Published in final edited form as:

Cytotherapy. 2018 December ; 20(12): 1437–1444. doi:10.1016/j.jcyt.2018.10.002.

Immunogenic potential of human bone marrow mesenchymal stromal cells is enhanced by hyperthermia

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Abstract

Background aims: Bone marrow—derived mesenchymal stromal cells (MSCs) have been reported to suppress T-cell proliferation and used to alleviate the symptoms of graft-versus-host disease (GVHD). MSCs are a mixed cell population and at this time there are no tools to isolate the cells responsible for the T-cell suppression. We wanted to find a way to enhance the immunomodulatory actions of MSCs and tried varying the temperature at which they were cultured.

Methods: We cultured human MSCs derived from healthy volunteers at different temperatures and tested their ability to switch macrophage character from pro-inflammatory to anti-inflammatory (M1 type to M2 type). Using an enzyme-linked immunosorbent assay (ELISA), we showed that when MSCs are cultured at higher temperatures their ability to induce co-cultured macrophages to produce more interleukin-10, (IL-10) (an anti-inflammatory cytokine) and less tumor necrosis factor alpha, (TNF α) (a pro-inflammatory cytokine) is increased. We performed Western blots and immunocytochemistry to screen for changes that might underlie this effect.

Results: We found that in hyperthermia the heat shock protein, HSF1, translocated into the nucleus of MSCs. It appears to induce the COX₂/PGE₂ (Cyclooxygenase2/Prostaglandin E₂) pathway described earlier as a major mechanism of MSC-directed immune-suppression.

Conclusion: Hyperthermia increases the efficacy of MSC-driven immune-suppression. We propose that changing the time of MSC administration to patients to mid-to-late afternoon when the body temperature is naturally highest might be beneficial. Warming the patient could also be considered.

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Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcyt.2018.10.002>.

Keywords

high temperature; human bone marrow stromal cells; mesenchymal stromal cells; priming mesenchymal stromal cells; pro- and anti-inflammatory macrophages (M1, M2)

Introduction

Since their first success as cellular therapy in 2004 [1], bone marrow mesenchymal stromal cells (BMSCs or MSCs) have become the focus of possible cellular therapy in a variety of disease states. Researchers and clinicians have explored the use of MSCs as a cellular treatment for autoimmune diseases, such as graft-versus-host disease (GVHD), sepsis and Crohn's disease [2-4]. Given the heterogeneity of MSC populations [5] and donor variability, researchers focused on trying to isolate the specific population that is responsible for the immune effects. Another option was to try to work with the mixed population of cultured MSCs but improve their immune-suppressive potential using priming and try to match donors with patients to achieve optimal results [6-13].

As one of the four classical signs of inflammation, fever plays an important role in innate immunity. The human body must regulate temperature changes tightly to effectively fight pathogens. Here, we explore the use of heat as a conditioning agent to enhance the immune-modulatory ability of MSCs to make them more efficient in switching pro-inflammatory macrophages (M1) to anti-inflammatory (M2) phenotype. M1 cells primarily make pro-inflammatory cytokines (such as tumor necrosis factor [TNF α]), whereas M2 makes more anti-inflammatory cytokines (such as interleukin [IL]-10). We observed that in lipopolysaccharides (LPS)-stimulated co-cultures of MSCs and macrophages, the macrophages reduce pro-inflammatory TNF- α production and increase anti-inflammatory IL-10 production. Our laboratory published this result in a mouse model of sepsis and uncovered the underlying mechanism involving the prostaglandin pathway [14]. We wanted to know whether heat could increase the efficiency of MSCs as immunotherapeutic agents.

Methods

Cell culture

Cryopreserved, clinical-grade adult human MSCs aspirated from the iliac crest of healthy donors were obtained from the Bone Marrow Stromal Cell Transplantation Center of the National Institutes of Health (NIH) and cultured as described earlier [15] (institutional review board [IRB] — approved protocol [NCT01071577](#)).

The cells, derived from healthy volunteers, were expanded and cryopreserved in freezing medium in aliquots of one to four million cells at passage three in liquid nitrogen. Aliquots were thawed as needed and cultured in Minimal Essential Medium (MEM- α), supplemented with 10% fetal bovine serum (FBS), 1% GlutaMax, and 1% PenStrep ("MSC medium"). THP1 monocytes were obtained from the American Type Culture Collection (ATCC) and cultured in Roswell Park Memorial Institute medium (RPMI-1640), supplemented with 10% FBS, 1% PenStrep and 1.75 μ L of 2-mercaptoethanol per 500 mL ("macrophage medium"). Freezing medium consisted of 50% MSC or macrophage medium depending on the cell

type, 40% FBS and 10% percent dimethyl sulfoxide (DMSO). Cells were expanded in culture in 5% CO₂ and 20% O₂ at 37°C. All cell types were cultured independently on tissue-treated culture plates until confluent.

Pilot experiments were carried out to decide the optimal range of temperature used in the experiments. Although 37°C seemed to be a logical starting point, when we heated the cells to 39–40°C, the morphology of the cells started to deteriorate and significant cell death occurred. Thus, to try to avoid this problem, we ran an experiment to test if we could use a similar range starting at a lower temperature (Supplementary Figure 1) and decided to use a range between 36°C and 38.5°C in all experiments. We also tested if just preheating the MSCs before they were placed in co-culture with THP1 cells would increase their immunomodulatory ability. We found that only the temperature of the co-culture has the effect; preheating did not seem to be satisfactory (Supplementary Figure 1C and 1D).

Enzyme-linked immunosorbent assay

For enzyme-linked immunosorbent assay (ELISA) experiments, cells were transferred to 96-well tissue culture plates at a concentration of 10 000 MSCs and 100,000 macrophages (using the THP1 human monocytic cell line, ATCC TIB-202) per well in 200 μ L of macrophage medium. Each sample covered one column of a plate, or eight wells. Cells were allowed to adhere overnight, and half of the samples were stimulated with 1 μ g/mL of LPS the next morning. Plates for TNF- α were incubated for 6 h, and plates for IL-10 were incubated for 24 h. These were found in pilot experiments to be the optimal time for detecting changes in these cytokines. To harvest the samples, the plates were centrifuged, and the supernatants were transferred to low-absorbance plates either for temporary storage at –20°C or immediate use in an ELISA.

ELISAs for human IL-10 and TNF- α were performed using DuoSet ELISA kits (R&D Systems; DY217B, DY210) according to the manufacturer's instructions. The plates were analyzed using a Turner BioSystems Modulus Microplate Reader at 450 nm using 3,3',5,5'-Tetramethylbenzidine (TMB) as a substrate.

Immunocytochemistry

MSCs were seeded at 37°C in eight-chamber slides at a density of 5–10 000 cells per chamber. The chamber slides were later placed at 38.5°C and 40°C for 1, 3 and 6 h before being fixed with 4% buffered formaldehyde, washed in phosphate-buffered saline (PBS) and stained. For immunostaining, the slides were blocked for 1 h with 1% bovine serum albumin (BSA) and 0.05% Tween in 1X PBS. Immunostainings were performed immediately using antibodies as shown in Supplementary Table 1.

Primary antibody activity was visualized using species-specific secondary antibodies (Jackson ImmunoResearch; 712-586-153, 715-546-151, 715-586-151) and a widefield DMI6000 inverted Leica fluorescent microscope. Control stainings were performed without primary antibody incubation.

Western blot

Protein lysates were prepared from heat-treated MSCs using freshly made RIPA and NP-40 buffers. Protein quantification was performed using the BioRad DC Protein Assay (BioRad, 5000111). Protein samples were mixed with loading buffer and added to an 8% gel. Antibody staining was performed with the same antibodies used in immunocyto-chemistry shown in Supplementary Table 1.

RNA sequencing

RNA samples from MSCs cultured for 1 and 6 h, at 36°C and 38.5°C, with and without LPS stimulation, were prepared by TRIZOL extraction (Fisher Scientific; 15-596-018) following the manufacturer's recommendations. RNA integrity was assessed using a Fragment Analyzer (Advanced Analytical) and sequencing libraries were prepared using the Illumina TruSeq method (Illumina). Libraries were sequenced on an Illumina HiSeq 1500, on 126bp paired-end mode. Raw sequences underwent initial quality control (QC) analysis and were subsequently aligned to the human hg38 genome version with STAR v2.5.2a. Raw gene read counts produced using STAR were filtered to remove low-expressing genes (56 395 initial genes; 28 970 after filtering) and were further processed in R (see <https://www.R-project.org/>) using the EdgeR package [16,17]. A subset of genes involved in inflammatory pathways of interest was examined with both RNA sequencing and quantitative reverse transcription PCR (RT-qPCR) to yield multiple, cross-supporting data sets.

RT-qPCR

RT-qPCRs were performed using the Qiagen QuantiNova SYBR Green PCR Kit (Qiagen; 208054) according to the manufacturer's instructions. The custom-designed primers used are shown in Supplementary Table 2.

Thermo-cycling was performed in an Applied Biosystems StepOnePlus machine for 2 min of initial heat activation at 95°C, followed by 40 cycles of 5-sec denaturation at 95°C and 10-sec combined annealing and extension at 60°C. A final melt curve confirmed completion of the reactions. Data were analyzed with the StepOnePlus software, normalized to a housekeeping gene. Several HK genes were tested to find ones that were heat-stable, and we chose glucuronidase beta (GUSB), 14-3-3 protein zeta/delta (YWHAZ) and glyceral-dehyde 3-phosphate dehydrogenase (GAPDH). Double delta cycle threshold (CT) calculations were performed to determine fold changes.

Results

ELISA

We measured cytokine production in the supernatants of MSC-macrophage (THP1 cell line) co-cultures using ELISA. THP1-derived IL-10 production after LPS stimulation is undetectable at 37°C and is barely detectable at 38.5°C if MSCs are not present. Co-cultured with MSCs, stimulated with LPS and placed at various temperatures, the THP1 macrophages increase their IL-10 production by 5-fold (36°C) to 10-fold (38.5°C) ($P < 0.001$ and $P < 0.0001$, respectively; Figure 1). Heat alone reduces TNF α levels in LPS-stimulated THP1 cultures by 10–20% (Figure 1), which is a statistically significant decrease ($P < 0.002$).

However, when MSCs are also present, the TNF- α production by the macrophages decreases by 40–80% ($P < 0.0001$).

THP1 macrophages not stimulated with LPS produced no IL-10 or negligible TNF- α and are not included in the graph. MSCs do not produce either cytokine.

RNAseq

Cell lysates were prepared from three different donor MSCs treated at 36°C and 38.5°C for two timepoints (1 and 6 h). To identify genes responsive to high temperature (fever), we performed RNAseq on MSCs derived from three independent adult donors (as biological replicates) and submitted to incubation at different temperatures for 1 h (short) or 6 h (long) or maintained at 37°C (normal). Initial characterization of the resulting gene expression profiles by principal component analysis (PCA) revealed profound intrinsic differences among the three donors (Supplementary Figure 2 PCA12 plot). This in turn overwhelmed all efforts to identify differentially expressed genes by standard methods (DESeq2, edgeR and Limma-Voom, not shown). Further analysis of other principal components revealed that while PC1 (Supplementary Figure 3A) and PC2 (Supplementary Figure 3B) were strongly correlated with the donor variable, PC3 (Supplementary Figure 3C) was significantly correlated with the “time” experimental variable and PC4 (Supplementary Figure 3D) with “temperature”. Therefore, by analyzing PC factor loading on the PC dimensions 3 and 4, we obtained a list of dimensionally correlated genes (50 positive and 50 negative) responsive to time and temperature (Supplementary Figure 4A and 4B).

Although the RNAseq did not pinpoint specific genes of interest that consistently changed in all donors in the same environment (heat), when we performed a pathway analysis looking for enrichment among the 50 positive and 50 negative genes shown in Supplementary Figure 4, four pathways were identified, the elements of which seemed to be significantly different if time and temperature (PC3 and PC4) were considered. These included the interleukin signaling pathway and the inflammation mediated by chemokine and cytokine signaling pathway (Supplementary Figure 5).

qPCR analysis

We focused on factors/genes that have been suggested in the literature to be involved in the immune-suppressive behavior of MSCs. We used MSCs from the same donors at the same passage (P4) that were used for the RNAseq experiments. We noticed changes in expression in several of the genes that were reported to affect MSC-driven immune suppression. At first, we looked at the members of the prostaglandin pathway and detected increased expression in prostaglandin synthase (PTGES) and cyclooxygenase 1 (PTGS1 or COX1) and 2 (PTGS2 or COX2). There was also an increase in hepatic growth factor (HGF), programmed cell death ligand 1 (PDL-1), heme oxygenase 1 (HMOX1), heme oxygenase 2 (HMOX2), proenkephalin (PENK) and microfibrillar-associated protein 5 (MFAP5). Most of these changes were detected after 1 and 3 h of gradual heat treatment and they disappeared by 6 h. Following heat treatment, we found increased expression (between 2.5- and 4-fold increases) in most of the above genes (Figure 2). TNF-stimulated gene 6 (TSG6) showed a 20-fold increase (Supplementary Figure 6), but this was only detectable at the 3-h timepoint

in one of three donors. Because we could not confirm the presence of the protein (due to the lack of available antibodies), this data is shown separately in the Supplementary Material.

Western blotting

To demonstrate the presence of the encoded peptides/proteins, we performed Western blotting for target proteins when an appropriate antibody was available. Here we confirm the presence of heat shock factor1 (HSF1), COX2, PTGES, HMOX1 and HGF in the MSC lysates (Figure 3).

Immunocytochemistry

Next, we used chamber slides to culture cells from the same donors that were used for the gene expression studies. When the cells were about 80% confluent, we applied the heat treatment and fixed the cells. HSF1 was present in the cytoplasm of MSCs before heat treatment (Figure 4A and 4B). After 1 h of heat shock, the staining disappeared in the cytoplasm and the nuclei became positive for HSF1 (Figure 4C and 4D).

Using human-specific antibodies, we stained for COX2, PTGES, HGF, PDL-1, HMOX-1 and Indoleamine 2, 3-dioxygenase (IDO). All antibodies stained, and some showed a clear change in staining intensity as the heat treatment progressed in time (Figure 5). COX2 and PTGES seemed to be the most abundant at 3 h, whereas the others were somewhat more intense at 6 h.

Discussion

MSCs have been studied and used for cellular therapy in a variety of human diseases and numerous clinical trials are ongoing to test their usefulness in patients. Several underlying mechanisms of how the MSCs effect the immune system were suggested and some proven in the literature during the last decade [18]. MSCs convert pro-inflammatory macrophages into anti-inflammatory cells by activating the prostaglandin pathway [14,19]. MSCs respond to LPS and TNF α via their TLR4 and the TNFR-1 receptors, respectively, activating NF- κ B and increasing COX-2. COX-2 converts arachidonic acid to prostaglandin H₂ (PGH₂), which is then made into prostaglandin E₂ (PGE₂) by prostaglandin E synthase (PTGES). The PGE₂ is then released by the MSCs and binds to the prostaglandin EP2 and EP4 receptors on macrophages. The stimulation of E2 and E4 results in the reduction of inflammatory (TNF- α) and an increase of anti-inflammatory (IL-10) cytokine production by the macrophage. Cell-to-cell contact appears necessary for this effect to occur [14]. Because HSF1 was shown to regulate COX-2 expression in human cells [20], we wanted to know if translocation of HSF1 into the nucleus of MSCs occurs in heat-treated MSCs. This was apparent after the cells were cultured for 1 h at 38.5°C. When the cells were cultured at 36°C, HSF1 remained in the cytoplasm. We then asked how well heat-treated MSCs shifted cytokine production by macrophages. We found that in response to increasing the temperature of the co-culture of MSCs and macrophages by 2.5°C, this results in a significantly more efficient induction of macrophage-produced IL-10 and reduction of TNF- α release. These cytokines are considered to be specific markers for anti-inflammatory (M2) or proinflammatory (M1) cells, respectively. Taken together, these data are in good

agreement with the observation by Rossi *et al.* [20] that heat shock should activate the COX-2 pathway in MSCs to modulate the MSC-macrophage interaction contributing to the changes seen in the IL-10 and TNF- α levels from the co-culture media. While heat-induced changes in genes related directly to the prostaglandin pathway are clearly responsible for some of the effects of hyperthermia on MSC immunogenicity, other genes may play a role as well. Our findings suggest that heat causes significant changes in MSC expression of HGF that has been reported to also activate the COX-2 gene [21]. In addition to the prostaglandin pathway, we found that a variety of other factors that were reported in the literature to contribute to the immune-suppressive behavior of human MSCs also changed with heat treatment. These included PDL-1, which is made and secreted by MSCs and triggers T-cell suppression in response to inflammatory cytokines [22]. Our results suggest that PDL-1 is also increased by heat and might play a role in the character change of macrophages. Recently, hemoxigenases [23,24] and TSG-6 [25] were also shown to play a role in MSC-derived immune suppression. We observed that heat treatment induces HEMOX-1, HEMOX-2 and in one of the three donors the expression of TSG-6 was also highly elevated. So was IDO, which is known to be a regulator of dendritic cell function [26]. MSCs are known to increase IDO production in response to interferon-gamma stimulation and were one of the first molecules found to be used by human MSC—derived immune-suppression [25,27,28].

Finally, we observed the heat-induced up-regulation of two genes present in MSCs, MFAP5 and PENK, reported to attenuate inflammation and down-regulate macrophage activation [29].

Many research groups have sought to increase the immunogenicity of MSCs by priming them for use in a clinical setting [5,18,25,28,30]. Using drug or cytokine stimulation can require special permissions and protocols to pre-treat clinical-grade cells.

Based on our observations, heat may offer an easily exploitable alternative method of cell conditioning. The heat-induced increase in MSC immunomodulatory activity appears to result partly from effects on prostaglandins that lead to suppression of pro-inflammatory macrophages in sepsis [14], but it may also rely on other factors. We suggest that giving MSCs to patients when their body temperatures are highest (in the afternoon), or warming patients up by 2°C for a brief period, might increase treatment efficacy. This would require no manipulation of the cells *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

The work was supported by the National Institutes of Health (NIH) intramural research funds of the National Institute of Dental and Craniofacial Research. The RNAseq was performed at the Genomics and Combinational Bioinformatics Core of the National Institute of Deafness and other Communication Diseases. The human MSCs were prepared and supplied by the NIH Bone Marrow Stromal Cell Transplantation Center.

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Cytokine levels in the medium of BMSC co-culture with THP1 monocytes following LPS stimulation

Figure 1.

Cytokine measurements of the cell culture medium of THP1 and THP1/MSC co-cultures (both stimulated with LPS) demonstrating that heat increases the efficiency of MSCs to induce pro-inflammatory macrophage (M1) conversion toward anti-inflammatory macrophages (M2). Data shows the average of three subjects; the assays were run in triplicates. Two-way analysis of variance (ANOVA) was performed, and the statistical significance is as follows: *** $P < 0.001$; **** $P < 0.0001$.

Figure 2. qPCR showing the change in messenger RNA (mRNA) expression of factors made by the MSCs that induce the macrophages to change character from M1 (pro-inflammatory) to M2 (anti-inflammatory) in response to heat treatment. The data are normalized to control temperature (36°C) and show fold differences.

Figure 3.
Western blots showing protein expression in MSCs following heat treatment.

Figure 4. Translocation of HSF into the nucleus. Cytoplasmic staining is observed in the BMSCs before heat treatment (A and B). Following treatment, the staining disappears from the cytoplasm and the nuclei (marked by DAPI staining) are stained positive (C and D). Scale, 20 μ m. DAPI (4',6-diamidino-2-phenylindole).

Figure 5.

Using human-specific antibodies, we immunostained MSCs for COX2, PTGES, HGF, PDL-1, HMOX-1 and IDO. All antibodies stained; some showed a clear increase in staining intensity as the heat treatment progressed in time. COX2 and PTGES seemed to be the most intense at 3 h, whereas the others were more intense at 6 h. Control slides with no primary antibody showed no immunostaining (not shown). Scale, 50 μ m.