Daintain/AIF-1 (Allograft Inflammatory Factor-1) Promotes Erythrocyte Lysis and Heme Release Probably via Binding to Hemoglobin

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Free heme is potentially cytotoxic, particularly in the presence of oxidants or activated phagocytes. Daintain/AIF-1 (allograft inflammatory factor-1) is a macrophage factor that has been implicated in the regulation of inflammation. In the present study, daintain/AIF-1 was found to induce cytolysis of erythrocytes, resulting in heme release *in vitro*. Furthermore, the interacting protein of daintain/AIF-1 was purified by daintain/AIF-1-6 histidine antigen fusion protein nickel affinity chromatography. MALDI-TOF-MS analysis identified hemoglobin subunit β-1 as an interacting protein of daintain/AIF-1. These data suggest that daintain/AIF-1 may be involved in heme-associated diseases.

Key words: Daintain/AIF-1, Heme, Erythrocyte Lysis, Hemoglobin Subunit β-1

Introduction

Heme is present in the hemoglobin in blood. Extracellular hemoglobin is easily oxidized and readily releases heme. Free heme can be quite cytotoxic, particularly in the presence of oxidants or activated phagocytes. Because of the hydrophobic nature of heme, it can rapidly intercalate with cell membranes and cause severe damage. Hemoglobin-derived heme has been demonstrated to act as a catalyst for the oxidation of low-density lipoprotein (LDL), which in turn causes atherosclerosis (Jeney *et al*., 2002).

In the mid 1990s, Utans and coworkers and our team identified a novel macrophage factor from different systems and named it allograft inflammatory factor-1 (AIF-1) and daintain, respectively (Utans *et al*., 1995; Chen *et al*., 1994, 1997). When aligned, the amino acid sequences of daintain and AIF-1 are highly similar. Therefore, we call the polypeptide daintain/AIF-1. During the last 10 years, this peptide has rapidly gained interest by a fast growing group of scientists. To date, an overwhelming body of evidence indicates that endogenous daintain/AIF-1 and its related proteins (Deininger *et al*., 2002; Ohsawa *et al*., 1997) affect numerous critical cellular functions including the survival and pro-inflammatory activity of macrophages (Watano *et al*., 2001), the augmentation of the production of cytokines in a mouse macrophage cell line (Yang *et al*., 2005), the promotion of vascular smooth muscle cell proliferation and migration, the association with neointimal hyperplasia and p38 kinase activity (Autieri *et al*., 2003; Sommerville *et al*., 2009; Chen *et al*., 2004), as well as the regulation of endothelial cell activation, signal transduction, and vasculogenesis (Ying *et al*., 2009).

But the potential role of daintain/AIF-1 in blood and the mechanism by which it acts are not clear. Often it is possible to deduce the function of a protein by identification of its binding partners. In the present study, we have used this approach to uncover the role of daintain/AIF-1 in blood and its mechanism of action.

Materials and Methods

Materials

Kunming mice, 18–20 g in weight, were obtained from the standard animal centre of the Hubei Province, China. The Sepharose-Ni affinity col-

umn was purchased from Amersham Pharmacia (Uppsala, Sweden).

Erythrocyte cytolysis and heme assay

Fresh erythrocytes obtained from Kunming mice were washed three times with saline and diluted to $1 \cdot 10^7$ cells/mL for use. Daintain/AIF-1 (Chen *et al.*, 1997) was then added to 100 μL of the cell suspension to a final concentration of 3 and 30 μM, respectively. For the controls, 3 and 30 μM bovine serum albumin (BSA), and saline alone were used, respectively. All samples were incubated at 37 °C followed by observation after 0, 15, 30 min under a microscope to check cell lysis. Free heme in the cell supernatants was determined by the absorbance at 405 nm (Tsai *et al.*, 1993).

Affinity chromatography

Daintain/AIF-1-6 histidine was expressed and purified as we reported previously (Wang *et al.*, 2010). Kunming mice were bled into evacuated tubes, and the blood was diluted in cell lysis solution, then stirred for 5 min. The lysate was centrifuged at 13,000 x *g* at 4 °C for 10 min, and the clear supernatant was subjected to affinity purification. Five mg ligand daintain/AIF-1-6 histidine were coupled to 1 mL Sepharose-Ni affinity column according to the manufacturer's protocol, and Sepharose-Ni beads not coupled with daintain/AIF-1-6 histidine were used as control. The complex of daintain/AIF-1 and its binding protein was eluted with 100 mM imidazole. Proteins eluted from the affinity column were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% PA). The band on the gel was digested with trypsin, and matrix-assisted laser desorption/ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) (Micromass, Manchester, UK) was used to chemically characterize the protein fragments. The obtained data were submitted to the database Matrixscience using the MASCOT software (http://www.matrixscience.com) for identifying the binding protein.

Results

Erythrocyte cytolysis and heme assay

When erythrocytes from Kunming mice were incubated with 30 μM daintain/AIF-1 in saline at 37 °C, pH 7.4, hemolysis was obvious after 30 min, while no change was observed with 3 μM daintain/AIF-1 or 3 or 30 μM BSA (Fig. 1). This observation that an endogenous peptide produced by macrophages can induce erythrocyte lysis was made for the first time. The concentration of heme in the supernatant from cells treated with 30 μM BSA was low, and no change was detected after 30 min. The concentration of heme in the supernatant from cells treated with 30 μM daintain/AIF-1 was considerably elevated, with a peak value at 30 min (Fig. 2).

Affinity chromatography

Daintain/AIF-1 was coupled to the Sepharose-Ni affinity column through a tail of six histidines. When proteins from the erythrocyte lysates, which had bound to the affinity column and were eluted with imidazole, were separated by SDS-PAGE, a protein band was found in the daintain/AIF-1 sample which was absent from the control (Fig. 3). The protein was hydrolyzed with trypsin and identified by MALDI-TOF-MS as hemoglobin subunit β-1.

Discussion

In this study, we extended our earlier observations on the activities of daintain/AIF-1 produced by macrophages (Chen *et al.*, 1994, 1997). When erythrocytes were exposed to daintain/AIF-1, they lysed, resulting in heme release. Because heme is lipophilic, it can easily intercalate in the membrane and impair lipid bilayers and organelles, such as mitochondria and nuclei, and destabilize the cytoskeleton (Balla *et al.*, 1991; Beri and Chandra, 1993; Ryter and Tyrrell, 2000).

To investigate the mechanism of how daintain/AIF-1 promotes heme release, the factor inter-

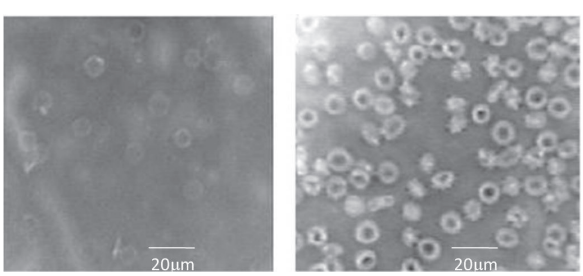

Fig. 1. Erythrocytes after a 30-min incubation with either 30 μM daintain/AIF-1 (left) or 30 μM BSA (right).

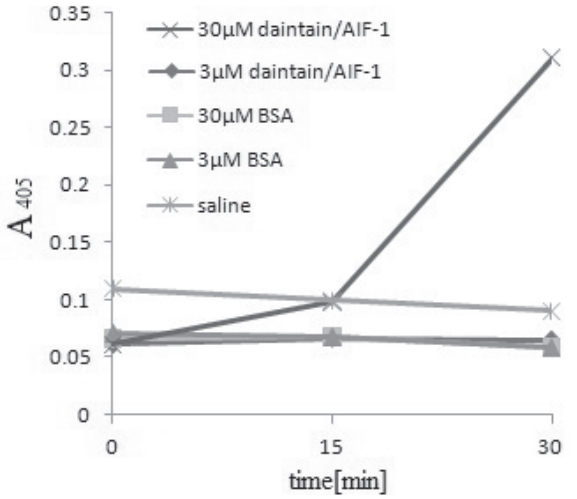

Fig. 2. Daintain/AIF-1-induced heme release. Heme absorbance in the supernatant was determined after incubation of cells with 3 and 30 μM daintain/AIF-1, or 3 and 30 μM BSA, respectively, or saline alone.

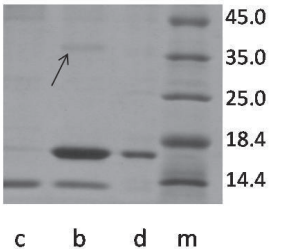

Fig. 3. Identification of daintain/AIF-1 binding protein. Proteins eluting from the daintain/AIF-1 affinity column were separated by SDS-PAGE. c, control; b, daintain/AIF-1 treatment; d, daintain/AIF-1 standard; m, protein markers (kDa). The band marked with an arrow was subjected to MALDI-TOF-MS.

acting with daintain/AIF-1 was purified and characterized as hemoglobin subunit β-1.

There is accumulating evidence that an excess of free heme can cause cell damage and tissue injury, since heme catalyzes the formation of reactive oxygen species (ROS), resulting in oxidative stress (Vercellotti *et al.*, 1994; Jeney *et al.*, 2002). Various pathologic conditions, such as hemorrhage, hemolysis, and cell injury, are characterized by the release of large amounts of heme, thus daintain/AIF-1 may be involved in the mentioned pathologic processes.

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