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Research paper

In vitro allogeneic immune cell response to mesenchymal stromal cells derived from human adipose in patients with rheumatoid arthritis

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ABSTRACT

We investigated the regulatory activity of human adipose-derived mesenchymal stromal cells (MSCs) (n = 10) towards immune cells in a cohort of 84 rheumatoid arthritis (RA) patients, 36 apparently healthy controls. We co-cultured MSCs with lymphocyte subsets of T, B, and T regulatory cells (Tregs). Levels of the pro- and anti-inflammatory markers (tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin-10 (IL-10)) were estimated in serum and co-culture supernatants. The study revealed a two-fold increase in the proportion of Tregs and an increased level of $CD4^+CD25^+$FoxP3. MSCs altered T cell, B cell, and Treg cytokine production during an anti-inflammatory immune response. The MSCs inhibited CD3+T cell-mediated TNF-α secretion, upregulated IL-10, and suppressed the production of autoantibodies against citrullinated protein antigens produced by B cells. These data offer insight into the interactions between allogeneic MSCs and immune cells, and elucidate the dose-dependent modulation of MSCs.

1. Introduction

Rheumatoid arthritis (RA) involves systemic complications, aberrant joint fibroblast activation, progressive tissue destruction with a loss of function, and, potentially, death if not adequately treated [1–3]. Disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and various biological agents have transformed the treatment of RA [4]. However, several unmet needs remain because current therapies have not yielded a complete cure. Prolonged and continuous usage of conventional therapies to alleviate the disease symptoms has met with only a partial response. This underlines the need to develop new therapeutic approaches that are safe and reliable, and elicit a sustained response.

Several studies have shown that mesenchymal stromal cells (MSCs) have anti-inflammatory properties and release paracrine factors. Recent trials have produced encouraging results wherein MSCs inhibit human T cell proliferation and decrease the severity of collagen-induced arthritis (CIA) in mouse models [5–6]. Human MSCs obtained from abdominal adipose tissue have emerged as an important source for cell therapy [7–10]. MSCs can easily be obtained in large numbers from lipo-aspirates, and can be expanded rapidly *in vitro* to generate a clinically effective dose. The authors of recent studies have reported that human adipose-derived MSCs (ADSCs) have immunomodulatory properties similar to those of bone marrow [11–13]. The pathophysiology of RA includes details of our current understanding of the intricacy of cytokines and immune mediators involved in disease pathogenesis [14]. MSCs also alter the cytokine secretion profiles of naive and effector T and B cells to induce a more profound anti-inflammatory or tolerant phenotype [15]. T regulatory cells (Tregs) maintain self-tolerance and act as immunosuppressive cells in which TGF-β plays a complex and intertwined role in inflammation, T cell lineage commitment, antibody generation, and immune suppression [16]. Anti-citrullinated protein antibody (ACPA) is a specific marker for RA [17–20]. Both ACPAs and rheumatoid

Abbreviations: DMARDs, disease-modifying anti-rheumatic drugs; NSAIDs, nonsteroidal anti-inflammatory drugs; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; MSCs, human adipose-derived stem cells; ACPA, anti-citrullinated protein antibody.

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arthritis factors (RFs; types IgG and IgM) have been detected in RA patients, and are likely to play a key role in the pathogenesis and associated vasculitis of RA [21–23]. Not much is known about the immunomodulatory effect of MSCs on B cells with respect to the production of autoantibodies in RA. Therefore, in the present experiment, B cells from patients with RA and from normal volunteers were cultured to test for the production of antibodies against cyclic citrullinated peptide (CCP) and RFs IgG and IgM in serum and co-culture experiment supernatants. Having shown that *in vitro* autoantibody production can be detected under culture conditions [24], we used this method to evaluate the ability of MSCs to suppress autoantibodies and B cell responses in patients with RA.

In this study, we describe the dose-dependent effects of MSCs on the responses of B cells, T cells, and Tregs in co-culture experiments. Furthermore, the study explores the inflammatory cytokine levels in serum and culture supernatants arising from MSC dose modulation *in vitro*. This should help in formulating an effective therapeutic approach for the treatment of RA using MSCs.

2. Methods

2.1. Patient classification criteria and clinical manifestations

Prior written informed consent was obtained from all participants in the study cohort. The experimental protocol was carried out in accordance with the guidelines of the Declaration of Helsinki, and was approved by the Institutional Ethics Committee of Global Hospitals, Hyderabad (Ref. # GMERF/BS/SAC/IEC/IC_SCR 2014/02R3).

The study cohort comprised 120 subjects: 84 RA patients that had been clinically diagnosed as per the American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) classification; and 36 gender-, age-, and ethnicity-matched healthy controls. We isolated MSCs from the adipose tissue provided by 10 apparently healthy donors not included in the *in vitro* study. The classification parameters for RA were joint involvement; serology (RFs IgG and IgM, and anti-cyclic citrullinated peptide (anti-CCP)); levels of acute-phase reactants; and the duration of symptoms. Individuals with a history of another autoimmune antibody were excluded from the study. Patients included in the study were taking pain-relieving drugs. Peripheral blood (PB) samples were collected at Aware Global Hospitals, Hyderabad. Serum was separated for the estimate of the cytokine levels at the baseline to be compared with the culture supernatants. All reagents and consumables were of high grade and were purchased from Sigma (USA), GIBCO (USA), R&D Systems (USA), and Corning (USA).

Abdominal adipose tissue was collected from morbidly obese donors ranging in age from 18 to 62 years during routine bariatric surgery. Tissue samples were collected in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with antibiotics, and were processed within 3–4 h.

2.2. In vitro culture of MSCs

To isolate the MSCs, we washed omentum tissue fragments intensively in phosphate-buffered saline and digested them with collagenase type I (1 mg/mL) for 30–60 min in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C with gentle agitation. The digested tissue was filtered through a 40-μm cell strainer and the cells were centrifuged at 600*g* for 5 min. The cell pellet was re-suspended in L-DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO®), penicillin (100 IU/mL), streptomycin (100 IU/mL), gentamycin (50 IU/mL), amphotericin B (2.5 μg/mL), and bFGF (10 ng/mL). The cells were placed in polystyrene T25 culture flasks at a concentration of $1 \times 10^5/cm^2$, and incubated in a 5% CO_2 atmosphere at 37 °C for 48 h. Nonadherent cells were discarded and adherent cells were cultured in complete medium for 10 days, with the medium replaced every 3 days. The MSCs were harvested at 80% confluence based on plastic adherence. We carried out cell surface antigen phenotyping by flow cytometry at the third passage to characterize the MSCs. We used Cell Quest Software (Becton Dickinson) to establish the positive and negative expression of certain epitopes: CD90 (fluorescein isothiocyanate (FITC)), CD34 (phycoerythrin (PE)), CD73 (allophycocyanin (APC)), CD45 (FITC), and CD105 (peridinin chlorophyll (PerCP)).

2.3. Isolation of lymphocyte subsets by magnetic-activated cell sorting (MACS)

Peripheral blood mononuclear cells (PBMCs) were separated from the samples provided by the 120 subjects by density gradient centrifugation over Ficoll-Hypaque according to the manufacturer's instructions (Sigma). The PBMCs were further segregated into $CD3^+$ T cells, $CD19^+$ B cells, and a Treg cell subset ($CD4^+CD25^+$) using a MACS column according to the proprietary method [25]. The purity of the segregated cells was checked by flow cytometry.

2.4. MSC-immune cell co-culture and the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay

MSCs were plated in triplicate onto 96-well plates at 2×10^4 cells/mL in 100 μL L-DMEM media and allowed to adhere to the plates for 2–4 h. B cells T cells or Tregs were added to wells containing the MSCs of patients and to wells without MSCs (which served as controls). The MSCs were allowed to proliferate. The MSC to immune cell ratios were 100:1 10:1 1:1 1:10 and 1:100. After incubation for 7 days a BrdU label was added to each well 2 h prior to culture termination. The optical density (OD) values captured at 450/550 nm indicate the amount of BrdU incorporated in the proliferating cells. The co-culture experiments were conducted in two formats i.e. Transwell co-culture and direct co-culture to evaluate the effect of MSCs on B cells T cells and T cell subsets and are further described below

2.4.1. Transwell co-culture

The MSCs were placed in the lower chambers of 24-well Transwell plates with 0.3-μm pore size membranes (Corning) in a dose-dependent manner, i.e., 1×10^5, 1×10^4, and 1×10^3 cells/mL in complete DMEM supplemented with 10% FBS. The volume of the supplemented cell culture media was 3.0 mL. The following day, purified $CD3^+$ T cells were added to the cultures in the upper chambers, at different concentrations, i.e., 1×10^3, 1×10^4, and 1×10^5 cells/mL, and were cultured under the same conditions for 7 days. The MSC to T cell titration ratios were 100:1, 10:1, 1:1, 1:10, and 1:100. Cultures with phytohemagglutinin (PHA) (2.5 μg/mL) and lipopolysaccharide (LPS) served as controls. The cultures were terminated on Day 7 for further evaluation. Parallel experiments to that performed with T cells were carried out using $CD19^+$ B and Treg cells.

2.4.2. Direct co-culture

(a) Co-culture of MSCs and CD4+CD25+ Tregs: The MSCs were co-cultured with CD4+CD25+ Tregs in triplicate in a dose-dependent manner, i.e., 1×10^5, 1×10^4, and 1×10^3 cells/well using DMEM medium on 96-well culture plates. A PHA-stimulated culture (2 μg/mL PHA) served as a positive control, and a Treg culture without MSCs served as a negative control. The cultures were maintained in a humid 5% CO_2 incubator at 37 °C and were terminated on Day 7. The supernatant was then collected to estimate the cytokine levels for inflammatory