 h. The macrophages were washed and fixed with 4% paraformaldehyde, and stained with oil red O to detect intracellular neutral lipids. The numbers of oil red O-positive lipid droplets were counted, and cells with greater than 10 lipid droplets per cell ($n = 5$) were scored as "lipid-laden foam cells," as described by [14].

To test the SRA on the cultured PMA-derived U937 as described above, the cells were collected and followed by ultrasonicated to prepare the protein sample. The lysates from 1×10^5 cells/ml were applied to 12% polyacrylamide gel electrophoreses under reducing conditions, then transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat powdered milk in TBST buffer (0.1 mol/L Tris-HCl, pH 8.0, containing 1.5 mol/L NaCl and 0.5% Triton X-100). Membranes were incubated with a 1:1,000 dilution of SRA antibody and a 1:10,000 dilution of goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, California).

Statistics

t-test is used to analyze the data. The results will be considered significant when P values ≤ 0.05 .

Results

Location of daintain/AIF-1 in CHD artery

The coronary artery vessel specimens from 17 CHD were tested by immunohistochemical stains. The vessel segments with atherosclerotic plaque were selected as experimental group, and the vessel segments which are far from atherosclerotic plaque were selected as control group. The stained results revealed an abundant expression and a brown staining of daintain/AIF-1 in the tunica intima and media of the coronary artery vessel specimens with atherosclerotic plaque, and fewer expression in control group (Fig. 1).

C-reactive protein increased and activity of superoxide dismutase inhibited by Daintain

The average level of C-reactive protein in BALB/c mice plasma was 0.90 ± 0.45 mg/L, but the level in the plasma of mice injected with daintain/AIF-1 increased to 2.32 ± 0.47 mg/L (Fig. 2A). These results show that daintain/AIF-1 significantly increased the concentrations of C-reactive protein *in vivo*. Simultaneously, using the conventional assay kits, we determined that SOD activity in the plasma of mice injected with daintain/AIF-1 was 278 ± 6 U/mL compared with 341 ± 17 U/mL in the plasma of those injected with saline (Fig. 2B). The SOD activity was decreased by 18.5% of the injected saline mice. This outcome ascertains that daintain/AIF-1 significantly impairs the activity of SOD *in vivo*. Finally using DTT reduction method, we determined the oxidative capacity of the blood of mice injected with daintain/AIF-1 and the mice injected with saline respectively. The results showed that the blood from the mice injected with daintain/AIF-1 consumed 96% DTT compared with

Fig. 2. Effects of daintain/AIF-1 on CRP, oxidative capacity and SOD *in vivo*. A. CRP level. B. SOD activity. (Black and white in A and C indicate the results injected with daintain/AIF-1 and saline respectively, $P < 0.01$, $n = 10$). C. Contents of DTT in the plasma. The bars indicate DTT in HPLC: 1. DTT in 0.1% trifluoroacetic acid; 2. DTT in the blood of mice injected with saline; 3. DTT in the blood of mice injected with daintain/AIF-1.

Fig. 3. Effects of daintain/AIF-1 on U937 macrophages uptaking ox-LDL. A. The detection of intracellular neutral lipids with oil red O at concentrations of 0, 10, 100 and 1000 ng/mL daintain/AIF-1 in 1, 2, 3 and 4 respectively. B. The counted number of oil red O positive cells expressed as % of total cells ($*P < 0.05$, $**P < 0.01$, $n = 5$). C. SRA expression, stimulated by 0, 1, 10 and 100 ng/mL daintain/AIF-1. Glyceraldehyde 3-phosphate dehydrogenase GAPDH as internal calibration.

80% DTT consumed in the mice injected with saline (Fig. 2C). This result indicates that daintain/AIF-1 can enhance oxidative capacity *in vivo*.

Daintain/AIF-1 promotes U937 macrophages uptaking ox-LDL

When U937 macrophages incubated in ox-LDL (20 $\mu\text{g/mL}$) were exposed to concentration gradients of daintain/AIF-1 of 0, 1, 10, 100 ng/mL respectively, we observed that daintain/AIF-1 at 10 ng/mL intensely stimulated U937 macrophages to uptake ox-LDL, and accumulation of lipid droplets in the U937 cytoplasm, and foam cells were largely formed in the medium (Fig. 3A and 3B). These results showed that daintain/AIF-1 concentration at 1 ng/mL had no significant effect on uptake of ox-LDL vs the control medium. At a concentration of 100 ng/mL daintain/AIF-1, the effect on uptaking was not significantly different from the 10 ng/mL concentration (Fig. 3B).

To explore the influence of daintain/AIF-1 on U937 uptaking ox-LDL, we investigated whether daintain/AIF-1 could up-regulate the expression of SRA on U937 macrophages, which play a pivotal role for phagocytosis of ox-LDL and further foam cell formation [15].

Equal number of phorbol-12-myristate-13-acetate (PMA) -derived U937 cells was seeded into 24-well plates. By western blot, we inspected that daintain/AIF-1 potentially promoted SRA over expression on U937 macrophages, and the expression was gradually increased by the increased concentrations of daintain/AIF-1 (Fig. 3C).

Discussion

On account of inflammatory characteristics of polypeptide daintain/AIF-1, and atherosclerosis as a kind of chronic inflammatory condition, we further investigated how daintain/AIF-1 is associated with atherogenesis. By using immunohistochemical approaches, we firstly found a dense daintain/AIF-1 distribution in the tunica intima and media of the artery segment with atherosclerotic plaque from the Chinese individuals with CHD, and almost not in the artery segment without atherosclerotic plaque, which strongly suggests that daintain/AIF-1 plays a role in atherogenesis, special the formation of atherosclerotic plaque. We also *in vivo* investigated the effects of daintain/AIF-1 on production of CRP in BALB/c mice. The result showed that daintain/AIF-1 significantly increased the concentration of CRP in blood. Whether CRP is a causal factor in the pathogenesis of atherosclerosis is still a debated topic [16]. However, it is accepted that the elevated CRP in blood is a risk factor, which has been adopted as an indicator for diagnosis of cardiovascular disease [17]. Our results have demonstrated that daintain/AIF-1 is one of upstream elements which influences the level of CRP in organism.

Using DTT reduction method, we find that daintain/AIF-1 can notably enhance the oxidative capacity in blood of BALB/c mice. Growing evidences indicate that the oxidative capacity in blood can directly damage artery vessels through several pathways. For example, it constitutes the essential microenvironment for overproduction of reactive oxygen species leading to oxidative stress, playing further roles for atherogenesis [18, 19]. The stronger oxidative capacity in blood also promotes oxidation of LDL-C into ox-LDL-C, the latter is the substrate for formation of foam cells [20]. Daintain/AIF-1 can notably enhance the oxidative capacity in blood, implying that daintain/AIF-1 takes roles in the above biochemical reactions leading to atherogenesis in organisms. Furthermore, daintain/AIF-1 can impair the activity of plasma SOD *in vivo*. It has been demonstrated that SOD activity in arterial vessel is required not only to prevent deleterious effects of superoxide anion, but also to preserve NO activity and prevent peroxynitrite production [21].

It is well known that foam cells formed and accumulated in artery are the key events for atherogenesis [22]. Using oil red O to detect the intracellular neutral lipids, we discovered that *in vitro*, daintain/AIF-1 largely promoted the foam cell formation by its stimulating overexpression of SRA on U937 macrophages. In the meantime, the overexpressed SRA largely facilitated ingestion of ox-LDL by U937. It has been verified that SRA plays essential roles in the phagocytosis of macrophages for foam cell formation. It is inferable that daintain/AIF-1 could facilitate foam cell formation by upregulating expression of SRA on macrophages in the inflammatory region of artery.

Taken together, the data showed that the overexpressed daintain/AIF-1, the novel inflammatory polypeptide derived from macrophage lineages, is closely associated with atherogenesis via affecting the blood composition and promoting macrophage uptake and foam cell formation, and suggested that daintain/AIF-1 could be further characterized as a novel risk factor for atherogenesis.

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