Mesenchymal Stem Cells Inhibit Complement Activation by Secreting Factor H

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Mesenchymal stem cells (MSCs) possess potent and broad immunosuppressive capabilities, and have shown promise in clinical trials treating many inflammatory diseases. Previous studies have found that MSCs inhibit dendritic cell, T-cell, and B-cell activities in the adaptive immunity; however, whether MSCs inhibit complement in the innate immunity, and if so, by which mechanism, have not been established. In this report, we found that MSCs constitutively secrete factor H, which potently inhibits complement activation. Depletion of factor H in the MSC-conditioned serum-free media abolishes their complement inhibitory activities. In addition, production of factor H by MSCs is augmented by inflammatory cytokines TNF- α and interferon- γ (IFN- γ) in dose- and time-dependent manners, while IL-6 does not have a significant effect. Furthermore, the factor H production from MSCs is significantly suppressed by the prostaglandin E2 (PGE2) synthesis inhibitor indomethacin and the indoleamine 2,3-dioxygenase (IDO) inhibitor 1-methyl-D-tryptophan (1-MT), both of which inhibitors are known to efficiently dampen MSCs immunosuppressive activity. These results indicate that MSCs inhibit complement activation by producing factor H, which could be another mechanism underlying MSCs broad immunosuppressive capabilities.

Introduction

COMPLEMENT IS A PIVOTAL part of the innate immunity whose primary roles are fighting infection and clearing out immune complexes. To maintain immune homeostasis, complement activation is tightly controlled by its regulators to prevent complement-mediated self-tissue injury [1]. It has been well established that excessive complement activation contributes to the pathogenesis of many autoimmune diseases including rheumatoid arthritis [2,3], type I diabetes [4,5], myasthenia gravis [6,7], glomerulonephritis [8,9], as well as transplanted grafts rejection [10,11].

Factor H is the primary fluid phase complement regulator and it is mainly produced by hepatocytes in the liver [12]. Factor H inhibits complement activation by prohibiting the assembly of and accelerating the decay of C3/C5 convertases, and by serving as a co-factor for factor I to inactivate C3b and C4b [13–15]. Patients with factor H deficiency exhibit excessive complement activation, which leads to many disorders including glomerulonephritis, systemic lupus erythematosus, and bacterial infections [16–18].

Mesenchymal stem cells (MSCs) are adult stem cells that possess potent and broad immunosuppressive activity [19].

Previous studies have shown that MSCs inhibit the differentiation/maturation of dendritic cells [20,21] and suppress the proliferation/function of T cells [22] and B cells [23] in adaptive immunity through different mechanisms. However, whether MSCs inhibit complement in the innate immunity, and if so, by which mechanism, have not been established. In this report, we show for the first time that MSCs constitutively produce factor H, thereby inhibiting complement activation, which could contribute to MSCs broad immunosuppressive activities. The production of factor H by MSCs is significantly augmented by TNF- α and interferon- γ (IFN- γ), inflammatory cytokines that are up-regulated in many disorders that MSC-based therapies are being tested in, and suppressed by indomethacin and 1-MT, the 2 inhibitors that dampen MSCs immunosuppressive activity.

Reagents and Methods

MSCs and reagents

Human MSCs (passage 3) were kindly provided by Tulane University Center of Gene Therapy and maintained in a 6-well plate in MEM with 10% fetal bovine serum (FBS;

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Invitrogen, Carlsbad, CA) following the provided protocols. 24 h before each experiment, complete MEM was aspirated; MSCs were washed; and MesenCult-ACS (Stemcells, Vancouver, Canada) serum-free media (SFM) was added for cell culture. MSCs with passage numbers 4–10 were used in all experiments. Purified human properdin, C3b, factor B, factor D, factor H, factor H-depleted serum, and goat anti-human factor H serum were purchased from Complement Tech (Tyler, TX). Biotinylated mouse anti-human factor H IgG (clone OX-24) was ordered from Cedarlane Biolabs (Burlington, NC) and streptavidin–agarose was purchased from Biomeda (Foster City, CA). Recombinant human TNF- α , IFN- γ , and IL-6 were ordered from Biolegend (San Jose, CA). The prostaglandin E2 (PGE2) synthesis inhibitor indomethacin was purchased from Cayman Chemical (Ann Arbor, MI), and the indoleamine 2,3-dioxygenase (IDO) inhibitor 1-methyl-D-tryptophan (1-MT) was from Sigma (St. Louis, MO).

Sheep erythrocytes hemolytic assay

Complement inhibitory activity in MSC-conditioned SFM was assessed by a modified sheep erythrocyte-based hemolytic assay following the protocol described before [24]. In brief, 4×10^6 sheep erythrocytes were mixed with 30% factor H-depleted serum in GVB EGTA/Mg²⁺ buffer in a 100 μ L volume with 20% MSC-conditioned SFM or 20% control SFM followed by 30 min of incubation at 37°C. Complement-mediated hemolysis was quantitated by measuring OD₅₄₁ and the percentage of hemolysis was calculated using the formula: % hemolysis = (A–C)/(B–C) $\times$ 100%, where A is the experimental sample, B is the complete lysis (erythrocytes incubated with water), and C is the spontaneous hemolysis (EDTA).

Alternative pathway C3 convertase decay assay

The decay of the preassembled alternative pathway C3 convertase (C3bBb) was assessed using an ELISA-based assay as described before [25]. In brief, C3bBb convertases were pre-assembled on a C3b-coated microtiter plate by adding 800 ng/mL of factor B, 300 ng/mL factor D, and 1,000 ng/mL properdin in phosphate buffer supplemented with 75 mM NaCl, 10 mM MgCl₂, 4% BSA, and 0.05% Tween-20. After this, MSC-conditioned SFM or control SFM was added (1:5 dilution) and incubated for another 1 h at 37°C. The decay of the preassembled C3 convertases was assessed by measuring the remaining plate-bound C3 convertases using ELISA with an anti-factor B antibody [25].

Western blot of factor H

Proteins in MSC-conditioned or control SFM were separated by a 7.5% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, IN). The presence of factor H was detected with goat anti-factor H serum (1:5000) following standard western blot protocols [26].

Factor H concentration measurement

Concentrations of factor H in MSC-conditioned or control SFM were measured by standard enzyme-linked immunosorbent assay (ELISA) as described before [27]. In brief, 96-well ELISA plates were coated with 10 μ g/mL goat anti-

factor H serum overnight at 4°C. After blocking, factor H standards or MSC-conditioned SFM were added and incubated for another 2 h at room temperature. Following washing, 100 ng/mL biotinylated anti-factor H IgG was added and after which, the plates were developed and measured at OD₄₀₅ using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Factor H depletion in MSC-conditioned SFM

To deplete factor H, 2 μ g/mL biotinylated mouse anti-factor H IgG (Clone OX-24) was mixed with MSC-conditioned SFM and incubated for 1 h with rotation. After this, 10 μ g/mL streptavidin–agarose was added and incubated for another 1 h. The biotinylated, anti-factor H/streptavidin–agarose complexes were then removed by centrifuging at 10,000 rpm at 4°C for 5 min. Nonspecific mouse IgGs were used as controls.

Cytokine treatments

The 1.5×10^5 MSCs were cultured in SFM in 12-well plates and different concentrations (0, 10, and 100 ng/mL) of recombinant human TNF- α , IFN- γ , or IL-6 were added into each well. MSC-conditioned SFM were collected at 0, 12, 24, and 48 h, and concentrations of factor H in the supernatants were measured by ELISA.

Statistics

All experiments were repeated at least twice. Results were compared using the Student's *t*-test. A *P* value <0.05 was considered significant.

FIG. 1. Mesenchymal stem cell (MSC)-conditioned serum-free media (SFM) possess complement inhibitory activity. **(A)** MSC-conditioned SFM (20%) collected at 12, 24, and 48 h of culture inhibit complement-mediated sheep erythrocytes hemolysis in a time-dependent manner. **(B)** MSC-conditioned SFM (20%) collected at 12, 24, and 48 h accelerate the decay of the alternative pathway C3 convertase (C3bBb) in a time-dependent manner (representative results of 3 experiments, values shown are mean $\pm$ SD of triplicate samples, **P* < 0.05).

Results

MSC-conditioned SFM has complement activation inhibitory activity

To examine whether MSCs could inhibit complement activation, we assayed MSC-conditioned SFM collected at 0, 12, 24, and 48 h using a modified sheep erythrocytes hemolytic assay [24]. These assays (Fig. 1A) showed that MSC-conditioned SFM efficiently inhibited complement-mediated hemolysis in a time-dependent manner. Even with a 5-fold dilution, MSC-conditioned SFM collected at the 24-h time point inhibited complement-mediated hemolysis by 20% and the SFM collected at the 48-h time point inhibited the hemolysis by >40%. Additional alternative pathway C3 convertase decay assays (Fig. 1B) showed that the MSC-conditioned SFM accelerated the decay of the alternative pathway C3 convertases C3bBb. These data indicate that MSCs constitutively produce complement inhibitor(s), which protects sheep erythrocytes from complement-mediated lysis at least in part by accelerating the decay of the alternative pathway C3 convertases.

Factor H is present in the MSC-conditioned SFM

To explore the underlying mechanism of the complement inhibitory activity detected in the above assays, we examined the existence of factor H in the MSC-conditioned SFM by Western blot. These experiments (Fig. 2A) showed that factor H was present in the MSC-conditioned SFM, indicating that MSCs constitutively express and secrete factor H. We next quantitated the factor H concentrations by ELISA. These assays (Fig. 2B) showed that 1.5×10^5 MSCs constitutively produced 33 ± 2 ng/mL after 12 h, 130 ± 28 ng/mL after 24 h, and 283 ± 4 ng/mL of factor H after 48 h of culturing in the SFM.

FIG. 2. Factor H is present in the mesenchymal stem cell (MSC)-conditioned serum-free media (SFM). (A) Detection of factor H in MSC-conditioned SFM collected at 0, 12, 24, and 48 h, showing that MSCs constitutively produce factor H in a time-dependent manner. (B) Measurements of factor H concentrations in MSC-conditioned SFM collected at different time points. Values shown are mean $\pm$ SD of triplicate samples.

Depletion of factor H in MSC-conditioned SFM abolishes its complement inhibitory activity

To determine that factor H is the primary complement regulator in the MSC-conditioned SFM, we depleted factor H in the conditioned SFM collected at the 48-h time point by adding biotinylated anti-factor H IgG, then streptavidin-agarose, followed by high-speed centrifugation to remove the biotinylated anti-factor H IgG-factor H/streptavidin-agarose complexes. Western blot showed that no factor H protein could be detected in the MSC-conditioned SFM media after these procedures (Fig. 3A). In similar complement-mediated sheep erythrocytes hemolytic assays, complement inhibitory activity of the MSC-conditioned SFM was abolished after factor H depletion (Fig. 3B). Consistent with these results, in alternative pathway C3 convertase decay assays, MSC-conditioned SFM after factor H depletion failed to accelerate the decay of the pre-assembled C3bBb (Fig. 3C).

FIG. 3. Factor H is the primary complement inhibitor in mesenchymal stem cell (MSC)-conditioned serum-free media (SFM). (A) Detection of factor H by Western blot in MSC-conditioned SFM with and without factor H depletion, showing that factor H is not detectable after biotinylated anti-factor H IgG and streptavidin-agarose treatment. (B) Complement-mediated hemolytic assays with 20% MSC-conditioned SFM and 20% factor H-depleted, MSC-conditioned SFM, showing that after factor H depletion, MSC-conditioned SFM failed to inhibit complement-mediated hemolysis. The 300 ng of purified human factor H was included as positive control. (C) C3 convertase decay assays with 20% MSC-conditioned SFM and 20% factor H-depleted, MSC-conditioned SFM, showing that after factor H depletion, MSC-conditioned SFM failed to accelerate the decay of the alternative pathway C3 convertase C3bBb. The 300 ng of purified human factor H was included as positive control (representative results of 3 experiments, values shown are mean $\pm$ SD of triplicate samples, * $P < 0.05$).

FIG. 4. Inflammatory cytokines TNF- α and interferon- γ (IFN- γ) but not IL-6 up-regulate production of factor H by mesenchymal stem cells (MSCs). The 1.5×10^5 MSCs were cultured in serum-free media (SFM) and different concentrations of TNF- α , IFN- γ , or IL-6 were added. After 12, 24, and 48 h of culture, MSC-conditioned media were collected and concentrations of factor H in them were measured by ELISA. These assays showed that TNF- α (**A**) and IFN- γ (**B**) significantly up-regulated factor H production by MSCs while IL-6 does not have a significant impact under the culture conditions (**C**) (representative results of 3 experiments, values shown are mean $\pm$ SD of triplicate samples, * $P < 0.05$).

These results indicate that factor H is the primary complement inhibitor secreted by MSCs.

Inflammatory cytokines TNF- α and IFN- γ but not IL-6 up-regulate the production of factor H by MSCs

Because MSCs are being tested in clinical trials for treating inflammatory diseases, we next studied how the production of factor H by MSCs could be regulated by inflammatory cytokines. We added different amounts of TNF- α , IFN- γ , or IL-6 into MSCs cultured in SFM, and collected supernatants at 12, 24, and 48 h for factor H concentration measurements by ELISA. These assays (Fig. 4) showed that at a concentration of 10 ng/mL, only IFN- γ significantly up-regulated MSC factor H production after 48 h of incubation; at a concentration of 100 ng/mL, both TNF- α and IFN- γ significantly up-regulated factor H production from MSCs after 12, 24, or 48 h of incubation. However, at all of the concentrations tested, IL-6 did not appear to have a significant impact on factor H production by MSCs.

Both the PGE2 and IDO inhibitors suppress the production of factor H by MSCs

Previous studies done by others have demonstrated that blocking PGE2 or IDO activities by their inhibitors

indomethacin or 1-MT suppresses the immunosuppressive activity of MSCs [20,28]. To assess whether these inhibitors also suppress MSC factor H production, we incubated MSCs in the presence of 10 μ M of indomethacin or 1 mM of 1-MT with and without 100 ng/mL of TNF- α or IFN- γ . We collected the supernatants at 12, 24, and 48 h, and measured their levels of factor H by ELISA. These assays (Fig. 5) showed that at all the time points tested, both indomethacin and 1-MT significantly inhibited the constitutively expressed factor H production, and the TNF- α - and IFN- γ -induced factor H production.

Discussion

In this report, we showed for the first time that MSC-conditioned media accelerate the decay of the alternative pathway C3 convertase C3bBb and protect sheep erythrocytes from complement-mediated lysis. MSCs constitutively produce factor H, which is the primary complement inhibitor produced by MSCs. Production of factor H by MSCs is up-regulated by inflammatory cytokines TNF- α and IFN- γ in both dose- and time-dependent manners, while IL-6 does not have a significant impact on MSC factor H production. Furthermore, PGE2 and IDO inhibitors that dampen MSC immunosuppressive activities also inhibit factor H production from MSCs with or without cytokine treatments.

FIG. 5. Prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) inhibitors suppress the production of factor H by mesenchymal stem cells (MSCs). The 1.5×10^5 MSCs were cultured in serum-free media (SFM) and ng/mL of TNF- α or interferon- γ (IFN- γ) was added. After 12, 24, and 48 h of culture, MSC-conditioned media were collected and concentrations of factor H in them were measured by ELISA. These assays showed that the PGE2 inhibitor indomethacin and the IDO inhibitor 1-MT significantly inhibited constitutive factor H production by MSCs as well as TNF- α - and IFN- γ -stimulated factor H production (representative results of 2 experiments, values shown are mean $\pm$ SD of triplicate samples, * $P < 0.05$).

Excessive complement activation contributes to the pathogenesis of many inflammatory diseases. After activation, complement can not only cause direct cell injury by forming membrane attack complexes (MACs) on cell surfaces, but also recruit inflammatory cells and stimulate the production of chemokines/cytokines [29]. Previous studies have shown that MSCs possess potent and broad immunosuppressive capabilities including inhibiting dendritic cells development/maturation, suppressing T-cell and B-cell activation/proliferation in the adaptive immunity through cell-cell contact, and/or secreting soluble factors. However, it was unclear whether MSCs could also inhibit complement activation in the innate immunity, and if so, by which mechanism. The results that MSC-conditioned SFM inhibits complement-mediated hemolysis and that depleting factor H in the conditioned SFM abolishes its complement inhibitory activity indicate that MSCs inhibit complement activation in the innate immunity, and it do so primarily by producing factor H. Because excessive complement activation participates in the pathogenesis of many immune-mediated diseases, inhibition of complement by MSCs could contribute to their broad immunosuppressive activity.

We chose to use SFM instead of complete medium with FBS because even though the FBS in cell cultures is heat-inactivated to remove complement activities, bovine factor H in the FBS could still be functional after heat inactivation [24], which therefore could comprise our assays. Using component-defined SFM eliminates the complexity of possible contamination by bovine factor H.

Factor H is the primary complement inhibitor in the serum. Mutations or deletions in factor H gene, which result in decreased or abolished factor H activity, have been causally linked to many autoimmune diseases including atypical hemolytic uremic syndrome [30,31] and membranoproliferative glomerulonephritis [32,33]. In these patients, lack of factor H complement inhibitory activity leads to augmented complement activation, which results in elevated inflammation and direct MACs-mediated cell injury. Recent

studies also found that certain polymorphisms in factor H genes significantly increase risks for age-related macular degeneration (AMD) [34,35]. However, the precise underlying mechanism remains elusive.

Although factor H is primarily produced in the liver, previous work has shown that it is also constitutively expressed by other cells in addition to hepatocytes including vascular endothelial [36] and proximal tubular epithelial cells [37]. Our results indicate that MSCs are another type of cells that constitutively produce factor H, and that their complement inhibitory activity is primarily dependent on the factor H that they produce. Compared with the rate of ~ 40 ng/ 10^6 cell/day of factor H produced by human umbilical vein endothelial cells (HUVAC) [27], the MSCs that we tested in this study express factor H at a rate of ~ 300 ng/ 10^6 cells/day. In addition, previous studies have shown that factor H production from endothelial or epithelial cells are markedly up-regulated by inflammatory cytokines like IFN- γ [36,37]. Our results indicate that, similar to these previous findings, factor H expression by MSCs can be up-regulated by IFN- γ and TNF- α but not IL-6.

In clinical trials, infusion of MSCs effectively dampens inflammation and ameliorates clinical symptoms in various immune-mediated inflammatory diseases [reviewed in ref. [38]] including graft versus host diseases (GVHD), type I diabetes and rheumatic arthritis, in which levels of TNF- α or IFN- γ are markedly up-regulated. Although the precise mechanism underlying MSCs beneficial effects in these diseases is still under investigation, it has been established that soluble factors produced by MSCs such as IDO [39,40] and PGE2 [20,41] inhibit activities of dendritic cells, T cells, and/or B cells in the adaptive immunity because IDO or PGE2 inhibitors greatly dampen the immunosuppressive activity of MSCs [20,28]. Interestingly, excessive complement activation from the innate immunity has also been found to be integrally involved in the pathogenesis of all these diseases [42–45]. Our results that both the IDO and PGE2 inhibitors also significantly inhibited MSC factor H production suggest that factor H produced locally by the MSCs could contribute

to their broad immunosuppressive activities. Although the amount of factor H produced by MSCs is insignificant when compared with systemic plasma factor H produced by hepatocytes, these locally produced factor H in the MSC niche environment outside the vasculature could reach high local concentrations and be of physiological significance. In a similar situation, we [46] and others [47] have recently shown that local complement produced by interacting antigen-presenting cells and T cells but not systemic complement produced by hepatocytes is important in modulating T-cell responses and autoimmunity. In addition, previous studies done by others have shown that synovial cells that contain MSCs from rheumatic arthritis patients produce factor H, which helps to control local inflammation [48,49].

In summary, we have found that MSCs inhibit complement activation by producing factor H, and that the production of factor H by MSCs is augmented by inflammatory cytokines IFN- γ and TNF- α , and suppressed by IDO and PGE2 inhibitors. Our results, together with the previous reports that complement is integrally involved in the pathogenesis of many inflammatory diseases [42–45], suggest that complement inhibition by MSCs produced factor H, especially under inflammatory conditions, could be another mechanism underlying MSCs broad and potent immunosuppressive effects. The complement inhibitory activity of MSCs could contribute to their efficacies in ameliorating inflammatory disorders in which complement contributes to the pathogenesis.

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Author Disclosure Statement

The authors indicate no potential conflicts of interest.

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