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Statin treatment reduces matrix degradation capacity of proinflammatory polarized macrophages

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A B S T R A C T

Background and aims: Macrophages are versatile immune cells involved in tissue degradation and remodeling. Proinflammatory macrophages have the highest capacity of matrix degradation and proteolysis. Within atherosclerotic lesions, proinflammatory macrophages are associated with unstable plaques. Statins have been demonstrated to increase plaque stability. Possible changes of polarized macrophage tissue degradation behavior under statin treatment are currently unknown.

Methods: Polarized macrophages were tested in vitro for matrix degradation capacity with or without statin treatment.

Results: Proinflammatory macrophages show high matrix degradation capacity, which is lost after statin treatment. Statin concentrations were within a physiological range and did not influence overall macrophage polarization. Proinflammatory macrophages showed however a loss of filopodia where activators of MMPs are located. Loss of matrix degradation in proinflammatory macrophages was associated with changes of MMP14 activation and loss of uPAR localization at filopodia. Supplementation of mevalonate restored localization of uPAR to cellular protrusions and matrix degradation capacity.

Conclusion: Statins reduce the matrix degradation potential of proinflammatory macrophages by reducing uPAR localization to cellular filopodia and reducing intracellular MMP14 activation.

1. Introduction

Macrophages are required to react to environmental cues and can thus polarize into specific subsets in order to fulfill their pathophysiological tasks. This polarization is mediated by external stimuli and leads to phenotypical changes in macrophage behavior. Polarization states can be defined by certain marker proteins. The two most drastic polarization conditions are a proinflammatory state and an alternatively activated tissue repair state [1–3]. In general, macrophage subsets can be characterized by the expression of pro and anti-inflammatory proteins, their capacity to engulf lipids and their matrix degradation capability [4]. In order to study macrophage polarization in vitro, macrophages can be polarized towards two distinctively characteristic phenotypes. Using lipopolysaccharide (LPS) and interferon-γ (IFN-γ) macrophages are polarized into a highly proinflammatory M (LPS + IFN) subset. To obtain an anti-inflammatory macrophage subset, macrophages are polarized using interleukin (IL)-4 and IL-13 to obtain M(IL4 + IL13) [2]. Within atherosclerotic lesions, macrophages have been described to present both in a proinflammatory polarization state as well as in an anti-inflammatory state depending on the tissue environmental cues [5]. These conditions can occur within the same atherosclerotic plaque, with a higher tendency of proinflammatory macrophages being located to the vulnerable shoulder region of a plaque [6].

A hallmark of proinflammatory macrophages is their enhanced tissue degradation capacity via membrane bound matrix metalloproteinase (MMP) activators including MMP14 and urokinase plasminogen activator (uPA) and its receptor uPAR [7]. Pro-MMPs are activated at filopodia by either MMP14 or uPA bound to uPAR and enhance tissue degradation capacity of these proinflammatory macrophages [7]. Whereas uPA is secreted in an already active form to process pro-MMPs to their active form, MMP14 is activated intracellularly via furin which leads to the active form of MMP14 capable of activating pro-MMPs [8]. The increased matrix degradation capacity of proinflammatory

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macrophages originates from the ability to activate multiple MMPs via MMP14 and uPA-uPAR as protein levels of pro-MMPs are similar for all polarization conditions [7]. Due to these increased tissue destabilizing properties, proinflammatory macrophages were associated with vulnerable plaques [9].

Standard treatment in patients with atherosclerosis includes lipid lowering using statins [10]. Reduced plaque rupture and reduced matrix degradation in atherosclerotic lesions are among the beneficial effects associated with statin therapy. Besides their lipid lowering capacities, statins have been reported to possess anti-inflammatory properties as well as to influence intermediates of the cholesterol synthesis pathway that serve as important lipid attachment for the posttranslational modification of a variety of proteins [11,12]. However, the direct influence of statins on matrix degradation capacity of proinflammatory polarized macrophages has so far not been determined.

Therefore, the aim of our study was to identify a possible regulation of matrix degradation capacities of in vitro polarized macrophage subsets under a physiologically relevant concentration of statins [13]. We show here a reduction of matrix degradation of proinflammatory macrophages due to a loss of MMP14 activation and uPAR receptor loss at filopodia of polarized macrophages.

2. Methods

2.1. Generation of human macrophages

Human macrophages were generated as published recently [7]. In short, monocytes were isolated by adhesion to plastic and then differentiated to macrophages in RPMI supplemented with 10% fetal calf serum (FCS) and penicillin, streptomycin, glutamine, and fungizone with a supplement of 100 ng/ml macrophage colony stimulating factor (Thermo Scientific, MA, USA). Macrophages were polarized to M (LPS + IFN) using 100 ng/ml LPS (Sigma Aldrich, MO, USA) and 100 ng/ml IFN-γ (Thermo Scientific) and to M(IL4 + IL13) using 20 ng/ml IL-4 (Thermo Scientific) and 20 ng/ml IL-13 (Santa Cruz, CA, USA) for 48 h. Macrophages were treated with 0.5 μM atorvastatin (Pfizer, Sandwich, UK) during polarization. In indicated experiments mevalonate (Sigma Aldrich) was added at a concentration of 100 μM as published previously [14]. Human blood samples were obtained from the Clinic for Blood Group Serology and Transfusion Medicine of the Medical University of Vienna including informed consent. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the study protocol has been approved by the institution's ethics committee.

2.2. Flow cytometry

Cell surface proteins uPAR, CD80 and CD206 were stained with flow cytometry antibodies (all Thermo Scientific) using standard protocols as reported previously [15] on a FACS Canto II (BD, CA, USA) system using FACS Diva software (BD). MMP14 antibody (Santa Cruz) was directly labeled with fluorescein (Abcam, UK) and MMP14 was analyzed as published previously [7].

2.3. Protein determination

To determine protein concentrations we used commercially available ELISAs for IL-6 (R&D, MN, USA), IL-10 (Thermo Scientific), and uPA (R&D) as indicated by the manufacturer. MMP14 in complex with furin was evaluated as published previously [16]. In short, Triton X-100 lysed cellular extracts were incubated in MMP14 antibody (Santa Cruz) pre-coated wells. A secondary antibody versus furin (Santa Cruz) was used to identify MMP14-furin complexes. Detection was enhanced using a biotinylated antibody (Abcam, UK) and biotin (R&D). TMB substrate (Roche, Switzerland) was applied until satisfactory color development and stopped using 1 M H_2SO_4 (Sigma Aldrich). Wells were read on a plate reader (Biotek, VT, USA). To correct for different protein concentrations, total protein was measured using a NanoDrop system (Thermo Scientific) and results were calculated accordingly.

2.4. Matrix degradation assay

Matrix degradation capability of macrophages was evaluated using a commercially available QCM gelatin invadopodia assay (Merck, Germany). CY-3 labeled gelatin coated chamber slides (Thermo Scientific) were seeded with macrophages and macrophages were polarized for 48 h in the absence or presence of 0.5 μM atorvastation.

2.5. Immunofluorescence staining

For immunofluorescence staining macrophages were grown on coverslips and staining was performed as described previously [17]. To evaluate the distribution of MMP14 and uPAR, cells were stained using an MMP14 antibody (Santa Cruz), or a uPAR antibody (Sekisui, Germany) with a secondary CY3 labeled antibody (Abcam). Filopodia were visualized using phalloidin staining (Abcam). Slides were embedded in ProLong Gold antifade (Thermo Scientific) and visualized on a Zeiss Axiovision (Zeiss, Germany) microscope equipped with an AxioCam MRc5 and ZEN blue software.

2.6. Statistics

Sample groups were compared using Student's *t*-Test using SPSS 21 (IBM, CA, USA). p-Values of $p \leq 0.05$ were considered statistically significant.

3. Results

We used LPS and IFN-γ to polarize macrophages into proinflammatory M(LPS + IFN) and IL-4 and IL-13 to polarize macrophages into the alternatively activated M(IL4 + IL13). To determine the effect of a physiological dose of statins on macrophage polarization markers we included 0.5 μM atorvastatin in both polarization states. Statin treatment did not affect surface protein levels of the characteristic M (LPS + IFN) marker CD80 or the M(IL4 + IL13) marker CD206. In addition, protein secretion of the proinflammatory marker IL-6 in M (LPS + IFN) and of the alternatively activated IL-10 in M(IL4 + IL13) was not altered (Fig. 1A and B). This data shows that a concentration of atorvastatin corresponding to physiologic plasma levels does not influence the basic polarization program of macrophages.

In order to determine functional changes after polarization under statin treatment, we analyzed the matrix degradation capacity of polarized macrophage subsets. Confirming previous results, we were able to demonstrate the highest matrix degradation capacity for M (LPS + IFN) polarization [7]. However, under statin treatment M (LPS + IFN) lost their capability to degrade a gelatin matrix with no consequences for the other polarization conditions (Fig. 2A). This loss of function was accompanied by a loss of filopodia in M(LPS + IFN) treated with atorvastatin (Fig. 2B).

To further characterize the loss of matrix degradation activity after statin treatment in M(LPS + IFN) macrophages we analyzed the presence of MMP14 at cell protrusions. As exemplified in Fig. 3A, localization of MMP14 was intact in M(LPS + IFN) cells treated with statins. In addition, cellular MMP14 levels did not change with statin treatment (Fig. 3B). However, intracellular complex formation of MMP14 with furin was reduced after statin treatment (Fig. 3C).

Besides MMP14, M(LPS + IFN) use the uPA-uPAR system for activation of MMPs and subsequent matrix degradation [7]. Treatment with statins had no effect on the protein levels of uPAR (Fig. 4A). Similarly, expression of uPA remained unchanged regardless of statin treatment (Fig. 4B). Of note, also M0 and M(IL4 + IL13) macrophages