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Painting factor H onto mesenchymal stem cells protects the cells from complement- and neutrophil-mediated damage

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ABSTRACT

Mesenchymal stem cells (MSCs) are undergoing intensive testing in clinical trials as a promising new therapy for many inflammatory diseases and for regenerative medicine, but further optimization of current MSC-based therapies is required. In this study, we found that in addition to direct complement −mediated attack through the assembly of membrane attack complexes (MACs) that we and others have recently reported, of the released complement activation products, C5a, but not C3a, activates neutrophils in the blood to further damage MSCs through oxidative burst. In addition, we have developed a simple method for painting factor H, a native complement inhibitor, onto MSCs to locally inhibit complement activation on MSCs. MSCs painted with factor H are protected from both MAC- and neutrophil-mediated attack and are significantly more effective in inhibiting antigen-specific T cell responses than the mock-painted MSCs both in vitro and in vivo.

1. Introduction

Mesenchymal stem cells (MSCs), adult stem/progenitor cells found in many tissues, such as bone marrow, adipose tissue, and umbilical cord, can differentiate into different types of cells, including adipocytes, osteoblasts, and chondrocytes [1]. In addition to this property, which makes them potentially useful in regenerative medicine, MSCs are also strongly immunosuppressive and can inhibit cells both in the adaptive immune system, including T and B cells [2], and in the innate immune system, including NK cells and γδ T cells [3]. Because of their potent immunosuppressive activity, MSCs are being extensively tested in clinical studies as a potential therapy for many inflammatory diseases, such as graft versus host disease (GVHD) [4], multiple sclerosis [5], diabetes [6], colitis [7], and transplant rejection [8]. Despite all the efforts over the years and more than 400 registered MSC-based clinical trials (www.clinicaltrials.gov), there has been no approved MSC product in the US for treating inflammatory diseases. However, even though the results from a recently completed Phase III clinical trial using MSCs to treat GVHD failed to meet the primary endpoints when all patients were considered [9], subsequent analyses demonstrated treatment efficacy in the pediatric subgroup [10]. Although the results of these clinical studies and promising results from previous in vitro studies and in vivo studies in animals strongly argue that MSCs have potential for development as a new therapy for inflammatory diseases, current MSC-based therapies need to be improved in order to be successful.

Complement is an important component of the innate immune system and its primary role is to defend the host from infection [11]. Complement factors are abundant in the blood and, after activation, can assemble membrane attack complexes (MACs) on target cells to lyse or damage them. At the same time, the small molecular weight components, C3a and C5a, known as anaphylatoxins, are generated and released from the site of complement activation and bind to their respective receptors, C3aR and C5aR, both of which are G protein-coupled receptors present on different types of cells, especially myeloid cells. This results in chemoattraction and activation of nearby cells, such as neutrophils, triggering inflammation to damage the invading pathogens (foreign cells) [12].

Previous studies have suggested that MSCs are immunoprivileged and can escape from host immune system surveillance partially due to their potent immunosuppressive activities [13]. Because of this belief and other advantages, such as cost and

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convenience, many MSCs in clinical development are allogeneic MSCs. We and others previously reported that, although MSCs seem to be able to escape from adaptive immune surveillance, infused MSCs can be recognized by complement from the innate immune system [14–16]. Using serum as a source of complement, we further demonstrated that MACs are assembled on MSCs and directly damage the cells, leading to cellular injury and impaired function[14].

In practice, when MSCs are administrated, they encounter not only complement, but also other cells in the blood. In this study, we investigated the impact of neutrophils, the most abundant circulating leukocytes in the blood in mammals, on the fate of MSCs after administration and the underlying mechanisms. In addition, we also studied the effects of locally "painting" MSCs with complement factor H (CFH) [17,18], a native complement inhibitor, on their viability and function after administration. We found that MAC generation and activation of neutrophils by C5a synergistically damage MSCs, leading to reduced MSC viability and impaired function. We also demonstrated that the simple CFH painting approach protects MSCs from both MAC-mediated and neutrophil-mediated attack, leading to significantly improved MSC survival and function both in vitro and in vivo.

2. Reagents and methods

2.1. MSCs, mice and normal human serum

Primary human MSCs were isolated from bone marrow from healthy donors at the MSC Core Facility at Case Western Reserve University as described previously [14] and were used at passage numbers 4–7 were used. MSCs were cryopreserved in a liquid nitrogen and before each experiment, MSCs were thawed and cultured for ~1 week in complete media under standard culturing conditions. Pooled normal human serum (NHS) were ordered from Innovative Research Inc (Novi, MI), and aliquoted NHS were stored in a −80 °C freezer until needed. OT-II mice, C3 knockout mice (both are on C57BL/6 background) and C57BL/6 mice, purchased from the Jackson Laboratory (Bar Harbor, Maine), were maintained in the animal facility at the Cleveland Clinic. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees of the Cleveland Clinic.

2.2. In vivo neutrophil activation assay

1×10^6 MSCs in 300 μl of sterile PBS or PBS alone was injected into WT or C3 KO mice by tail vein injection, then, after 40 min, activation of neutrophils in the blood was assessed by staining peripheral blood cells for reactive oxygen species (oxidative burst) with 10 μM dihydrorhodamine 123 [19] (DHR123; Sigma, St. Louis, MO) for 20 min at room temperature. After washing and lysis of red blood cells, neutrophil activation ($DHR123^+$) was measured using a flow cytometer (FACSCalibur BD Bioscience).

2.3. In vivo neutrophil depletion

Neutrophils were depleted by intraperitoneal injection of mice with an anti-neutrophil monoclonal antibody (NIMP-R14; kindly provided by Dr. Eric Pearlman, Case Western Reserve University) (0.5 mg/mouse) 24 h before the experiment following a previously published protocol [20–22] and neutrophil depletion was confirmed by staining the peripheral white blood cells with phycoerythrin (PE)-labeled anti-mouse Gr-1 monoclonal antibody (mAb) (2 μg/ml Biolegend, San Diego, CA) for 15 min at 4 °C, followed by flow cytometry analysis.

2.4. Complement-mediated MSC damage assay

MSC damage after incubation with serum was assessed using a 2′, 7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) leakage assay [14]. In brief, 2×10^4 MSCs were labeled by incubation for 30 min at 37 °C with 5 μM BCECF-AM (ThermoFisher, Waltham, MA). After washing, the labeled MSCs were incubated 30 min at 37 °C with different concentrations of pooled normal human serum (NHS) in 100 μl of GVB++ buffer (0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.3, Complement technology Inc, Tyler, TX), then released BCECF in the supernatants was measured by a fluorescence GEMINI XS Microplate Reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 485 nm and 538 nm, respectively. To calculate the percentage of BCECF release (complement-mediated injury), the following equation was used as reported previously [23,24]: percentage of BCECF release (cell damage) = [(A−B)/(C−B)] ×100%, where A represents the mean experimental BCECF release, B represents the mean spontaneous BCECF release, and C represents the mean maximum BCECF release induced by incubating cells with 0.1% Triton X-100.

2.5. Neutrophil-mediated MSC damage assay

Human neutrophils, either purified from healthy donor peripheral blood mononuclear cells (PBMCs) by Ficoll centrifugation (provided by the Hematopoietic Stem Cells Core Facility at Case Western Reserve University) or differentiated from the neutrophil progenitor cell line HL-60 (ATCC, Manassas, VA) [25], were used. In some experiments, neutrophil-mediated MSC damage was assessed using a similar BCECF-based assay to that described above [14]. In brief, 2×10^4 MSCs were labeled with BCECF-AM as described above, then, after washing, the labeled MSC were incubated for 30 min at 37 °C with different numbers of neutrophils in the presence or absence of 30% NHS in 100 μl of GVB++ buffer with various concentrations of the neutrophil function inhibitor wortmannin that inhibits oxidative burst [26] (Cayman Chemical, Ann Arbor, MI) and released BCECF in the supernatants was measured as described above. The percentage BCECF release (cell injury) was calculated as above. In some experiments, a C5aR antagonist (JPE1375,Calbiochem, Billerica, MA) [27] or a C3aR antagonist (SB 290157,Calbiochem, Billerica, MA) [28] were added, both at 10 μM, to assess the role of C3aR and C5aR on neutrophils, while, in other experiments, NHS was omitted and 50 ng/ml of purified C5a (Complement technology Inc, Tyler, TX) added instead.

2.6. Painting purified CFH onto MSCs

Primary MSCs were painted with purified human CFH (Complement technology Inc, Tyler, TX) after 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-mediated activation of carboxyl groups. In brief, 2 mM EDC and 5 mM N-hydroxysulfosuccinimide (ThermoFisher, Waltham, MA) were added to CFH solution at the indicated concentration and allowed to react for 15 min at room temperature to create activated carboxyl groups, then 20 mM β-mercaptoethanol was added to quench the excess EDC and the activated CFH was separated using a desalting column (ThermoFisher, Waltham, MA) equilibrated with PBS. MSCs (2×10^4) were then incubated with different concentrations of activated CFH for 30 min at 37 °C in 100 μl PBS, then the cells were washed with PBS and used for experiments.

2.7. Detection of painted CFH on MSCs

Painted CFH on MSCs were detected by flow cytometric analysis.