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ZIP8 induces monocyte adhesion to the aortas ex-vivo by regulating zinc influx

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ABSTRACT

Monocytes recruited and adhering to the inflamed arteries are crucial for atherosclerosis development. Here, we report the role of zinc (Zn^{2+}) homeostasis in monocyte adhesion and recruitment. By comparing the expression levels of Zn^{2+} transporters between non-adhering and adhering monocytes, we found that the Zn^{2+} importer ZIP8 was specifically upregulated in monocytes adhering to the aortas ex-vivo. Although the overexpression of ZIP8 increased the absorption of Zn^{2+}, Fe^{2+} and Cd^{2+} in monocytes, only Zn^{2+} supplementation was demonstrated capable of promoting the adhesion of monocytes to endothelial monolayers in vitro. In addition, we confirmed the role of ZIP8-dependent Zn^{2+} influx in promoting monocyte adhesion to the aortas ex-vivo. More importantly, the enforced expression of ZIP8 increased monocyte adhesion and recruitment to the nascent atherosclerotic lesions in $ApoE^{-/-}$ mice. Overall, our results suggest that the Zn^{2+} influx in monocytes regulated by ZIP8 is a novel factor determining their adhesion and recruitment to atherosclerotic lesions, and that targeting ZIP8 or Zn^{2+} homeostasis may represent a novel strategy to interfere these activities.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of arteries and represents the major cause for cardiovascular diseases [1]. Monocytes recruited to the inflamed arteries and plaques play a central role for the development and eventual rupture of atherosclerotic lesions [2,3]. In the early phases of atherosclerosis, the inflamed artery endothelium increases the expression of cell adhesion molecules and the secretion of chemoattractants to recruit leukocytes including mainly monocytes [4]. Monocytes adhere to the endothelium and then transmigrate into the arterial intima, where they proliferate and differentiate into distinct subsets of macrophages that contribute to inflammation or resolution [3]. It has been reported that two main circulating monocyte populations, classical monocytes ($Ly6C^{high}$) and patrolling monocytes ($Ly6C^{low}$), are recruited to the inflamed arteries with different frequencies [5,6]. In addition, the proliferation of monocytes and their derived macrophages is also important for determining their number in the plaque [7]. To date, less is known about how the adhesive behavior of monocytes is regulated.

Zinc (Zn^{2+}) is the second most abundant trace element that is indispensable for many physiological and pathological processes [8]. Zn^{2+} not only serves as an integrate activator or coactivator for many proteins by providing a structural scaffold [9], but also presents in a labile form in cytoplasm and some intracellular compartments [10]. Normal cellular activities require tightly regulated Zn^{2+} homeostasis, and its disruption has been linked to abnormal embryonic development, immune dysfunction and increased mortality, etc. [11]. Zn^{2+} homeostasis is primarily controlled by Zn^{2+} transporters consisting of 10 members (ZNT1-ZNT10) of exporters and 14 members (ZIP1-ZIP14) of importers [12]. The ZNT family mediates Zn^{2+} efflux from cells or influx into intracellular compartments from the cytosol, and oppositely, the ZIP family promotes Zn^{2+} influx from the extracellular environment or release from intracellular compartments into the cytoplasm [13].

Recently, the functional abnormalities of Zn^{2+} transporters and Zn^{2+} homeostasis have been implicated in immunity, such as modulating inflammation, oxidative stress and signal transduction [10,14–16]. However, their roles in atherosclerosis are largely undetermined. In this study, we found that the ZIP8-mediated Zn^{2+} influx promoted monocyte adhesion to aortas ex-vivo and recruitment to the

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nascent atherosclerotic lesions in vivo, thereby uncovering a previously unappreciated link between ZIP8-mediated Zn^{2+} transport and atherosclerosis.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies and reagents were purchased from the sources as follow: ZIP8 (Proteintech, 20459-1-AP), β-actin (AC-15) (Santa Cruz, sc-69879), SM22α (C-12) (Santa Cruz, sc-373928), γ-Tubulin (GUT-88) (abcam, ab11316), PECAM1 (Novus, NB100-2284), Ki-67-PE-Cy7 (BD Pharmingen, 561283), Ly6C-BV421 (BD Pharmingen, 562727), Goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004), Goat anti-mouse IgG-HRP (Santa Cruz, sc-2302), Goat anti-rat IgG-HRP (Millipore, AP136P). Collagenase II, BCECF, paraformaldehyde, $ZnCl_2$, $FeCl_2$, $CdCl_2$, $MnCl_2$, $CoCl_2$ and TPEN were purchased from Sigma-Aldrich.

2.2. Animals and diet

$ApoE^{-/-}$ mice (on a C57BL/6J background) and C57BL/6J wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained under a specific pathogen-free condition at the facilities of The first Affiliated Hospital of Xi'an Jiaotong University. All procedures of animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of The first Affiliated Hospital of Xi'an Jiaotong University for animal welfare. Generally, mice were kept on a chow diet (CD) (PicoLab Rodent Chow 5010; LabDiet, St. Louis, MO). To prepare aortas used for ex-vivo assay, $ApoE^{-/-}$ mice were fed a western high-fat diet (WD) (21% fat and 0.2% cholesterol, Harlan Teklad, Harlan Laboratories, Indianapolis, IN) for 16 weeks starting at 8 weeks of age and used at 24 weeks of age. To establish nascent atherosclerosis [17], $ApoE^{-/-}$ mice were fed a WD for 3 weeks starting at 8 weeks of age.

2.3. Isolation and culture of monocytes

Monocytes were isolated from peripheral blood of C57BL/6 wild-type mice by positive selection with anti-CD115-bio Abs and streptavidin-specific microbeads (Miltenyi Biotec) [18]. Monocytes were then maintained in gelatin-coated plates in complete DMEM medium (10% FBS, 10% HS, 20 mM HEPES, 4 mM glutamine, NEAA and penicillin-streptomycin), and incubated in a humidified incubator at 37 °C with 5% CO_2 for yielding long-term adherent cell cultures [19].

2.4. Adenoviruse infection and siRNA-mediated knockdown of monocytes

Adenoviruses expressing mouse *Zip8* (Ad-*Zip8*) (ADV-272407) or empty control (Ad-Ctrl) (1300) were purchased from Vector Biolabs. Isolated monocytes were cultured for 2 days as described before. Cells were then infected with Ad-C or Ad-*Zip8* for 12 h, followed by 2-day culture for adenovirus-mediated expression and further analyses. For siRNA transfection, monocytes were transfected with 40 nM small interfering RNA targeting luciferase (siCtrl) or *ZIP8* (si*ZIP8*) using reagent lipofectamine RNAimax (Invitrogen). After transfection, monocytes were further cultured for 2 days according to the manufacturer's instructions.

2.5. Ex-vivo cell adhesion assay

The ex-vivo monocyte adhesion assay was performed as previously described [18]. Briefly, aortas from $ApoE^{-/-}$ mice fed a WD for 16 weeks were dissected, longitudinally cut open and pinned to sterile agarose gel in serum-free DMEM medium. Meanwhile, peripheral blood monocytes marked with CD115 were isolated as described before and labeled with 10 μM BCECF. BCECF-labeled monocytes were co-cultured with pinned aortas in DMEM for 6 h. Non-adhering monocytes were washed off with PBS, and then collected in tubes or directly discarded according to experimental purposes. The adhering monocytes were imaged under a fluorescent microscope (Zeiss AxioObserver Z1; Magnification at 40/NA 0.6). The number of bound cells was counted in 20 random fields per condition using ImageJ software. For analyzing protein and mRNA levels, the adhering monocytes were harvested by trypsin digestion.

2.6. Isolation and culture of mouse aortic endothelia cells

The isolation of mouse aortic endothelial cells (MAECs) from C57BL/6J wild-type mice (8–12 weeks of age) required selective enzymatic digestion as previously described [20,21]. Briefly, the aorta lumen was filled with 1 mg/mL collagenase II (Sigma) using a 24-gauge cannula, and incubated for 1 h at 37 °C. ECs were removed from the aorta by flushing with DMEM containing 20% FBS. After culture for 1 week in complete DMEM medium (20% FBS, penicillin-streptomycin, glutamine, NEAA, sodium pyruvate, HEPES, heparin and ECGS), ECs were purified with rat-anti-mouse-CD102 (BD Pharmingen) and sheep-anti-rat coated magnetic beads (Life Technologies). The purity of ECs was confirmed by specific expression of PECAM-1 via Western blotting analysis. The purified MAECs were then maintained in gelatin-coated plates in complete DMEM medium as described before.

2.7. Cell adhesion assay

WEHI78/24 is a mouse monocyte cell line that has been fully characterized and used in cell adhesion assay [22]. The monolayers of MAECs were cultured to reach confluent and co-cultured with BCECF-labeled primary monocytes or BCECF-labeled WEHI78/24 cells in static conditions with 5% CO_2 for 1 h at 37 °C. Unbound cells were washed and the remaining co-cultures at bottom were fixed with 4% paraformaldehyde. The adherent cells were imaged under a fluorescent microscope (Zeiss AxioObserver Z1; Magnification at 40/NA 0.6). The number of bound cells was counted in 20 random fields per condition using ImageJ software.

2.8. Measurement of intracellular metal ion levels

Before incubated with detection reagents, cells were washed with 0.1 M EDTA to remove nonspecifically bound metal ions and twice with ice-cold PBS. FluoZin-3AM, was used to measure the intracellular labile Zn^{2+} levels [23]. Briefly, cells were incubated with 1 μM FluoZin-3AM for 30 min at 37 °C and 5% CO_2 in the dark, and the fluorescence was measured using a Spectramax Gemini microplate fluorescence reader (Molecular Devices). Intracellular Fe^{2+} levels were measured using ferrozine assay [24,25]. Briefly, cells were lysed in lysis buffer (1% Triton X-100, 10% glycerol, 25 mM HEPES, pH 7.4 in PBS) for 30 min at 4 °C. The lysate supernatants, adjusted to equal amounts of protein, were incubated with Fe^{2+} detection reagents (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1 M ascorbic acid dissolved in water), and absorbance was read at 550 nm using a VersaMax microplate reader (Molecular Devices). Intracellular Mn^{2+} levels were measured using a cellular Fura-2 Mn^{2+} extraction assay [26]. Briefly, cell lysate supernatants were extracted with 0.1% Triton X-100 in PBS containing 0.5 mM Fura-2 (Enzo Life Sciences) at 33 °C for 1 h. Fura-2 fluorescence was measured with excitation at 355 nm and emission at 538 nm using a Spectramax Gemini microplate fluorescence reader (Molecular Devices). Intracellular Cd^{2+} levels were measured using Leadmium Green (Invitrogen) [27]. Briefly, cells were incubated with 0.1 mg/mL of Leadmium dye at 37 °C for 1 h. Fluorescence was measured using a Spectramax Gemini microplate fluorescence reader (Molecular Devices) with excitation at 488 nm, and emission at 530 nm. Intracellular Co^{2+} levels were detected using fluorescent probe SGPB1 [28]. Briefly, cell were incubated with 1 M SGPB1 at 37 °C for 20 min.