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Dynamic compaction of human mesenchymal stem/precursor cells (MSC) into spheres self-activates caspase-dependent IL1 signaling to enhance secretion of modulators of inflammation and immunity (PGE2, TSG6 and STC1)

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Abstract

Human mesenchymal stem/precursor cells (MSC) are similar to some other stem/progenitor cells in that they compact into spheres when cultured in hanging drops or on non-adherent surfaces. Assembly of MSC into spheres alters many of their properties, including enhanced secretion of factors that mediate inflammatory and immune responses. Here we demonstrated that MSC spontaneously aggregated into sphere-like structures after injection into a subcutaneous air pouch or the peritoneum of mice. The structures were similar to MSC spheres formed in cultures demonstrated by the increased expression of genes for inflammation-modulating factors TSG6, STC1, and COX2, a key enzyme in production of PGE2. To identify the signaling pathways involved, hanging drop cultures were used to follow the time-dependent changes in the cells as they compacted into spheres. Among the genes up-regulated were genes for the stress-activated signaling pathway for IL1 / , and the contact-dependent signaling pathway for Notch. An inhibitor of caspases reduced the up-regulation of IL1A/B expression, and inhibitors of IL1 signaling decreased production of PGE2, TSG6 and STC1. Also, inhibition of IL1A/B expression and secretion of PGE2 negated the anti-inflammatory effects of MSC spheres on stimulated macrophages. Experiments with -secretase inhibitors suggested that Notch signaling was also required for production of PGE2 but not TSG6 or STC1. The results indicated that assembly of MSC into spheres triggers caspase-dependent IL1 signaling and the secretion of modulators of inflammation and immunity. Similar aggregation *in vivo* may account for some of the effects observed with administration of the cells in animal models.

Keywords

MSC; IL1; PGE2; sphere; caspase; Notch

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Introduction

Mesenchymal stem/stromal cells (MSC) isolated from various tissues have produced promising results in animal models of human diseases that have prompted the use of the cells in clinical trials [1-3]. MSC are relatively easy to isolate, expand rapidly in culture, and differentiate into multiple cell types. Also they are not tumorigenic. The benefits of MSC administration into animal models are often observed without significant engraftment. Instead, the benefits have been attributed to paracrine effects, cell-to-cell contacts, and transfer of microvesicles or mitochondria that modulate inflammatory and immune reactions or enhance tissue regeneration [1-5]. MSC secrete a large number of cytokines and growth factors *in vitro* but they are activated to secrete many others when administered *in vivo* [1-6]. The activation *in vivo* is often attributed to cytokines and other factors released by injuries to tissues, but the mechanisms of activation have not been clearly defined.

MSC are similar to some but not all other stem/progenitor cells in that they first aggregate and then compact into tightly-packed spheres when cultured in hanging drops or on non-adherent surfaces [7-21]. The assembly of cells into spheres was first observed with neural cells and then with cells from a variety of normal tissues and cancers [22]. Assembly of cells into spheres does not necessarily provide an assay of stem cells. Instead, recent observations suggests that assays for sphere formation reflects the potential of both stem cells and the potential of progenitor cells such as transit amplifying cells to revert to an earlier phenotype under a given set of culture conditions [22]. When MSC assembled into spheres, they displayed many of these features [7-21,23]. Among the factors with increased production in MSC spheres formed in hanging drop cultures were prostaglandin E2 (PGE2) and tumor necrosis factor α -induced protein 6 (TSG6) that modulate the inflammatory responses and stanniocalcin 1 (STC-1), the calcium/phosphate regulating protein that reduces reactive oxygen species [15,16,23]. In a zymosan-induced model for peritonitis (peritoneal inflammation), injection of MSC spheres into the peritoneum suppressed the inflammation much more effectively than injection of MSC cultured as 2D monolayers [16]. In experiments with lipopolysaccharide (LPS)-activated macrophages, the PGE2 secreted by MSC spheres promoted a transition of the macrophages from a primarily pro-inflammatory M1 to a more anti-inflammatory M2 phenotype, phenomenon not observed with 2D monolayer MSC [15].

In the experiments described here, we first observed that MSC can spontaneously aggregate into sphere-like structures *in vivo* and in the process up-regulate expression of cyclooxygenase 2 (COX2) a key enzyme in production of PGE2, TSG6 and STC1. We then used hanging drop cultures of MSC to identify signaling pathways that drove the increased production of PGE2, TSG6 and STC1 as the cells assembled into spheres. The results demonstrated that both caspase-dependent interleukin 1 (IL1) signaling and Notch signaling were required for up-regulation of PGE2, but only caspase-dependent IL1 signaling was required for up-regulation of TSG6 and STC1.

Materials and Methods

MSC culture

Human MSC, isolated from bone marrow aspirates and cultured as previously described [16], were obtained as frozen vials in passage 1 from the Center for the Preparation and Distribution of Adult Stem Cells (<http://medicine.tamhsc.edu/irm/msc-distribution.html>). MSC were suspended in complete culture medium (CCM) consisting of α -Minimum Essential Medium (MEM, Gibco), 17% fetal bovine serum (FBS, Atlanta Biologicals), 100 units/ml penicillin (Gibco), 100 μ g/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco) and plated in a 152 cm² culture dish (Corning). After 24 h, cells were washed with

phosphate buffered saline (PBS) and harvested using 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA, Gibco) for 3-4 min at 37°C, plated at 100 cells/cm², and expanded for 7 days before freezing as passage 2 cells in α -MEM containing 30% FBS and 5% dimethylsulfoxide (DMSO, Sigma). For the experiments described here, a vial of passage 2 MSC were recovered by plating in CCM on a 152 cm² culture dish for a 24 h period, re-seeded at 100-150 cells/cm² (Adh Low), and incubated for 6-7 days in CCM. Culture medium was changed every 2-4 days and 1 day before harvest.

Fibroblast culture

Human adult dermal fibroblasts (DF) were obtained from 3 commercial sources (American Type Culture Collection (ATCC), Lonza, and Gibco). Frozen vials of the cells were thawed and plated on adherent 152 cm² culture dishes in CCM for up to 24 h. Cells were harvested with trypsin/EDTA for 3-4 min at 37°C and re-plated at 1500 cells/cm² for expansion. Medium was changed 3 days after plating and cells were harvested on day 4 at 70-90% confluence for assays.

MSC aggregate formation *in vivo*

Male C57BL/6J and BALB/c mice (Jackson Laboratories), 6-8 weeks of age, housed on a 12 hour light/dark cycle, were used in the experiments. All animal procedures were approved by the Animal Care and Use Committee of Texas A&M Health Science Center and in accordance with guidelines set forth by the National Institutes of Health. Total of 1-2 $\times 10^6$ MSC expressing green fluorescent protein (GFP) were injected into the peritoneal cavity of BALB/c and C57BL/6J mice with a 28G needle under isoflurane anesthesia. GFP-MSC were also injected into an air pouch, formed in C57BL/6J mice by repeated subcutaneous injections of sterile air into the back of the mouse (5 ml initially, then 3 ml on day 3 and 6). Either at 4 h or 72 h later, animals were euthanized and the cavities of peritoneum or air pouches were exposed. GFP-MSC aggregates were visualized with Illumatool Bright Light Systems LT 9900 (Lighttools Research) with EGFP filter set (470 nm excitation, 515 nm emission). Fluorescent and bright-field images were captured with Nikon Digital Sight DS-2Mv camera attached to SMZ800 dissecting microscope (Nikon, Japan). GFP-positive aggregates were collected with tweezers, centrifuged (500 $\times$ g for 5 min), and lysed in RLT buffer containing β -mercaptoethanol (Qiagen) for RNA isolation.

Sphere generation *in vitro* and inhibitor assays

To generate multi-cellular spheres [16], MSC or DF were suspended in CCM at 714 cells/ μ l and placing 35 μ l drops (25000 cells) on the inverted lid of a cell culture dish. The lid was then rapidly re-inverted onto the culture dish that contained PBS to prevent evaporation of the drops. The hanging drop cultures were incubated for 1-4 days at 37°C in a humidified atmosphere with 5% CO₂. In some experiments, MSC in hanging drops were cultured for 3 days in the presence of 10 μ M of the broad-spectrum pan caspase inhibitor Q-VD-OPh (EMD Millipore); 20-500 ng/ml IL1 receptor antagonist (IL1ra) (R&D Systems); neutralizing antibodies to IL1 α , IL1 β , and IL1 receptor 1 (IL1R1) (R&D Systems); IgG antibody (R&D Systems); 20 μ M of interleukin receptor associated kinase (IRAK) inhibitor N2B; 2-50 μ M of the α -secretase inhibitor DAPT (Cayman Chemical) or SMLY (Stemgent); 1 μ M of nuclear factor κ B (NF κ B) translocation inhibitor QNZ (Cayman Chemical).

Transfections with small interfering RNA (siRNA)

Reverse transfections in suspension were performed using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). MSC were harvested and washed with antibiotic-free CCM. Total of 4.5 nmol negative control siRNA duplex (Low and Medium GC content, Invitrogen), or IL1A and IL1B siRNA duplexes (Invitrogen) were

mixed with 15 ml of Opti-MEM medium (Gibco). For each reaction, 225 μ l of Lipofectamine RNAiMAX was added and the combination was gently mixed and incubated for 10 min in RT. Total of 3.1×10^6 MSC in 75 ml of antibiotic-free CCM were added for each reaction. The final reactions contained 50 nM of siRNAs, 1:400 of Lipofectamine RNAiMAX, 17% Opti-MEM, and 83% antibiotic-free CCM. The suspensions were mixed gently and MSC were plated at 5000 cells/cm² in 152 cm² dishes and incubated at 37°C and 5% CO₂. After 24 h, transfected cells were harvested and cultured in hanging drops for 3 days. Knock-down of IL1A and IL1B gene expression was validated by real-time PCR. From the three siRNAs tested for each gene, siRNAs with the best knockdown efficiency were used in further experiments.

Conditioned medium and cell lysate harvest

MSC and DF were plated at a high (5000 cells/cm², 25.5 cells/ μ l, Adh High) or very high density (200000 cells/cm², 714 cells/ μ l, Adh VH) on adherent dishes in CCM, or in hanging drops (714 cells/ μ l) in CCM. After 3 days, conditioned medium was harvested and centrifuged at 453 $\times$ g for 5-10 min. The supernatant was clarified by centrifugation at 10,000 $\times$ g for 10 min before using for assays or storage at -80°C.

For cell lysis, the cultures were washed twice with PBS and lysed on the adherent dishes with RLT buffer containing β -mercaptoethanol. To obtain sphere cell lysates, spheres were centrifuged at 453 $\times$ g for 5 min, washed with PBS, centrifuged at 453 $\times$ g for 5 min, and lysed with RLT buffer.

Assays for secreted proteins

PGE2, IL1 α , IL1 β , mouse tumor necrosis factor (α 1) (mTNF α), and mIL10 were assayed with ELISA kits (R&D Systems). TSG6 levels were determined as previously described [16] with some modifications. Briefly, wells (Costar) were coated overnight at 4°C with 10 μ g/ml of TSG6 monoclonal antibody (clone A38.1.20; Santa Cruz Biotechnology) in 100 μ l PBS. The wells were washed 3 times with 400 μ l of 1x wash buffer (R&D Systems), blocked with 100 μ l of PBS containing 0.5% bovine serum albumin (BSA, Thermo), and incubated with 100 μ l of sample or recombinant human TSG6 protein standards (R&D Systems) diluted in blocking buffer. After 2 h, wells were washed and incubated with 0.5 μ g/ml biotinylated anti-human TSG6 (R&D Systems) in 100 μ l of PBS. After 2 h, 100 μ l of streptavidin-horseradish peroxidase (HRP, R&D systems) was added. After 20 min, 100 μ l of substrate solution (R&D Systems) was added. The colorimetric reaction was terminated after 15 min with 2 N sulfuric acid (R&D systems). For all assays, optical density was determined on a plate reader (FLUOstar Omega; BMG Labtech) at an absorbance of 450 nm with wavelength correction at 540 nm.

Immunofluorescence of IL1 and COX2

MSC spheres were harvested from day 3 hanging drop cultures, washed twice with PBS, and fixed with 3% paraformaldehyde (USB Corporation) in PBS for 20 min. The fixed spheres were washed twice with PBS, centrifuged at 500 $\times$ g for 5 min, and incubated at 4°C overnight in 1 ml of 30% sucrose (Sigma) in 0.1 M phosphate buffer (Sigma). After 24 h, the spheres were collected in 800 μ l of 50% OCT (Sakura Finetek) and transferred into a histology mold. The mold was frozen in isopentane (Sigma) chilled by liquid nitrogen and stored at -80°C. Cryosections (6 μ m) were prepared with a Microm HM560 cryostat and incubated for 20 min. The sections were post-fixed for 10 min in 3% paraformaldehyde, washed twice in TBS, and permeabilized using a 0.2% triton x-100 solution (Sigma). Non-specific antibody binding was blocked by incubating samples for 45 min in tris-buffered saline with tween-20 (TBST, Cell Signaling), 1% BSA (Thermo Scientific), and 5% normal serum (Thermo Scientific). Following 2 washes in TBST, samples were incubated for 2 h

with 1 µg/ml of primary antibodies to human IL1 (R&D systems), IL1 (R&D systems), and COX2 (Abcam). Sections were washed 3 times in TBST, incubated for 1 h with Alexa 488 or Alexa 594 conjugated secondary antibodies, then counterstained with DAPI for 10 min. The sections were washed 3 times in TBS and mounted in Prolong Gold antifade reagent (Life Technologies) overnight. Images were acquired on a Nikon Eclipse 80i upright microscope and processed using NiS Elements AR3.0 software.

Caspase activity assay

Caspase activity was determined on sphere MSC derived from 1-3 day hanging drop cultures using Vybrant FAM poly caspase activity kit (Life Technologies). Spheres were incubated with trypsin/EDTA at 37°C for approximately 10 min with pipetting every 3 min. When no cell aggregates were visible, the sphere cells were collected by centrifugation at 453 ×g for 10 min, and resuspended at 1000 cells/µl in CCM. Total of 300000 cells were incubated for 1 h at 37 °C with 1x FLICA reagent. MSC were washed twice in 6 ml PBS then resuspended in PBS containing 2% FBS and 5 µg/ml 7AAD (Sigma). Caspase activity was analyzed on an FC500 benchtop analyzer (Beckman Coulter).

Macrophage inflammatory assay

Mouse macrophages (J774A.1, ATCC) were expanded as adherent cultures on 15 cm diameter petri dishes (Falcon) in high glucose Dulbecco's Modified Eagle Medium (Invitrogen), 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Subcultures were prepared every 2-3 days by washing the cells from the dishes and re-plating at 1:6-1:12. For the inflammatory assay macrophages were centrifuged at 250 ×g for 5 min and stimulated with LPS (Sigma). After a 5-10 min equilibration period, the stimulated macrophages were transferred at 25000 cells/cm² onto 12-well culture plates containing a 1:300 dilution of sphere-conditioned medium. The final concentration of LPS was 100 ng/ml. After 18–24 h, conditioned medium was collected and centrifuged at 500 ×g for 5 min.

Microarrays

For the time course microarray assays, MSC (Adh Low) were incubated in hanging drops for 2 h (Sph 2h), 8 h (Sph 8h), 24 h (Sph 24h), 48 h (Sph 48h), or 72 h (Sph 72h). Cells were harvested and lysed, RNA isolated with RNeasy Mini Kit, and the isolated RNA was quantified with Nanodrop spectrophotometer (Thermo Scientific). RNA from 3 experiments were pooled at equal amounts (100 ng each) for total of 300 ng for each time point sample. Labeled amplified RNA (aRNA) was prepared according to manufacturer's instructions for GeneChip 3' IVT Express Kit (Affymetrix). Total of 15 µg of labeled aRNA was fragmented and hybridized (GeneChip Hybridization Oven 640, Affymetrix) onto human arrays (HG-U133 Plus 2.0, Affymetrix) followed by washing and staining (GeneChip Fluidics Station 450, Affymetrix) with GeneChip Wash and Stain Kit (Affymetrix). Arrays were scanned with GeneChip Scanner (Affymetrix) and raw data files (CEL-files) were transferred into Partek Genomics Suite 6.4 (Partek). Data were normalized using robust multi-array (RMA) algorithm and gene level analysis and comparisons were done using the Partek software. For hierarchical clustering, genes were filtered based on significant changes (at least 2-fold up- or down-regulated) in the expression between the Sph 72h and Adh Low samples. This resulted in 5632 differentially expressed genes.

Previously published microarray data on MSC and DF cultured in hanging drops (Sph) and at different densities as monolayers (Adh Low, Adh High) were searched for IL1 signaling related genes [15,16].

Real-time PCR assays

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen) with DNase (RNase-Free DNase Set; Qiagen) digestion step and quantified with Nanodrop spectrophotometer. RNA was converted to cDNA with High-Capacity cDNA RT Kit (Applied Biosystems). Real-time PCR was performed in triplicate for expression of COX2, TSG6, STC1, IL1A, IL1B, IL1R1, IRAK2, delta-like 1 (DLL1), Notch homolog 2 (NOTCH2), hairy/enhancer of split related with YRPW motif 1 (HEY1), jagged 1 (JAG1), prostaglandin E synthase (PTGES), phospholipase A2 group IVA (PLA2G4A), and PLA2G4C using Taqman® Gene Expression Assays (Applied Biosystems) and Taqman® Fast Master Mix (Applied Biosystems). Total of 5-50 ng of cDNA was used for each 20 µl reaction. Thermal cycling was performed with 7900HT System (Applied Biosystems) by incubating the reactions at 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Data were analyzed with Sequence Detection Software V2.3 (Applied Biosystems) and relative quantities (RQs) were calculated with comparative critical threshold (C_T) method using RQ Manager V1.2 (Applied Biosystems).

Data analysis

Data are summarized as mean $\pm$ SD. One-way analysis of variance with Bonferroni's Multiple Comparison Test was used to calculate the levels of significance (ns, $p > .05$; *, $p < .05$; **, $p < .01$; ***, $p < .001$) for data with at least 3 samples. Unpaired two-tailed t-test was used when the data consisted of only 2 samples. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software).

Results

MSC aggregate into sphere-like structures *in vivo*

To test the hypothesis that MSC might aggregate and form spheres *in vivo*, we injected human GFP-MSC either into a non-inflamed subcutaneous air pouch or into the peritoneum of mice. The cells recovered after 4 h of injection into peritoneum of C57BL/6 mice had aggregated into sphere-like structures (Fig. 1A). Human specific real-time PCR assays of the recovered structures demonstrated up-regulated expression of COX2, TSG6, and STC1 (Fig. 1B-1D). Moreover, MSC aggregates could be recovered even after 72 h from the peritoneum, and the recovered aggregates demonstrated up-regulated expression of COX2, TSG6, and STC1 (Fig. 1E-1H). Similar results were obtained with a second strain of mice (BALB/c) (supporting information Fig. S1A-S1C). The cells recovered after 4 h of injection into air pouch of C57BL/6 mice had also aggregated into sphere-like structures and expressed high levels of COX2, TSG6, and STC1 (supporting information Fig. S1D-S1G). The results demonstrated therefore that aggregation of MSC can occur spontaneously *in vivo* and is accompanied by an increase in the expression of potentially therapeutic genes.

MSC in hanging drop cultures undergo dynamic changes in their transcriptome

To identify signaling pathways that drove expression of COX2, TSG6 and STC1, we cultured MSC in hanging drops under the conditions previously employed [15,16]. Time-lapse microscopy demonstrated that the MSC first aggregated into a series of sphere-like structures and then the separate sphere-like structures coalesced into a single sphere (Fig. 2A, supporting information Fig. S2 and video). In the process, some cells were shed from spheres along with cellular debris, suggesting organization of the cells during assembly into spheres in hanging drops (Supporting information video) [16]. Also, the cells ceased proliferating even though the medium contained 17% fetal calf serum pre-selected for rapid growth of human MSC in monolayer [16]. As the spheres formed, the cells compacted with a marked decrease in the relative amount of cytoplasm and a decrease in average cell

volume from approximately 4,000 μm^3 to less than 1,000 μm^3 [16]. Of note was that the transcript levels of the three genes of interest, COX2, TSG6, and STC1, increased and were expressed at peak levels at 72-96 h, a time at which the cells are fully compacted into a single sphere. Microarray assays demonstrated that the morphological changes of the cells were accompanied by dynamic changes in their transcriptome (Fig. 2C). The expression pattern of the 5632 differentially expressed genes (genes that were up- or down-regulated at least 2-fold between the 72 h sphere sample and baseline monolayer culture) started to change already after 2 h, however, the major changes occurred as the cells assembled into a sphere over the next 24-72 h.

MSC spheres self-activate IL1 and Notch signaling

To identify possible signaling pathways, we searched the microarray data for up-regulation of stress-activated genes and for genes signaling through cell-to-cell contacts. Large gene expression changes were not detected in interferon (IFN) or toll like receptor (TLR) pathways but the expression of a series of genes for IL1 signaling were up-regulated in a time-dependent manner (supporting information Table S1) including the ligands IL1A (31-fold) and IL1B (154-fold), the receptor for IL1 (IL1R1, 4-fold) and the IL1 receptor-associated kinase 2 (IRAK2, 8-fold) (Fig. 2D, supporting information Table S1). Of note was that one of the most highly activated genes was the gene for pro-IL1 that is processed by caspase 1 during inflammasome activation [24].

Up-regulation of IL1 signaling in the MSC spheres was confirmed with real-time RT-PCR assays that demonstrated significant increases in IL1A (>1000-fold), IL1B (>2000-fold), IL1R1 (5-fold), and IRAK2 (>25-fold) (supporting information Fig. S3). The levels of the transcripts at 72 h were much higher than in MSC cultured on adherent dishes as monolayers at low, high, or very high densities.

In addition, several genes for Notch signaling were up-regulated in a time-dependent manner (Fig. 2E, supporting information Table S2). The expression of Notch ligand JAG1 was up-regulated within 2 h and then declined. The expression of a second ligand DLL1 and two Notch genes (NOTCH2 and NOTCH3) were up-regulated and maintained during sphere compaction. Real-time RT-PCR assays confirmed the higher expression of Notch signaling molecules in MSC spheres (supporting information Fig. S4). For example, transcripts of HEY1, one of the downstream transcription factors targeted by the pathway, increased and peaked within the first few hours (supporting information Fig. S4E). The initial peak probably reflected the process of cell lifting with trypsin/EDTA and the use of fresh medium to initiate the hanging drop cultures. However, the levels of the HEY1 remained high throughout the assembly of the spheres and they were higher than in MSC cultured in monolayers (supporting information Fig. S4C).

The increase in IL1 signaling molecules was confirmed by ELISAs that demonstrated secretion of IL1 and IL1 by MSC spheres but not by monolayer MSC (Fig. 3A, 3B). Also, immunofluorescence assays demonstrated the presence of IL1, IL1, and COX2 positive cells in the MSC spheres (Fig. 3C). These results raised the possibility that both IL1 and Notch signaling might drive the expression of COX2, TSG6, and STC1 in MSC spheres. However, it was of interest that the IL1, IL1, and COX2 positive cells were widely distributed in foci in the spheres, an observation that indicated the activation of the cells was initiated at many sites throughout the spheres (Fig. 3C).

Activation of caspases and NFkB are required for increased IL1 signaling

A small fraction of the MSC that assemble into spheres under the conditions employed here undergo apoptosis [16]. Therefore we tested the hypothesis that the activation of IL1

signaling in the spheres might be driven by caspases and NF- κ B activation. Changes in expression of genes for caspases were not prominent in the microarray data, but caspase activity was detected in MSC dissociated from spheres and the level did not change between days 1 and 3 (Fig. 3D). Addition of a broad-spectrum caspase inhibitor (Q-VD-OPh) to the hanging drops prevented the increase in transcripts for IL1A, IL1B, and IL1R1 during assembly of the spheres (Fig. 3E, 3F, 3G). As expected, the inhibitor reduced the secretion of IL1 α and IL1 β by the MSC spheres (supporting information Fig. S5A, S5B). Similarly, an inhibitor of NF- κ B transcriptional activation (QNZ) reduced the secretion of IL1 α and IL1 β by the MSC in spheres (supporting information Fig. S5C, S5D). These results indicated that both activated caspases and NF- κ B are required for the activation of IL1 signaling in MSC in spheres.

Increased IL1 signaling is required for increased secretion of PGE2

We next explored whether increased IL1 signaling was required for production of PGE2 by the MSC spheres. IL1 receptor antagonist (IL1ra), when added to the hanging drop cultures, inhibited up-regulation of the transcripts for a series of enzymes required for synthesis of PGE2: COX2, PTGES, and PLA2G4C (supporting information Fig. S6). IL1ra also decreased the secretion of PGE2 by MSC in the spheres (Fig. 4A). A mixture of antibodies to IL1 α , IL1 β , and the IL1R1 reduced the secretion of PGE2 by MSC spheres in a dose dependent manner (Fig. 4B) and inhibited the up-regulation of COX2, PTGES, PLA2G4A, and PLA2G4C (supporting information Fig. S7). Similar results were obtained when the IL1 blocking antibodies were used individually or in different combinations, suggesting an important role for activating IL1 signaling to produce PGE2 in MSC spheres (Fig. 4C, supporting information Fig. S8). Use of IL1A and IL1B siRNAs to knockdown the transcripts confirmed the role of IL1 α and IL1 β in production of PGE2 (Fig. 4D, supporting information Fig. S9). Furthermore, inhibiting IRAK decreased the production of PGE2 by MSC in spheres (Fig. 4E). Therefore the results indicated that activated IL1 signaling in MSC was required for increased production of PGE2.

Up-regulation of IL1 signaling is also required for modulation of macrophages

Previous results demonstrated that secretion of PGE2 accounted for the ability of MSC spheres to convert activated macrophages from an M1 to an M2 phenotype (Fig. 5A) [15]. To establish that IL1 signaling is required for the phenomenon, IL1 signaling in the MSC spheres was inhibited by IL1ra and neutralizing antibodies. As expected, inhibition of IL1 signaling in human MSC spheres with IL1ra negated the ability of conditioned medium from the spheres to convert LPS-activated macrophages from an M1 to an M2 phenotype as indicated by levels of mouse TNF α and mouse IL10 secreted by the mouse macrophages (Fig. 5B, 5C). Antibodies to direct targets in the pathway varied in their effectiveness but consistent results were obtained with a mixture of the antibodies and the effectiveness of the mixture was dose-dependent (Fig. 5D, 5E, supporting information Fig. S10). Moreover, inhibiting IRAK almost completely abolished the effects of the conditioned medium from MSC spheres on stimulated mouse macrophages (Fig. 5F, 5G). These results indicated that activation of IL1 signaling in MSC spheres was required for the anti-inflammatory effects of the conditioned medium on LPS stimulated macrophages.

Up-regulation of IL1 signaling was not observed in controls of human adult dermal fibroblast (DF) spheres

As one control for the requirement for IL1 signaling, we used DF, since they assemble into spheres in hanging drop cultures similar to MSC but they do not produce PGE2 and do not promote the M1 to M2 transition of LPS-stimulated macrophages [15]. Microarray data of MSC and DF cultures demonstrated much lower levels in the DF spheres of transcripts for three genes involved in IL1 signaling: IL1A, IL1B, and IRAK2 (Fig. 6A). The expression of

IL1 signaling genes were confirmed by real-time RT-PCR assays that demonstrated increases in the expression of IL1A, IL1B, IRAK2 (Fig. 6B, 6C, 6D), and IL1R1 (supporting information Fig. S11) in MSC spheres but not in DF spheres. Also, as expected, IL1 and IL1 secretion was extremely low or absent with spheres formed from three different DF donors (Fig. 6E, 6F). In contrast, spheres from four different preparations of MSC from four different donors consistently secreted higher levels of IL1 and IL1 (Fig. 6E, 6F). These results suggested that DF spheres do not produce PGE2 because IL1 signaling is not activated in them as they aggregate into spheres.

Activation of Notch signaling is also required for PGE2 production

The Notch signaling pathway has been implicated in a large number of developmental and metabolic pathways, including the production of many cytokines and inflammatory molecules [25-32]. Blocking Notch signaling in MSC spheres with two inhibitors of -secretase (SMLY and DAPT) decreased expression of COX2 (supporting information Fig. S12, S13A). Also, secretion of PGE2 by the MSC spheres was decreased in a dose-dependent manner (Fig. 7A, supporting information Fig. S13B). As expected, inhibition of Notch signaling with the -secretase inhibitors also negated the anti-inflammatory effect of condition medium from MSC spheres on LPS-stimulated macrophages as indicated by the mouse TNF and IL10 secretion (Fig. 7B, 7C, supporting information Fig. S13C, S13D). These results suggested that activation of Notch signaling in MSC spheres is required for the production of PGE2 and the anti-inflammatory effect of the conditioned medium on stimulated macrophages.

Production of TSG-6 and STC-1 also requires IL1 but not Notch signaling

Since the above experiments demonstrated that the production of PGE2 by MSC spheres was regulated through caspase-driven IL1 signaling, we sought to determine whether the same pathway was required for up-regulation of TSG6 and STC1. The results demonstrated that caspase-dependent IL1 signaling was essential since the secretion of both TSG6 and STC1 was inhibited by the broad-spectrum caspase inhibitor and by IL1ra (Fig. 7D, 7E). Also secretion of both TSG6 and STC1 was decreased by an inhibitor of NF κ B, a downstream target of IL1 signaling (Fig. 7D, 7E) [33,34]. However, decreasing Notch signaling with an inhibitor of -secretase had no effect (Fig. 7D, 7E). Therefore activation of caspase-dependent IL1 was essential for up-regulation of TSG6 and STC1 in the MSC spheres but activation of Notch signaling was not (Fig 7F).

Discussion

The results here demonstrate that one fate of MSC infused into non-inflamed hollow spaces such as a subcutaneous air pouch or the peritoneum of mice was to aggregate into sphere-like structures. The aggregation of MSC coincided with rapid increases in the expression of genes for COX2, a key enzyme in production of PGE2, TSG6, and STC1. Besides the systemic pro-inflammatory effects of PGE2, locally PGE2 is one of the most potent activators of anti-inflammatory M2 macrophages and thus promotes resolution of inflammation. TSG6, on the other hand, can modulate inflammatory reactions whereas STC1 can reduce reactive oxygen species. These genes are the same ones whose expressions are up-regulated as the cells aggregate and compact into structures referred to as spheres or spheroids when cultured in hanging drops under the conditions we employed previously [15,16]. Therefore these observations offer a second and probably complementary explanation for the beneficial effects observed after administration of MSC to animal models for human diseases. In addition to being activated by signals (e.g. cytokines) from injured tissues and cells [1-3], MSC can be activated by aggregation into sphere-like structures *in vivo* and increase the production of therapeutic proteins. Therefore self-

activation of MSC by assembly into spheres may explain some of the beneficial effects observed with injection of the cells into confined spaces such as the peritoneum [35], or the knee joint [36,37]. It is unclear whether sphere formation also occurs after intravenous administration. Most of the cells are trapped in lung after intravenous administration and they appear to form clusters in afferent blood vessels [38,39]. The number of cells in the clusters is smaller but might be enough to cause self-activation, as cells are still in very close contact with each other, and result in production of therapeutic molecules, such as PGE2, TSG6, and STC1. In addition, intravenously infused MSC may also be activated by TNF and other signals generated as a result of the micro-emboli they produce in the lung [39]. Therefore, sphere formation is probably beneficial in most cases, perhaps prolonging the retention and survival of the cells, but mainly in activating the cells to produce therapeutic proteins. However, in the current study we cannot rule out that mouse immune cells and cytokines partially contribute to the gene expression increases detected in MSC aggregates.

To identify the signaling pathways that are activated as MSC assemble into spheres, we exploited the ready accessibility of hanging drop cultures of MSC [8-10,15,16,19]. In the hanging drop cultures, MSC rapidly aggregate and the aggregates coalesce/assemble into a single sphere in a drop with dramatic changes in their transcriptome. During the assembly process, some cells and debris are shed from the forming spheres suggesting active assembly and organization of the cells in the spheres [16]. These features are very different to more passive pellet cultures of MSC often employed to induce chondrogenic differentiation where the cells are forced to aggregate by centrifugation and many gene expression changes are initiated by induction medium rather than aggregation. In hanging drop cultures of MSC, the time required for the changes was slower than the up-regulation of COX2, TSG6 and STC1 *in vivo*, an observation suggesting that the experimental conditions did not fully replicate aggregation of the cells *in vivo*. The spheres formed in the current work share some similarities to aggregates/spheres/spheroids generated in other studies using human MSCs [8-10,12,14-18,23], but are very different from mesospheres generated from a rare population of mouse MSCs that were nestin positive, proliferated in spheres, and expressed a set of specialized genes [40]

The results of the current work demonstrated that up-regulation of IL1 signaling was essential for production of PGE2, TSG6 and STC1. The conclusive role of IL1 signaling was confirmed by multiple protocols for inhibiting the pathway in culture. The amount of IL1 / secreted by MSC spheres is small (4-10 pg/ml in 72 h in hanging drops) suggesting that the self-secreted IL1 / may not have many deleterious effects on other properties of MSCs or enhance inflammation *in vivo*. For example, we previously showed that MSC dissociated from spheres were able to undergo efficient osteogenic and adipogenic differentiation comparable to MSC obtained from 2D monolayer cultures [16]. Moreover, enhanced chondrogenic differentiation of MSC aggregates has been demonstrated previously. Therefore the cells apparently retain most of their therapeutic potentials [23].

Experiments with an inhibitor demonstrated that the activity of caspases was required for up-regulation of IL1 signaling. Surprisingly, the single spheres formed in the hanging drops did not have central cores of IL1 signaling and apoptosis. Instead IL1 signaling was widely distributed in multiple foci. The results suggested that centers of apoptosis formed within the small spheres that formed during day 1 and continued to be activated as the small spheres aggregated in the large single sphere. This suggestion is consistent with the observation that low levels of apoptosis and caspase activity were present on day 1 and did not change as the large single spheres formed [16]. Experiments with two inhibitors of -secretase indicated that Notch signaling was required for secretion of PGE2 but not for TSG6 and STC1. The separate role for Notch signaling is consistent with the complex role of the signaling pathway as important regulator in both embryonic and adult tissues of multiple cellular

events that include cell proliferation, differentiation fate, determination, and stem/progenitor cell self-renewal [25-31].

The results here indicated caspase-dependent IL1 signaling as a novel mechanism that self-activates MSC to secrete therapeutically beneficial molecules (Figure 7F, supporting information Fig. S14). As suggested previously [7-21], culture of MSC as spheres may enhance their therapeutic potentials *in vivo* because they express the appropriate genes and avoid the lag period required to up-regulate expression of the genes in MSC cultured as monolayers, a lag period during which many of cells undergo necrosis and apoptosis [38].

Conclusion

The results demonstrated that human MSC self-activate caspase-dependent IL1 signaling as they compact into spheres and the IL1 signaling drives production of inflammation modulating molecules TSG6, STC1, and PGE2. Furthermore, they demonstrated that self-activation of Notch signaling in MSC spheres is also required for the production of PGE2. The same self-activation mechanism in MSC may enhance expression of potentially therapeutic genes *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MSC form sphere-like structures *in vivo* with increased expression of COX2, TSG6, and STC1

(A) Images of MSC aggregates in a peritoneum of a C57BL/6 mouse 4 h after intra-peritoneal injection of GFP-MSC. Scale bar 500 μ m. (B-D) RT-PCR assays demonstrating increased expression of COX2, TSG6, and STC1 in the GFP-MSC aggregates harvested from the peritoneum 4 h after MSC injection. (E) Images of MSC aggregates in a peritoneum of a C57BL/6 mouse 72 h after intra-peritoneal injection of GFP-MSC. Scale bar 1 mm. (F-H) RT-PCR assays demonstrating increased expression of COX2, TSG6, and STC1 in the GFP-MSC aggregates harvested from the peritoneum 72 h after MSC injection. Controls: GFP-MSC before injection. Values are mean $\pm$ SD ($n = 3$). *, $p < .05$; **, $p < .01$; ***, $p < .001$ compared to control MSC. Abbreviations: GFP, green fluorescent protein; RQ, relative quantity.

Figure 2. MSC undergo dynamic changes in their transcriptome as they compact into spheres in hanging drop cultures

(A) Image of a hanging drop culture plate and a 3 day MSC sphere in a hanging drop. Scale bar 500 μ m. (B) MSC up-regulated the expression of TSG6, COX2, and STC1 as the cells compact into a single sphere. (C) Hierarchical clustering of microarray data from MSC as they aggregated into spheres using the 5632 differentially expressed genes. Differentially expressed genes were obtained by comparing the Sph 72h and Adh Low samples and searching for genes that had at least 2-fold up- or down-regulated gene expression levels. (D) Relative gene expression levels of IL1 signaling molecules from the MSC sphere microarray data. (E) Relative gene expression levels of Notch signaling molecules from the MSC sphere microarray data. Abbreviations: Adh Low, adherent monolayer MSC plated at low density (100-150 cells/cm²) and incubated for 7 days; RQ, relative quantity; Sph, sphere MSC from hanging drop cultures.

Figure 3. Activation of caspase-dependent IL1 signaling in MSC spheres

(A,B) MSC in spheres increased the secretion of IL1 α and IL1 β . Values are mean $\pm$ SD ($n = 3$). (C) Immunofluorescence of sections of MSC spheres labeled with antibodies for IL1 α , IL1 β , and COX2. Scale bar 50 μ m. (D) Flow cytometry for caspase activity in MSC spheres incubated in hanging drops for 1, 2, and 3 days (1d, 2d and 3d). Representative density plots are shown. Values are mean $\pm$ SD ($n = 4$). (E-G) Caspase inhibition (10 μ M Q-VD-OPh) in MSC spheres reduces the expression of IL1A, IL1B, and IL1R1. Adh Low sample was used as a baseline. Values are mean $\pm$ SD ($n = 3$). **, $p < .01$ compared to vehicle control MSC spheres (DMSO). Abbreviations: 7AAD, 7-amino-actinomycin D; Adh High, adherent monolayer MSC plated at high density (5000 cells/cm²) and incubated for 3 days; Adh Low, adherent monolayer MSC plated at low density (100-150 cells/cm²) and incubated for 7 days; Adh VH, adherent monolayer MSC plated at very high density (200000 cells/cm²) and incubated for 3 days; Casp, caspase; DMSO, dimethyl sulfoxide; inh, inhibitor; RQ, relative quantity; Sph, sphere MSC from 3 day hanging drop cultures.

Figure 4. Activation of IL1 signaling In MSC spheres is required for increased secretion of PGE2
(A) Blocking IL1 signaling in MSC spheres with IL1ra (20-500 ng/ml) decreases secretion of PGE2. **(B)** Mixture of antibodies to IL1 , IL1 , and IL1R1 (0.33, 1, 3, or 6 µg/ml) decreases production of PGE2 by MSC spheres. **(C)** Antibodies to IL1 , IL1 , and IL1R1 (6 µg/ml) decrease production of PGE2 by MSC spheres. **(D)** Inhibition of IL1 signaling with siRNAs for IL1A and IL1B reduces PGE2 production in MSC spheres. **(E)** Inhibitor of IRAK (20 µM N2B) abolishes the production of PGE2 by MSC spheres. Values are mean ± SD (*n* = 3). ns, *p* .05; *, *p* < .05; **, *p* < .01; ***, *p* < .001 compared to PBS vehicle control (0) in (A), compared to appropriate amount of control antibody (IgG) in (B) and (C), compared to control siRNA (Scr) in (D), and compared to vehicle control (DMSO) in (E). Abbreviations: Ab, antibody; DMSO, dimethyl sulfoxide; inh, inhibitor; RQ, relative quantity; Scr, negative control for siRNA; Sph, sphere MSC from 3 day hanging drop cultures.

Figure 5. Activation of IL1 signaling in MSC spheres is required for their anti-inflammatory effect on stimulated macrophages

(A) Schematic of the macrophage assay. MSC sphere conditioned medium, through PGE2, can change stimulated macrophages from M1 to M2 phenotype. (B,C) Blocking IL1 signaling in MSC spheres with IL1ra (20-500 ng/ml) reduces their anti-inflammatory effect on LPS stimulated macrophages measured as mTNF and mIL10 secretion. (D,E) IL1 antibodies (1-18 μg/ml) inhibit the anti-inflammatory effect of MSC spheres on activated macrophages as reflected by mTNF and mIL10 secretion. Control IgG antibody was used at 18 μg/ml. (F,G) Inhibition of IRAK (20 μM N2B) abolishes the anti-inflammatory effects of MSC spheres measured as mTNF and mIL10 secretion. Values are mean ± SD ($n = 3$). ns, $p > .05$; ***, $p < .001$ compared to PBS vehicle control (0) in (B) and (C), compared to control antibody (IgG) in (D) and (E), and compared to vehicle control (DMSO) in (F) and (G). Abbreviations: Ab, antibody; CCM, complete culture medium; CM, conditioned medium; DMSO, dimethyl sulfoxide; inh, inhibitor; LPS, lipopolysaccharide; M1, pro-inflammatory macrophage; M2, anti-inflammatory macrophage; M, macrophage; PBS, phosphate buffered saline; sM, stimulated macrophage; Sph, sphere MSC from 3 day hanging drop cultures.

Figure 6. IL1 signaling is not up-regulated in DF spheres

(A) Microarray assays of expression of IL1 signaling genes in MSC spheres and DF spheres. (B-D) Real-time PCR data demonstrated higher levels of expression of IL1A, IL1B, and IRAK2 in MSC spheres than in DF spheres. MSC Adh Low sample was used as a baseline. Values are mean $\pm$ SD ($n = 3$). (E,F) Spheres from 4 MSC donors (D1-4) produced similar levels of IL1 α and IL1 β whereas the production was much lower in spheres from 3 DF donors. Values are mean $\pm$ SD ($n = 4$). Abbreviations: Adh High, adherent monolayer MSC plated at high density (5000 cells/cm²) and incubated for 3 days; Adh Low, adherent monolayer MSC plated at low density (100-150 cells/cm²) and incubated for 7 days; Adh VH, adherent monolayer MSC plated at very high density (200000 cells/cm²) and incubated for 3 days; Di, donor i; RQ, relative quantity; Sph, sphere MSC from 3 day hanging drop cultures.

Figure 7. Activation of Notch signaling is required for PGE2 but not TSG6 or STC1 production by MSC spheres

(A) Notch signaling blocking with -secretase inhibitor (2-50 μ M SMLY) in MSC spheres reduces the production of PGE2. (B,C) Blocking Notch signaling in MSC spheres also reduces their anti-inflammatory effect on LPS-stimulated macrophages measured as mTNF and mIL10 secretion. (D,E) Inhibiting caspase activation, NF κ B translocation, or IL1 signaling reduces the production of TSG6 and STC1 by MSC spheres. Notch signaling blocking (DAPT) in MSC spheres has no effect on TSG6 or STC1 secretion. Inhibitor/blocker doses: caspase inhibitor, 10 μ M Q-VD-OPh; NF κ B inhibitor, 1 μ M QNZ; IL1ra, 500 ng/ml; Notch inhibitor, 50 μ M DAPT. Values are mean $\pm$ SD ($n = 3$). (F) The proposed signaling in sphere MSC resulting in production of TSG6, STC1, and PGE2. ns, $p > .05$; *, $p < .05$; **, $p < .01$; ***, $p < .001$ compared to DMSO vehicle control (0) in (A), (B), and (C), and compared to appropriate vehicle control (DMSO or PBS) in (D) and (E). Abbreviations: CCM, complete culture medium; DMSO, dimethyl sulfoxide; inh, inhibitor; M Φ , macrophage; PBS, phosphate buffered saline; ROS, reactive oxygen species; Sph, sphere MSC from 3 day hanging drop cultures.