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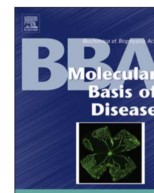

5-Lipoxygenase in monocytes emerges as a therapeutic target for intimal hyperplasia in a murine wire-injured femoral artery

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ABSTRACT

Given the importance of leukotrienes in vascular inflammation induced by local tissue injury, this study investigated the role for 5-lipoxygenase (5-LO) in monocytes in the development of intimal hyperplasia. As a mechanistic study, the importance of monocyte 5-LO in monocyte-macrophage differentiation with subsequent infiltration in neointima was evaluated. In a mouse model of wire-injured femoral artery, intimal hyperplasia started as early as 2 wks after injury, and luminal area and blood flow were reduced due to increased neointima formation. Time-dependent increases in macrophage infiltration were observed in neointima and showed a positive relationship with neointima volume. In 5-LO-deficient (KO) mice or wild-type (WT) mice treated with an inhibitor of 5-LO activating protein (MK886, 1 and 10 mg/kg), intimal hyperplasia and macrophage infiltration into neointima were reduced, but monocyte adhesion to injured luminal surface was not inhibited, which suggested 5-LO participates in monocyte-macrophage differentiation. In an *in vitro* study, monocyte-macrophage differentiation was found to be increased by high mobility group box 1 protein (HMGB1), but this effect was attenuated in cells isolated from 5-LO-KO mice. Furthermore, macrophage infiltration and intimal hyperplasia were more prominent in 5-LO-KO mice transplanted with monocytes from WT mice than in 5-LO-KO mice transplanted with monocytes from 5-LO-KO mice. Taken together, it was suggested that 5-LO in monocytes played a pivotal role in monocyte-macrophage differentiation and subsequent infiltration of macrophage in neointima, leading to vascular remodeling after vascular injury.

1. Introduction

Vascular restenosis is defined as a recurrent narrowing of lumen in patients that have undergone percutaneous intervention of coronary, carotid, or peripheral arteries, and is characterized by the infiltration of inflammatory cells in the neointima [1,2]. Endoluminal vascular interventional procedures cause stretching and injury of the vascular wall [3], and subsequently release endogenous damage-associated molecular patterns (DAMPs) including high-mobility group box 1 (HMGB1) [4]. Recently, HMGB1 and its receptor were suggested to be possible therapeutic targets for the attenuation of DAMP-mediated vascular inflammatory responses [5]. However, the precise role played by HMGB1 in the vascular remodeling process after vascular injury remains unclear.

During vascular inflammatory processes [6], the recruitment of monocytes to the injured tissues, their subsequent transformation into macrophages, and the overproduction of inflammatory cytokines are major steps [7,8]. These events then stimulate vascular smooth muscle cell (VSMC) accumulation and extracellular matrix (ECM) deposition and result in intimal hyperplasia and vascular occlusion [9]. However, our incomplete understanding of how vascular inflammation contributes to vascular remodeling explains the incapability of current treatments to prevent post-angioplasty restenosis.

Leukotrienes (LTs) exert their actions via four subclasses of receptors including BLT1 and BLT2 receptors for LTB4, and CysLT1 and CysLT2 receptors activated by the cysteinyl-leukotrienes [10], and mediate inflammatory responses in various cardiovascular diseases [11]. Several key proteins within the leukotriene cascades, such as 5-LO

Abbreviations: 5-LO, 5-lipoxygenase; WT, Wild-type; FLAP, 5-lipoxygenase activating protein; LT, leukotriene; ROS, reactive oxygen species; MMP, matrix metalloproteinases; DAMP, damage-associated molecular patterns; HMGB1, high mobility group box 1 protein; VSMC, vascular smooth muscle cell; BMDM, bone marrow derived monocyte; PBMC, peripheral blood mononuclear cell; LDPI, laser Doppler perfusion imaging; H & E, hematoxylin and Eosin; α-SMA, alpha-smooth muscle actin; DAPI, 4′6-diamidino-2-phenylindole; BLT receptor, receptor for leukotriene B4; CysLT receptor, receptor for cysteinyl-leukotrienes

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and FLAP (arachidonate 5-lipoxygenase activating protein), and LT receptors are highly expressed in human atherosclerotic plaques [12,13]. In particular, LT receptor activation has been implicated in atherogenesis and vascular remodeling after angioplasty [10,14]. Our previous studies showed that 5-LO was involved in the pathologic mechanism of vascular remodeling diseases via the increased production of ROS (reactive oxygen species) and MMP (matrix metalloproteinases) [15,16,17]. During these studies, we also found that 5-LO importantly contributed to the development of atherosclerosis by increasing the expression of adhesion molecule on monocytes, and thus increasing monocyte adhesion to vascular endothelium [18]. In FLAP-deficient mice, neointimal hyperplasia in injured arteries was significantly attenuated by reducing inflammatory cytokine release from FLAP-deficient macrophages [19]. Although 5-LO was proposed as a therapeutic target in vascular remodeling diseases, the importance of 5-LO in monocytes in vascular inflammation with subsequent vascular remodeling in injured vasculatures remains unclear.

The importance of 5-LO pathways in neointima formation has been previously described [19], however, the molecular processes that initiate macrophage infiltration into injured vasculatures are not well understood. Given the importance of monocytes in vascular inflammation and subsequent neointima formation, we hypothesized that 5-LO in monocytes might directly influence macrophage infiltration into neointimal lesions. Accordingly, the importance of 5-LO in monocytes for intimal hyperplasia was investigated using 5-LO-deficient mice and 5-LO-deficient mice transplanted with monocytes from WT mice. In addition, the impact of monocyte 5-LO on monocyte-macrophage differentiation and subsequent macrophage infiltration into neointimal lesions was also evaluated as a mechanistic study.

2. Materials and methods

2.1. Ethics statement and animals

All animal procedures were conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, 2011 revision), and all animal-related experimental protocols were approved by the Pusan National University Institutional Animal Care and Use Committee. Genotyping, including that of 5-LO-deficient mice, was performed by PCR using a protocol provided by the Jackson Laboratory (Harlan Nossan, Italy). Wild-type (WT) control mice (C57BL/6J) were purchased from the Jackson Laboratory. The animals were housed in an air-conditioned room at 22–25 °C and kept under a 12-hour light/dark cycle. Food and water were provided *ad libitum*.

2.2. Vascular injury models and blood flow measurement

WT (C57BL/6J) and 5-LO-deficient mice (male, 7 wks-old) were subjected to right femoral artery injury using a 0.25 mm diameter angioplasty guide wire under chloral hydrate (450 mg/kg, i.p.) anesthesia and aseptic conditions, as previously described [20]. At 0, 1, 2, 3, and 4 wks after wire injury, femoral arteries of control and MK886 (1 and 10 mg/kg, i.p, daily)-treated mice were harvested, and then cross sectioned (4 μm). Tissue sections were stained with hematoxylin and eosin (H & E) and specific antibodies for immunohistological analyses. Femoral arterial blood flow was also measured using a laser Doppler perfusion imaging (LDPI) analyzer (Moor instruments, Devon, UK) at 0, 1, 2, 3, and 4 wks after femoral artery injury. The changes in blood flow was calculated on the basis of colored histogram pixels.

2.3. Chemicals and antibodies

MK 886 was purchased from EMD Serono (Rockland, MA, USA), high mobility group box 1 protein (HMGB1) from R & D systems (Minneapolis, MN, USA), alpha-smooth muscle actin (α-SMA) antibody from Sigma-Aldrich (St. Louis, MO), CD11b antibody from Abcam (Cambridge, MA), and CD14 antibody from Biorbyt (Cambridge, UK). 5-LO and CD36 antibodies were purchased from Santa Cruz Biotechnology Inc. (Beverly, MA), purified anti-mouse CD11b antibody (Cat No. 12-0112-81) and anti-mouse IgG isotype control antibody (Cat No. 16–4714) from eBioscience (San Diego, CA, USA), and R-phycoerythrin PE-conjugated mouse anti-human CD11b/Mac-1 (done ICRF44; BD) antibody (Cat No. 555388) and PE-conjugated mouse IgG isotype control (clone MOPC-21) antibody (Cat No. 555749) from BD (San Diego, CA, USA). Restriction enzymes were purchased from Promega (Madison, WI), and PCR primers from Bioneer (Seoul, KO).

2.4. Isolation of bone marrow-derived cells and culture

Bone marrow derived cells (BMDCs) were isolated from C57BL/6 mice (male, 7 wks-old) euthanized with carbon dioxide and cervical dislocation. Briefly, after bone marrow cells were harvested from femurs and tibiae, red blood cells were lysed using lysing buffer (Sigma-Aldrich Corp., St Louis, MO, USA), and the cell suspensions obtained were separated using Ficoll-Histopaque (Sigma-Aldrich Corp., St Louis, MO, USA). After centrifugation at 2000 rpm for 40 min, the cell pellets obtained were washed twice with PBS and resuspended in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS). BMDCs were maintained in RPMI 1640 containing 10% FBS and antibiotic-antimycotic (Gibco BRL) at 37 °C. Cells (5×10^5/mL) were seeded and cultured for 24 h in complete medium for further experiments.

2.5. En face immunohistochemistry

Tissue fixation and preparation were performed by systemic perfusion at a pressure of 180 mm Hg via left ventricle using 10 ml of normal saline followed by 4% buffered formalin. Femoral arteries were then carefully dissected to the lower region, and immersed in 4% buffered formalin overnight at 4 °C. Femoral arteries were divided into 3–5 mm segments and the proximal ends of segments were marked. Segments were then placed in 0.05% hydrogen peroxide in methanol for 20 min at room temperature, rinsed three times with rinse buffer, placed in boiling citrate buffer, and then cooled gradually under running water. Segments were incubated with anti-rat CD11b/Mac-1 monoclonal antibody (1:200), incubated with biotinylated goat anti-rat IgG, and reacted with horseradish peroxidase-conjugated streptavidin (DAKO). Images were visualized by microscopy, and stained cells were counted using Image J software (NIH, MD, USA).

2.6. Flow cytometric analysis

Cell differentiation was assessed by following morphological changes and the expression of cell surface marker CD11b by flow cytometry analysis as previously described [21]. To assess the surface expression of CD11b protein, BMDCs were collected from cultures and washed with fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% FCS and 0.05% NaN_3). Cells were incubated to a FcR blocking reagent (MACS Miltenyi Biotec, CA, USA) to increase the specificity of antibody to their target protein, and then incubated with PE-conjugated anti-CD11b (Clone M1/70) antibody (1:500). Analysis was performed using a FACS Calibur and CELLQUESTPRO software (BD). 1×10^4 cells were recorded per sample. Live cells were gated based on size (FSC) and granularity (SSC), and then CD11b expression was analyzed.

2.7. RT-qPCR analysis

Total RNA was isolated from cells using Qiazol reagent (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the Improm-II Reverse Transcription System (Promega). 5-LO gene expression was determined by real-time PCR using 1 ng of reverse-transcribed cDNA

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