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Contribution of monocytes Siglec-1 in stimulating T cells proliferation and activation in atherosclerosis

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ABSTRACT

Objective: Atherosclerosis (AS) is widely accepted as an inflammatory disease and monocytes are particularly important in inflammatory immune responses. As an important biomarker of monocytes activation, Siglec-1 is highly expressed on circulating monocytes and atherosclerotic plaques of coronary artery disease (CAD) patients, but the exact role of Siglec-1 has not been elucidated.
Methods: M-CSF, INF-α, IFN-γ, TNF-α and ox-LDL alone or in combination were used to stimulate Siglec-1 expression on monocytes, whereas small interfering RNA (si-RNA) or blocking antibody was used to down-regulate Siglec-1. Meanwhile, the role of Siglec-1 in chemokines secretion was determined. Then monocytes from CAD patients or healthy controls were cocultured with CD4+ or CD8+ T cells from a third healthy individual, and lymphocyte proliferation and activation were determined.
Results: All the stimuluses could enhance Siglec-1 expression on monocytes in a dose-dependent manner, and M-CSF could synergistically stimulate Siglec-1 expression with ox-LDL. Moreover, the secretion of MCP-1, MIP-1α and MIP-2 were enhanced when Siglec-1 was up-regulated and down to normal level when Siglec-1 was blocked. More importantly, increased Siglec-1 expression on monocytes was related to the increased T cell proliferation and pro-inflammatory cytokines secretion in CAD patients. However, down-regulation of Siglec-1 could attenuate proliferation and activation of cocultured CD4+ and CD8+ T cells.
Conclusion: Siglec-1 can promote chemokines and pro-inflammatory cytokines secretion and influence the inflammatory process of AS.

1. Introduction

Atherosclerosis (AS) is a multifactorial process and its outcome, coronary artery disease, is considered to be responsible for the greatest number of deaths worldwide. Immune responses including innate and adaptive immune systems play important roles in the pathogenesis of atherosclerosis [1,2]. Among the immune cells in atherosclerotic plaques, mononuclear phagocyte system (mps) may be particularly important in the initiation of immune responses to plaque antigens, especially lipid antigens. Macrophages can internalize lipid antigen such as oxidized LDL and process and present it to T cells, resulting in T cells recruit to the site of atherosclerotic plaques [3]. Subsequently, cytokines production and population expansion of activated CD4+ and CD8+ T cells could exacerbate the inflammatory response in atherosclerotic plaque [4], leading to a series of atherosclerotic vascular events gradually.

The Siglecs are a family of sialic-acid-binding immunoglobulin-like lectins that are thought to promote cell–cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition [5,6]. Human Siglec-1 is first cloned by Paul R. Crocker's group in the year 2001 [7]. It is expressed exclusively in tissue-resident macrophages and is considered as an important biomarker of the activation of

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monocyte/macrophage. Evidence is accumulating that Siglecs, particularly Siglec-1, are important players in the initiation and progression of inflammatory and autoimmune diseases. Early study showed that Siglec-1 positive macrophages and CD4+ or CD8+ T cells were seen in atherosclerotic plaque of apolipoprotein E3-Leiden (APOE3-Leiden) transgenic mice [8]. Our previous study [9] found that Siglec-1 was highly expressed on circulating monocytes in coronary artery disease (CAD) patients. But the exact role of Siglec-1 in the pathogenesis of AS has not been elucidated so far. As an important biomarker of the activation of monocyte/macrophage, Siglec-1 may provide a potential pathophysiological link between lipid internalization, antigen presentation and T cells activation in atherosclerosis.

So we desired to explore the role of Siglec-1 in monocytes chemokines secretion and T cells proliferation and activation in this study. Firstly, the expression of Siglec-1 in atherosclerotic plaque of high-fat diet-fed ApoE$^{-/-}$ mice was determined by immunohistochemistry. Secondly, various stimuluses (M-CSF, INF-α, IFN-γ, TNF-α and ox-LDL) were used alone or in combination to enhance Siglec-1 expression and si-RNA targeting Siglec-1 or Siglec-1 specific blocking antibody was used to down-regulate Siglec-1 expression. And the role of Siglec-1 in chemokines secretion was determined. Thirdly, CD14+ monocytes from patients and healthy controls, CD4+ and CD8+ T cells from a third healthy blood donor were isolated by magnetic-activated cell sorting (MACS). Then, monocytes-lymphocytes allogenic mixed leukocyte reactions (MLR) were done to evaluate the capacity of monocytes from different sources in stimulating T cells proliferation and pro-inflammatory cytokines production. Meanwhile, the role of Siglec-1 in this process was determined.

2. Methods

For detailed methodology, please refer to the data supplement.

2.1. Patients

This study was in compliance with the Declaration of Helsinki ethical principles. Approval was granted by our university ethics review board and written informed consent was obtained from study subjects. The study group (see Table s1) consisted of 60 patients (18 ACS and 42 SA) who fulfilled the criteria of American Heart Association (AHA) for the diagnosis of ACS and SA, respectively. Thirty-two healthy volunteers, age and sex matched to the enrolled patients, served as a control group.

2.2. Cell line

A mouse macrophage cell line RAW264.7 was purchased from American Type Culture Collection (ATCC) and maintained in complete DMEM high glucose medium (Gibco, Grand Island, NY). M-CSF, INF-α, IFN-γ, TNF-α (all from R&D Systems, Minneapolis, MN) and ox-LDL alone or in combination were used to up-regulate the expression of Siglec-1. To down-regulate Siglec-1, si-RNA was used (see Supplementary Data). Then Siglec-1 expression was determined by flow cytometry, western blot or quantitative RT-PCR. Forty-eight hours after stimulation, chemokines (MCP-1, MIP-1α and MIP-2) secretion was determined by ELISA.

2.3. Mice

ApoE$^{-/-}$ mice were purchased from Animal Center of Peking University and maintained in SPF condition. Protocols for animal experiments were approved by the institutional animal use committee of the Second Military Medical University. Six weeks old male mice were fed a high-fat high-cholesterol Western diet for further 16 weeks. Plaques of aortic arch were obtained and immunohistochemical staining for Siglec-1 was performed using goat anti-mouse Siglec-1 (sc-23594) in 1:20 dilution (Santa Cruz Biotechnology, Santa Cruz, CA).

2.4. Magnetic-activated cell sorting (MACS)

PBMCs were isolated from heparinized peripheral blood by standard Ficoll–Paque PLUS (GE Healthcare, Uppsala, Sweden) density centrifugation. CD14, CD4 and CD8 cells were purified from PBMCs by positive selection using CD14, CD4 and CD8 MicroBeads, respectively (Miltenyi Biotech, Bergisch-Gladbach, Germany).

2.5. Flow cytometry analysis

For Siglec-1 detection, 2×10^5 RAW264.7 cells in 100 μl staining buffer (PBS + 0.5% BSA + 0.05% sodium azide) were firstly Fc-blocked with 1 μg of mouse IgG for 15 min and incubated with rat monoclonal (3D6.112) to sialoadhesin (Abcam, Cambridge, UK) at a concentration of 10 μg/ml for 1 h. After wash, cells were resuspended in 100 μl staining buffer containing DyLight™ 549 conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) at a concentration of 7.5 μg/ml for 30 min. And then washed and resuspended in 500 μl PBS. Cells were analyzed by FC500 flow cytometer and CXP Software (Beckman Coulter, Fullerton, CA). Appropriate isotype-matched control antibodies were used in parallel.

2.6. Western blot

Cells were lyzed and cell membrane protein was extracted by using transmembrane protein extraction kit (Novagen) with Extraction Buffer 2A. Proteins were separated and then transferred to PVDF membrane. Blots were blocked and incubated separately in blocking solution containing primary antibodies for Siglec-1 (R&D Systems, 1 μg/ml) or Na/K ATPase (Cell Signaling Technology, 1:1000) overnight at 4 °C. The next day, membranes were washed in TBST prior to 1 h incubation with appropriate HRP-conjugated secondary antibody (Jackson ImmunoResearch, 1:5000–1:10000 dilution). Signal was detected by using ECL chemiluminescent substrate (Millipore) and X-ray films.

2.7. Allogenic mixed leukocyte reactions (MLR)

Freshly isolated CD14+ monocytes from patients and controls were cultured in 96-well flat-bottom culture plates. To up-regulate the expression of Siglec-1 on monocytes, IFN-α (5 ng/ml, R&D Systems) was added in the culture medium in some cases [10–12]. To down-regulate Siglec-1, si-RNA or blocking antibody against Siglec-1 (2 μg/ml, HSn 7D2, Santa Cruz) was used. Twenty-four hours later, the supernatant was removed and monocytes were mixed at different ratios (1:5, 1:10, 1:20, 1:40, and 1:80) with freshly isolated CD4+ or CD8+ T cells (2×10^5 cells/200 μl) (Fig. s1) from a third healthy individual [13]. Lymphocytes alone and monocytes alone were used as negative controls. At day 3, the culture supernatants were collected for cytokine detection and cells were cultured for another 2 days. At day 5, cells were incubated with CCK-8 (Dojindo, Kumamoto, Japan) diluted 1:10 in