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Daintain / AIF-1-Mediated Upregulation of HCY Expression and PAI-1 Secretion.

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ABSRTACT

Previous studies have confirmed that inflammatory cytokine Daintain/AIF-1 is widely present in atherosclerotic tissue. One of the mechanisms for its involvement in atherosclerosis is vascular lesions due to its mediated endothelial cell proliferation and migration. Daintain/AIF-1 could upregulate the levels of blood fibrinogen and C-reactive protein in Kunming mice. The effect of Daintain/AIF-1 on Serum Hcy and PAI-1 concentrations in Kunming mice was determined by using the cycle enzyme and quantitative double-antibody sandwich enzyme-linked immunosorbent assay. To further elucidate the effect of Daintain/AIF-1 on PAI-1 mRNA expression, we investigate whether Daintain/AIF-1 improve the expression of PAI-1 gene and protein by real-time RT-PCR and Western blot. Daintain/AIF-1 could significantly increase serum Hcy and PAI-1 levels in mice, and enhance PAI-1 mRNA and protein expression in HUVEC cells in a dose-dependent manner. We also find that Daintain/AIF-1 improve the expression of PAI-1 gene and protein in a time-dependent manner. Hcy (1000 μM) and Daintain/AIF-1 treatment could significantly increase PAI-1 expression in HUVEC cells, respectively, while co-treatment of HUVEC cells with 1000 μM Hcy and 0.1 μM Daintain/AIF-1 was unable to further enhance PAI-1 expression. These findings implicate Daintain/AIF-1 plays an important role in the process of AS occurrence and development, the development of therapeutic antibodies against the inflammatory cytokine may be a new way for AS prevention and treatment.

Keywords: Daintain/AIF-1; HCY; PAI-1; Atherosclerosis

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INTRODUCTION

Atherosclerosis (AS) is a vascular disease commonly occurred in middle and large arteries. It can induce a variety of diseases, leading to very serious outcomes[1-3].In recent years, with changes in China's economic development and people's life style, the incidence of AS in China continues to increase and AS has become one of the major threats to people's health. It has been well known that increased risk of atherosclerosis is associated with a variety of factors including age, sex, familial early-onset atherosclerosis history, smoking, obesity, high blood pressure, high level of low density lipoprotein, high cholesterol, and diabetes [4]. In addition, other factors related to coagulation-fibrinolytic system such as fibrinogen, tissue-type plasminogen activator, plasminogen activator inhibitor-1 (PAI-1) and coagulation factor VII are receiving more and more attentions. Moreover, C-reactive protein (CRP) and the presence of high homocysteine are also closely related to the occurrence of atherosclerosis and have independent predictive values.

Homocysteine (Hcy), also known as homocysteine, participates in the formation of atherosclerosis through a variety of ways such as reducing NO bioactivity, causing oxidative stress and promoting thrombosis vascular smooth muscle cell proliferation [5， 6]. PAI-1 is a single-chain glycoprotein with molecular weight of 50 kDa in family of serine protease inhibitors. Numerous data have indicated that PAI-1 is related to the development of atherosclerosis. Hamsten et al[7]found that enhancement of blood PAI 1-concentration is a risk factor, even a decisive factor of primary and recurrent acute myocardial infarction. In 1998, Thogersen et al [8] reported that high plasma PAI-1 concentration is strongly correlated with the incidence of myocardial infarction in middle-aged men and women and independent of other traditional risk factors. Lupu et al [9] found that PAI-1 concentration is increased in the atherosclerotic lesions of coronary arteries, which on one hand, could exacerbate the vessel wall injury, while on the other hand, the presence of plaque also enhances PAI-1 expression[10], suggesting that PAI-1 may be associated with plaque rupture and destabilization.

Daintain/AIF-1 is an inflammatory factor. It was first coloned from heterotopic cardiac allografts in rats [11]. Previous studies have shown that Daintain/AIF-1, on one hand, could promote the proliferation and migration of smooth muscle cells, thus playing an important role in cardiovascular diseases [12]. On the other hand, it can reduce blood superoxide dismutase (SOD) activity [13], thereby decreasing the body’s ability to timely remove metabolization-generated reactive oxygen species (ROS), and eventually causing endothelial damage. Wang et al reported that Daintain/AIF-1 is positive in atherosclerotic plaques, and can mediate LDL phagocytosis in macrophages and affect the expression of scavenger receptors [14]. The above evidences suggest that Daintain/AIF-1 may be a risk factor for AS. However, the effects of Daintain/AIF-1 on other risk factors affecting AS have not been reported. In the present study, we mainly investigated the regulation effects of Daintain/AIF-1 on Hcy and PAI-1.

METHODS

Mice assignment and treatment

Female Kunming mice at age of 6 weeks old from Hubei Provincial Disease Prevention and Control Center were randomly assigned into control and Daintain/AIF-1 groups. Mice in Daintain/AIF-1 group and control group were intravenously given either 10 uL of 1 μg/g Daintain/AIF-1 saline solution or saline, respectively, twice in 24 h of intervals.

Determination of serum Hcy concentration

24 h after the last injection, blood was collected from eyeballs and Hcy concentration was determined using the Hcy assay kit (cycle enzyme) from Ningbo Ruiyuan Bio-technology Co. Ltd. according to the operating instructions provided by the manufacturer.

Measurement of serum PAI-1 concentration

24 h after the last injection, blood was collected from eyeballs into a heparin containing tube and serum PAI-1 concentration was measured using a PAI-1 quantitative double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit from Shanghai Shenke Sheng Biotechnology Co. according to the protocol provided by the manufacturer.

PAI-1 gene detection in HUVEC cells

HUVEC cells were treated with or without Daintain/AIF-1 respectively, then the cells were collected and lyszed by 500 µL Trizol lysis reagent (Invitrogen) and mixed with equal volume of chloroform to remove proteins. After centrifugation, the supernatant was collected and mixed again with equal volume of chloroform. The supernatant was collected and mixed with 2 volumes of cold ethanol. The precipitation was dissolved using RNase-free water and reverse transcripted into cDNA using the RNA reverse transcription kit (Fermentas) according to the instructions provided by the manufacturer. cDNA was then used as templates to detect PAI-1 gene expression in HUVEC cells using the forward primer 5'-AGGGCTTCATGCCCCACTTCTTCA-3 and reverse primer 5'-AGTAGAGGG CATTCACCAGCACCA-3'. PCR products were subjected to agarose gel electrophoresis and analyzed using Bandscan 4.3 software to determine PAI-1 gene abundance.

Statistical analysis

Statistical analyses were performed using Origin software (version 6.1, OriginLab, USA). Experimental results were expressed as mean ± standard deviation (SD) for three different replicates at each test concentration. Statistical significance was established at $p<0.01$.

RESULTS

Serum Hcy and PAI-1 concentrations were enhanced in mice intravenously injected Daintain/AIF-1

As shown in Figure 1, serum Hcy and PAI-1 concentrations were 6.79±0.40 µmol/L and 14.9±2.33 ng/mL, respectively, in control mice while those were 9.56±0.58 µmol/L and 23.86±3.67 ng/mL, respectively, in mice intravenously injected Daintain/AIF-1, indicating that injecting Daintain/AIF-1 could significantly increase serum Hcy and PAI-1 levels in mice ($P<0.05$).

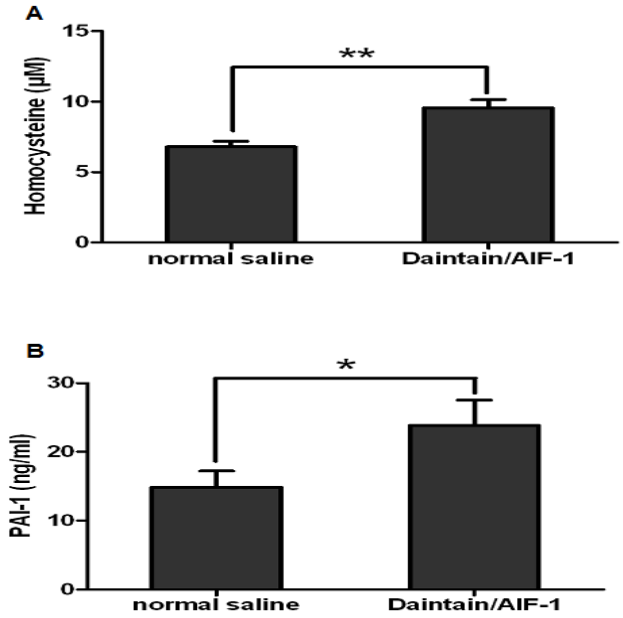

Fig 1: Daintain/AIF-1 via tail vein increased the concentration of Hcy (A) and PAI-1(B) in the blood of Kunming mice. (Data were presented as mean ± SD, n=5, ** P < 0.01, * P＜0.05)

Daintain/AIF-1 treatment dose-dependently increases PAI-1 gene and protein expression in HUVEC cells

Under physiological conditions, serum PAI-1 is mainly synthesized and secreted by endothelial cells. To determine whether Daintain/AIF-1 treatment is able to directly regulate the expression of PAI-1, we further investigated the effect of Daintain/AIF-1 on PAI-1 expression in cultured HUVEC cells. As shown in Figure 2, Daintain/AIF-1 treatment could enhance PAI-1 mRNA and protein expression in HUVEC cells in a dose-

dependent manner. We also found that Daintain/AIF-1 cound induce a peak PAI-1 expression at the concentration of 0.1 µmol/L which was increased by 45% compared with that of control in gene expression and about 2.9 fold compared with control in protein expression. However, a higher dose of Daintain/AIF-1 (5.0 µmol/L) had no significant effect on the expression of PAI-1 mRNA and protein in HUVEC cells.

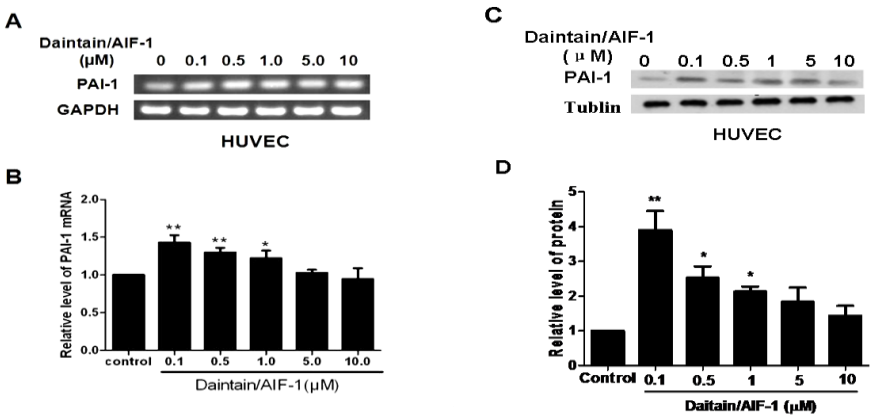

Fig 2: Daintain/AIF-1 enhanced the gene and protein expression of PAI-1 in HUVEC cells. HUVEC cells were treated with vary concentrations of Daintain/AIF-1 for 9 h, the levels of PAI-1 mRNA were evaluated by RT-PCR and western blot. A: The gene expression of PAI-1 were investigated by RT-PCR; B: Quantitative analysis of A; C, The protein expression of PAI-1 were investigated by western blot; D: Quantitative analysis of C (Data were presented as mean ± SD, n=3, ** P < 0.01, * P < 0.05).

Time effects of Daintain/AIF-1 treatment on PAI-1 gene and protein expression in HUVEC cells

To further elucidate the effect of Daintain/AIF-1 on PAI-1 mRNA expression, we investigate whether Daintain/AIF-1 improve the expression of PAI-1 gene and protein in a time-dependent manner. As shown in Figure 3, Daintain/AIF-1 treatment obviously promoted PAI-1 mRNA expression in HUVEC cells. After treatment with 0.1 µmol/L Daintain/AIF-1 for 3 h, PAI-1 mRNA expression reached its peak at 450% of that in control. Although treatment with 0.1 µmol/L Daintain/AIF-1 for 6 h, 9 h, 12 h could significantly increase PAI-1 mRNA expression, the level of PAI-1 mRNA in HUVEC cells showed a downtrend. Interestingly, treatment with 0.1 µmol/L Daintain/AIF-1 for 24h enhanced the expression of PAI-1 mRNA in HUVEC cells permantly. The results of western blot showed that 0.1 µmol/L Daintain/AIF-1 stimulation could largely increase PAI-1 protein expression. Whereas, there were no obvious increase on the PAI-1 protein expression after treatment HUVEC cells with 0.1 µmol/L Daintain/AIF-1 for 3 h.

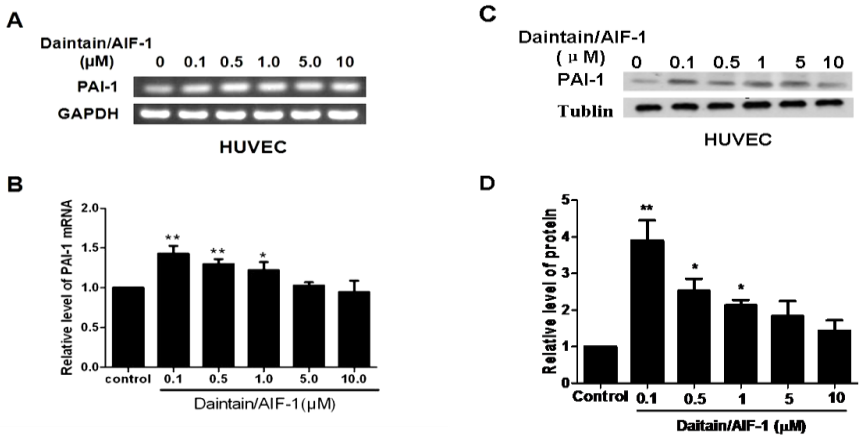

Fig 3: Daintain/AIF-1 enhanced the expression of PAI-1 mRNA in HUVEC cells. HUVEC cells were treated with 0.1µM Daintain/AIF-1 for 3 h, 6 h, 9 h, 12 h and 24 h, the levels of PAI-1 mRNA were tested by RT-PCR and western blot. A: The gene expression of PAI-1 were investigated by RT-PCR; B: Quantitative analysis of A; C: The protein expression of PAI-1 were investigated by western blot; D: Quantitative analysis of C. (Data were presented as mean ± SD, n=3, ** P < 0.01, * P < 0.05).

Effects of Daintain/AIF-1 and Hcy co-treatment on PAI-1 expression in HUVEC cells

It has been reported that high concentrations of Hcy could promote PAI-1 expression in HUVEC cells. Our results showed that Daintain/AIF-1 treatment could upregulate PAI-1 expression in HUVEC cells. Morever, injecting Daintain/AIF-1 by tail vein could increase serum Hcy concentration in BABC mice. Therefore, we further investigated whether co-treatment with Daintain/AIF-1 and Hcy could synergistically increase PAI-1 expression in HUVEC cells. Consistent with others, the results (Figure 4) showed that high concentration of Hcy (1000 μM) and Daintain/AIF-1 treatment could significantly increase PAI-1 expression in HUVEC cells, respectively, while co-treatment of HUVEC cells with 1000 μM Hcy and 0.1 μM Daintain/AIF-1 was unable to further enhance PAI-1 expression, indicating that Hcy and Daintain/AIF-1 has no synergistic effects on expression of PAI-1 in HUVEC cells.

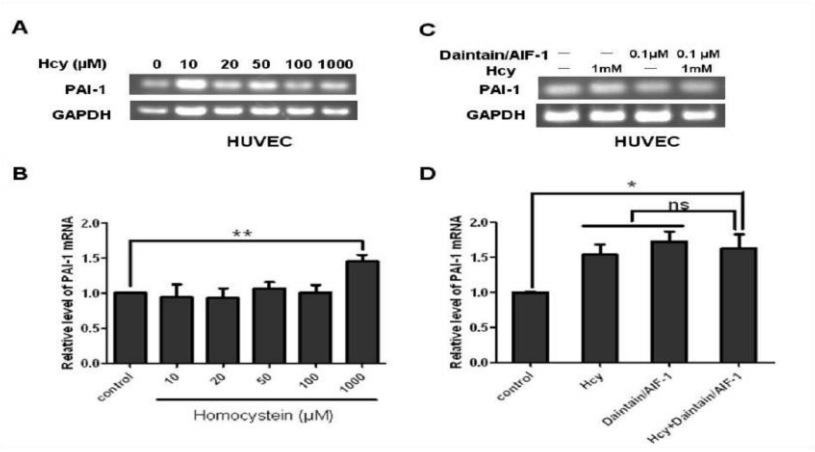

Fig 4: The synergistic effect of Hcy and Daintain/AIF-1 on PAI-1 expression in HUVEC cells. A: HUVEC cells were treated with various concentrations of Hcy for 9 h, then the PAI-1 mRNA levels were measured by RT-PCR. C: HUVEC cells were treated with 1mM Hcy, 0.1μM Daintain/AIF-1 and combination 1mM Hcy with 0.1 μM Daintain/AIF-1 for 9 h, respectively. The PAI-1 mRNA levels were measured by RT-PCR. B and D: Quantitative of A and C. (Data were presented as mean ± SD, n=3, ** P < 0.01, * P < 0.05).

DISCUSION

Atherosclerosis and its caused stroke, myocardial infarction and other secondary diseases are common diseases seriously threatening human health. Their pathogenesis is a complex process involving the interactions of multiple factors. Long-term epidemiological studies as well experimental and clinical investigations have indicated that numbers of factors are closely related with increased risks of atherosclerosis and appropriate interventions may reduce the risk of atherosclerosis. In recent years, in addition to traditional atherosclerosis risk factors such as high blood pressure, high cholesterol and high LDL, other factors such as high Hcy hyperlipidemia as well as factors associated with coagulation-fibrinolysis including fibrinogen, t-PA, PAI-1 and others have drawn increasingly more attention.

High Hcy concentration could induce oxidative stress, result in endothelial cell dysfunction, promote vascular smooth muscle cell migration and proliferation, activate platelet and imbalance coagulation-fibrinolysis system, thereby contributing to the development and progression of atherosclerosis [5]. It has been reported that serum Hcy concentration has a dose-response relationship with atherosclerosis [15]. Our results show that injecting Daintain/AIF-1by tail vein in female Kunming mice could increase serum Hcy concentration, suggesting that Daintain/AIF-1 may promote the occurrence and development of atherosclerosis by enhancing serum Hcy concentration. However, the underlying mechanisms need further study.

PAI-1 is an important physiological inhibitor of the fibrinolytic system. It is mainly present in blood and platelets, and released into the bloodstream by activated platelets. Excessive PAI-1 will decrease fibrinolytic activity, causing deposition of fibrin and formation of thrombosis, thereby contributing to the occurrence and development of atherosclerosis [16]. In this study, we also found that injecting Daintain/AIF-1 via tail vein increased serum PAI-1 concentration in female Kunming mice. In addition, treatment of HUVEC cells with low

rather than high concentrations of Daintain/AIF-1 could enhance PAI-1 mRNA expression. Moreover, treatment of HUVEC cells with 0.1 μmol/L Daintain/AIF-1 for 3 h, 6 h, 9 h, 12 h and 24 h could significantly enhance PAI-1 gene and protein expression, reaching its peak at 3 h of treatment. The above results suggest that Daintain/AIF-1 could decrease local plasmin activity by promoting PAI-1 expression and secretion in endothelial cells, thereby reducing matrix metalloproteinase activity and inhibiting matrix degradation. A large amount of matrix deposit will further lead to endometrial stromal thickening and luminal narrowing and reduction of local fibrinolytic activity can also affect TGF-β activity, thereby promoting smooth muscle cell proliferation and accelerating plaque formation and enlargement.

Consistent with a previous report [17], we also found that only high concentration Hcy could upregulate PAI-1 mRNA level in HUVEC cells. In addition, high dose Hcy and low dose Daintain/AIF-1 alone could stimulate the expression of PAI-1 mRNA in HUVEC cells. However, co-treatment with Hcy and Daintain/AIF-1 has no additive effect, suggesting that they may stimulate PAI-1 expression in endothelial cells through same pathway.

CONCLUSION

In summary, Daintain/AIF-1 could regulate multiple risk factors for AS. Although its exact mechanisms have yet to be studied in depth, it is certain that Daintain/AIF-1 plays an important role in the process of AS occurrence and developmen. Therefore, the development of therapeutic antibodies against the inflammatory cytokine may be a new way for AS prevention and treatment.

Conflicts of Interests

The authors confirm no external source of founding or conflicts of interest are present in this study.

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