Pretreatment with bone marrow–derived mesenchymal stromal cell–conditioned media confers pulmonary ischemic tolerance

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ABSTRACT

Objective: Mesenchymal stromal cell–based therapies have demonstrated efficacy in treating a variety of diseases. Despite the potential benefits, there are still significant hurdles that need to be overcome for clinical use. We describe a cell-free–based immunotherapy approach for inducing pulmonary ischemic tolerance by using mesenchymal stromal cell–conditioned media.

Methods: In our well-established lung ischemia–reperfusion model, we pretreated with mesenchymal stromal cell–conditioned media 30 minutes before injury. To determine the degree of lung injury, we assessed for changes in lung vascular permeability, proinflammatory cytokines and cellular infiltrates in bronchoalveolar lavage, and histopathology. Macrophage and T-cell subsets were assessed by immunohistochemistry.

Results: Pretreatment with mesenchymal stromal cell–conditioned media conferred protection against lung ischemia–reperfusion injury. This protection is characterized by a significant reduction in proinflammatory cytokines, a decrease in infiltrating inflammatory cells, and increases in M2-like macrophages and regulatory T cells.

Conclusions: Cell-free mesenchymal stromal cell–conditioned media therapy confers pulmonary ischemic tolerance. This therapy uses paracrine factors that provide beneficial protective effects by immunomodulating the inflammatory response in resident and infiltrating cell subsets. (*J Thorac Cardiovasc Surg* 2016;151:841-9)

MSC-CM confers pulmonary ischemic tolerance.

Central Message

MSC-CM–based therapy can be a substantial option for protecting or reducing the severity of lung IR injury.

Perspective

MSC-CM–based therapeutic strategy would set precedence in the field of lung IR injury.

See Editorial Commentary page 849.

Despite advances in preservation and supportive care, ischemia–reperfusion (IR) injury remains a major cause of primary graft failure after lung transplantation. Clinically significant reperfusion injury followed by major graft dysfunction develops in 15% to 30% of patients.¹⁻³ Patients with IR injury have a higher incidence of acute

graft rejection and are more prone to early-onset obliterative bronchiolitis and chronic rejection.¹⁻³

Mesenchymal stromal cells (MSCs) are a category of nonhematopoietic, stem-like cells found within the bone marrow niche and are capable of differentiating into a variety of cell types and immunomodulating cellular responses.⁴⁻⁸ Preclinical and clinical studies have reported that administration of MSCs before injury is protective against a variety of diseases.⁹⁻¹³ Most recently, MSCs have been shown to reduce the severity of lung injury in a variety of preclinical models of acute respiratory distress syndrome.⁹⁻¹³ Despite the potential benefits, there are still significant hurdles that need to be overcome to use MSC-based therapy clinically. This includes the requirement for large cell numbers for therapeutic use, lack of MSC quality control and characterization, and cell transformation after continuous in vitro passaging. Direct administration of MSCs has been effective in clinical trials, but if the beneficial effects of conditioned media are as good as those seen with cell-based therapy, this therapeutic strategy would be simpler with fewer potential limitations.

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Abbreviations and Acronyms

BAL	= bronchoalveolar lavage
IL	= interleukin
IR	= ischemia–reperfusion
MSC	= mesenchymal stromal cell
MSC-CM	= mesenchymal stromal cell–conditioned media
PBS	= phosphate-buffered saline

In the present study, we investigated a “cell-free therapy” approach for inducing ischemic tolerance by pretreating with mesenchymal stromal cell–conditioned media (MSC-CM). This therapeutic intervention would set precedence in this field by using the protective immunomodulatory paracrine factors of MSCs and avoiding limitations that have been reported in previous studies.

MATERIALS AND METHODS**Experimental Animals**

Pathogen-free, adult, male Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, Ind), weighing 275 to 300 g, were used for all in vivo experiments. Approval for all experimental protocols was granted by the University of Washington Animal Care Committee. Animals received humane care in compliance with the “Principles of Laboratory Animal Care” established by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” developed by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (Publication No. 86-23, revised 1996).

Isolation and Characterization of Bone Marrow**Mesenchymal Stromal Cells**

Clonal rat MSC lines were generated by Beverly Torok-Storb at Fred Hutchinson Cancer Research Center. Bone marrow from adult rats was obtained, and buffy coats were plated on plastic adherent tissue culture dishes at 1 to 2×10^6 cells/mL. Primary adherent cells were exposed to LXSN-16 E6E7 retrovirus for 2 hours and $4 \mu\text{g/mL}$ polybrene (BEV ref). Virus containing media was removed, and adherent cells were incubated with media containing polybrene for an additional 5 hours.

All clones expressed typical MSC markers: CD105, CD73, CD90, CD166, and CD146. Bone marrow MSC did not express hematopoietic markers CD45, CD14, and CD34. Mesenchymal stem cell Adipogenic and Osteogenic differentiation kits were used to assess the differentiation potential of all MSC clones (Trevigen, Gaithersburg, Md).

Conditioned Media Infusions

Seven clones were investigated in this study. All MSC lines differ in morphology, function, surface phenotype, and gene expression. Media used to grow these cells included Roswell Park Memorial Institute, 5% Pen/Strep, and 10% fetal bovine serum.

Different clonal rat MSC lines were expanded between passages 6 and 12, and conditioned media were collected for in vivo and in vitro experiments. Conditioned media were centrifuged at 1200 rpm for 10 minutes at 4°C to remove any cells and debris. A final volume of 200 μL was infused intratracheally in experimental rodents. Endotoxin studies on MSC-CM were performed using a Pierce LAL chromogenic endotoxin quantitation kit (ThermoScientific, Carlsbad, Calif).

Ischemia–Reperfusion Model

Pathogen-free Long-Evans rats (weight, 250–275 g) were used for all experiments. Rats were anesthetized with 2.5% isoflurane, and a 14-gauge angiocatheter was inserted into the trachea through a midline neck incision. Rats were connected to a CWE ventilator (CWE Inc, Ardmore, Pa), and settings were maintained at an inspired oxygen content of 60% with a positive end-expiratory pressure of 2 cm H₂O and respiratory rate of 80 breaths/min.

A left thoracotomy was performed, and the left lung was mobilized atraumatically. Heparin (50 units) was administered through the penile vein. After 5 minutes of circulation, a noncrushing clamp was placed across the left lung hilum. The clamp was removed after 90 minutes, and the left lung was reventilated and reperfused for 4 hours. A midline laparotomy and sternotomy were performed, and animals were euthanized by aortic transection. The heart–lung block was excised, and the pulmonary circulation was flushed with 20 mL of phosphate-buffered saline (PBS).

Mesenchymal Stromal Cell–Conditioned Media**Treatment Protocol and Experimental Groups**

Ten cohorts were studied. Negative control animals did not undergo pretreatment or surgical manipulation. Animals in the positive control groups received media only before IR. The remaining 7 cohorts received MSC-CM from different MSC clones. Conditioned media were infused intratracheally 30 minutes before undergoing IR.

Lung Permeability Index

Animals received Evans blue dye at a dose of 20 mg/kg of body weight intravenously 30 minutes before the end of the reperfusion. After the reperfusion, a midline abdominal incision was performed and the abdominal aorta and vein were severed. The left ventricle was vented with a small incision at the apex of the heart. The mitral apparatus was dilated with the left atria using a 14-gauge cannula passing through the mitral valve and into the left atrium to allow free flow of effluent blood from the lung. The pulmonary vasculature was flushed by injecting 10 mL of PBS with a 20-gauge cannula from the pulmonary artery. The left lung was excised and snap-frozen in liquid nitrogen. The frozen lung was homogenized in 2 mL of PBS, diluted with 4 mL of formamide, and incubated at 60°C for 24 hours. The homogenate was centrifuged at 8000 rpm for 5 minutes at room temperature. The supernatants were collected and measured by spectrophotometry at 620 nm.

Histology

Lungs from all experimental groups were biopsied and fixed in 4% paraformaldehyde. After fixation, lungs were embedded into paraffin, cut into 5- μm sections, and stained with hematoxylin–eosin.

Immunohistochemistry

Immunohistochemistry and histology were performed by the University of Washington Histopathology Shared Resource. Five-micron sections were cut, deparaffinized, and rehydrated in Dako Wash Buffer (Dako, Carpinteria, Calif). Slides were antigen retrieved in a Black and Decker (Baltimore, Md) steamer for 20 minutes in preheated Trilogy buffer (Cell Marque, Hot Springs, Ariz) and cooled for 20 minutes. Slides were rinsed 3 times in wash buffer, and all subsequent staining steps were performed at room temperature using the Dako Autostainer. Endogenous peroxidase activity was blocked using 3% H₂O₂ for 8 minutes followed by protein blocking. Slides were blocked in 15% goat serum and 5% canine serum in tris-buffered saline containing 1% bovine serum albumin for 10 minutes. Antibodies were used at 10 $\mu\text{g/mL}$ and incubated for 60 minutes and were detected using biotinylated goat anti-rat (112-065-167, Jackson ImmunoResearch Laboratories Inc, West Grove, Pa) at 1:200 for 30 minutes followed by Vector Elite ABC (Vector Laboratories, Burlingame, Calif).

FIGURE 1. Evaluation of lung injury. A, Vascular permeability measured by Evans blue dye in lung tissue. Groups were compared by Student *t* test. B, Proinflammatory cytokines IL-1 β , tumor necrosis factor- α , and anti-inflammatory IL-10 were measured in BAL supernatant by enzyme-linked immunosorbent assay. Group comparisons were analyzed by Student *t* test. *EBD*, Evans blue dye; *IR*, ischemia–reperfusion.

The staining for all slides was visualized with 3,3'-diaminobenzidine (Dako) for 8 minutes, and the sections were counterstained with hematoxylin (Dako) for 2 minutes. Concentration-matched isotype control slides were run for each tissue sample (Jackson ImmunoResearch Laboratories Inc).

Bronchoalveolar Lavage Procurement and Cell Counts

After 4 hours of reperfusion, a clamp was placed across the right hilum, and the left lung was lavaged with sterile saline. The recovered fluid was centrifuged at 1800 rpm for 10 minutes at 4°C. The supernatant was collected from each sample and assessed for cytokines. The cell pellet was resuspended and lysed for red blood cells. Cells were also washed at 1200 rpm for 10 minutes and resuspended in 1 mL of PBS. Total cell counts were assessed using a hemacytometer.

Bronchoalveolar lavage (BAL) samples were centrifuged at 1200 rpm for 10 minutes at room temperature, and cells were resuspended in 500 μ L of PBS. Cytospins were prepared from cells isolated from BAL and stained in Diff-Quik (Fisher Scientific, Pittsburgh, Pa) to assess for neutrophils, macrophages, and lymphocytes.

Protein Analysis for Cytokines

BAL supernatants were processed and collected as described earlier. Supernatants were analyzed using sandwich enzyme-linked immunosorbent kits (Thermoscientific, Carlsbad, Calif) for interleukin (IL)-10, tumor necrosis factor- α , and IL-1 β . Standards and samples were run in duplicate, and well-to-well variation did not exceed 5%.

Statistical Analysis

Graphical representations of the data are expressed as the mean $\pm$ standard error of the mean. Comparisons between 2 groups were made using the unpaired Student *t* test. All *P* values are 2 sided. No adjustment has been applied for multiple comparisons. The “Results” section includes median and range for datasets.

RESULTS

Effects of Mesenchymal Stromal Cell–Conditioned Media on Lung Ischemia–Reperfusion Injury

We evaluated the development of lung IR after pretreating with conditioned media. Of the 7 MSC-CM clones, 5 demonstrated some ability in reducing lung injury severity (Figure 1, A) compared with control rats receiving media only ($n = 15$) or SHAM ($n = 15$). Clones O ($n = 10$) and H ($n = 10$) showed the most significant reduction in lung injury at a median of 0.086 (0.0510–0.0920) by measure of lung permeability ($P = .0021$ and $P = .028$, respectively). Three clones demonstrated some reduction in lung injury severity but not to the degree seen with clones O and H. Clones N and E did not significantly reduce lung injury compared with controls ($P = .177$ and $P = .1126$, respectively).

FIGURE 2. Histopathologic assessment of lung injury. Hematoxylin–eosin-stained lung histology from representative animals. Lung sections were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5- μ m sections before staining. Pictures were taken using an Olympus BX51 microscope (Melville, NY) with a 20 $\times$ objective.

To further evaluate lung injury, cytokines were measured from BAL. Levels of IL-1 β ($n = 10$; median, 313.5, range, 55.6–486) and tumor necrosis factor- α ($n = 10$; median, 388.8; range, 55.1–644) were significantly reduced in animals pretreated with protective MSC-CM compared with media only ($n = 15$) and SHAM ($n = 15$) controls ($P < .0001$, $P < .0001$, respectively) (Figure 1, B). IL-10 levels were unchanged in the treatment groups that demonstrated protection against lung injury at a median of 7.25 (0.32–12) ($P = .1431$).

Histologic assessments of lung injury from IR control groups showed evidence of significant lung injury evident by a substantial increase in neutrophilic infiltrates, hemorrhage in bronchioles, and thickening of lung parenchyma. Lung biopsies taken from animals who received protective MSC-CM showed minimal infiltrates and tissue damage (Figure 2).

Mesenchymal Stromal Cell–Conditioned Media Pretreatment Significantly Reduced Inflammatory Cells to Lung Tissue

BAL cell analyses were performed on the same controls and experimental groups as previously described. Total cell counts from experimental groups conferring protection

(median, 9500; range, 7000–20,000) were not statistically significant when compared with controls (Figure 3). Absolute neutrophil counts from protected animals were significantly reduced at a median of 46,740 (6400–78,000), with macrophage (median, 76,950; range, 17,500–144,800) and T-cell counts (median, 6450; range, 2460–9500) significantly increased ($P = .0003$, $P = .025$, $P = .0043$, respectively) compared with controls.

Immune Cell Subsets in Mesenchymal Stromal Cell–Conditioned Media Conferred Protection

To better characterize the changes in cellular infiltrates that were associated with protection, immunohistochemistry was performed on lung sections taken from control and experimental groups. Samples were assessed for expression of MAC2, CD3, and FoxP3. Lung biopsies from animals receiving protective MSC-CM showed a significant increase in M2-like macrophages markers and a significant increase in overall FoxP3 expression (Figure 4), a marker for regulatory T cells.¹⁴

DISCUSSION

In this study, we demonstrate that pretreatment with conditioned media from bone marrow–derived MSCs

FIGURE 3. Cellular responses after pretreatment with conditioned media. BAL differentials were analyzed in the control and experimental groups. Samples were assessed for total leukocytes (A), neutrophils (B), macrophages (C), and lymphocytes (D). Total BAL cell counts were performed using a hemocytometer. *IR*, Ischemia–reperfusion.

confers protection in lung IR injury. Protection appears to be driven by a reduction in proinflammatory cytokine production, a decrease in infiltrating inflammatory cells, and increases in M2-like macrophages and regulatory T-cell subsets within the lung tissue. These data provide

evidence that paracrine factors in MSC-CM have beneficial protective effects in vivo by immunomodulating the inflammatory response in resident and infiltrating cell subsets.

Understanding the mechanism of action using MSC-CM has been suggestive but not fully defined. Paracrine factors

FIGURE 4. Immunomodulatory properties of MSC-CM. Immunohistochemistry stains were performed on lung biopsies taken from control and experimental groups. FoxP3, a marker for regulatory T cells, was assessed. Pictures were taken using an Olympus BX51 microscope at 20 $\times$ and 40 $\times$.

secreted by MSCs appear to immunomodulate several different cell types from the innate and adaptive immune system, reducing the production of proinflammatory cytokines and increasing regulatory cell subsets.² Several studies suggest that MSC paracrine factors block neutrophil function by suppressing the oxidative burst of resting and activated neutrophils while preserving their functions.¹⁵ Others suggest MSC-derived exosomes that drive tissue repair, decrease inflammatory mediators, and inhibit apoptotic events.^{16,17}

MSC-based therapeutic approaches seem to be promising in treating various numbers of diseases.^{9-13,18-20} These beneficial effects seem to be driven by multilineage differential potential and paracrine factors influencing various cell types. Effective migration and ability to home to sites of injury play a critical role in the therapeutic effects without engraftment of these cells.^{21,22} Despite positive outcomes, there are several challenges that need to be addressed, including clarification of MSC phenotypes, interactions with host cells, and quantification of the effect of MSCs in the microenvironment. There are also concerns about the safety and efficacy of using MSC-based therapies compared with cell-free MSC-CM. Developing a treatment strategy that uses the beneficial paracrine effects of MSCs without the uncertainties of using cells alone provides a more attractive platform and could eliminate these current challenges.²³

Study Limitations

The limitation of this study is the lack of understanding of how paracrine factors in MSC-CM immunomodulate cells that drive protection. The factors conferring therapeutic benefit will need additional investigation, both in vivo and in vitro, to further characterize and classify.

CONCLUSIONS

MSC-CM-based therapy for lung IR injury shows promise as a potential therapeutic strategy in protecting or reducing severity of lung injury. These data suggest that MSC-CM therapy is a substantial option for clinical translation.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: acute lung graft rejection, lung ischemia-reperfusion injury, mesenchymal stromal (stem) cell therapy, mesenchymal stromal (stem)-conditioned media

Discussion

Dr Hwang. I'm excited to have this opportunity to present some of our preclinical data looking at the use of MSC-CM for inducing ischemic tolerance. This work is in

collaboration with 2 institutions in Washington: Fred Hutchinson Cancer Research Center and University of Washington.

Lung IR occurs in a variety of cases (transplantation, shock, cardiopulmonary bypass), and approximately 25% of lung transplant recipients will experience some degree of graft dysfunction due to lung IR injury. It is a significant cause of morbidity and mortality, and current treatment is supportive. Some of the preclinical studies investigating different therapeutic strategies have included donor macrophage depletion, ischemic postconditioning, human recombinant apyrase therapy, and MSCs, which I'll go into more detail now.

Unfortunately, the term "MSC" has been used loosely in all these studies and has failed to be characterized properly before use. When properly identified and phenotyped, these cells are appealing for cell immunotherapy. MSCs are adherent nonhematopoietic stem cells that are immune privileged and able to vary in differentiation potentials. They modulate the function of host cells through cell-to-cell contact or paracrine mechanisms, so different cytokines and some general human MSC phenotype profiles are positive markers for CS105, CD90, and CD73. They're negative for CD45, CD34, CD14, CD19, CD11b, and human leukocyte antigen-DR. These cells secrete innumerable growth factors, cytokines, and exosomes, and are beneficial in reducing IR in various preclinical and clinical studies.

There are more than 2000 clinical trials using MSC-based therapy in the clinic for a variety of diseases. Despite the overwhelming enthusiasm for using these cells for therapy, there are pros and cons that need to be addressed. Potential benefits are that these cells are immune privileged. They have a low expression of MHC1 and MHC2, which suggests that you can use both autologous and allogeneic stem cell sources. Administration of these cells is safe and with few adverse effects, and they have anti-inflammatory, antifibrotic, antiapoptotic, and proreparative properties. Some of the negatives are that a lot of cells are required to do these therapies. There is not really a good standardized MSC quality control, and continuous in vitro passaging of these cells could result in cell transformation, which hasn't been demonstrated or actually looked at with other studies.

For our preclinical data, we're basically looking at a rat lung IR model as a preclinical study, and we pretreated the rats 30 minutes before IR, so we did it intratracheally, 90 minutes of ischemia and 4 hours of reperfusion. We assessed for injury or lung protection and did several different types of experiments, lung permeability assay, looking for lung injury, cytokine expression by enzyme-linked immunosorbent assay differentials on our BAL, immunohistochemistry, and

flow studies. In the study, we assessed 7 different MSC clones to determine which provided protection after injury, and this figure depicts lung injury data as measured by Evans blue dye, and along the X-axis, we have the different experimental groups, so we have our media IR control, sham, and conditioned media reperfusion, the experimental group. Along the Y-axis is the Evans blue dye arbitrary units, and as you look at this table here, we assessed all the clones, and 5 of the 7 clones that we tested showed some level of protection, whereas 2 clones that are actually depicted up here showed the most protection, so we used those for our further studies. If you compare it with the media IR control, it is significant. For lung histology, the lung tissue samples from 3 groups are represented here. We have the injured animals treated with the control media, and then clone H again and clone L, which are the 2 most protective clones that we used in these studies, and you can see there is significant infiltrate in the media IR group, and then in sharp contrast there is minimal to mild infiltrating cells and damage.

The cytokine profiles were assessed after injury from control and experimental animals by enzyme-linked immunosorbent assay for tumor necrosis factor- α , IL-1 β , and IL-10. Along the X-axis, we have picograms per milliliter, and these are the experimental groups that include the positive control, which is our media IR, the sham control, and our conditioned media IR, which again includes the 2 clones that are most protective, clones L and H.

In all the experiments, for tumor necrosis factor- α and IL-1 β , which are markers for lung injury, you can see there is a significant reduction in both cytokines after pretreating with conditioned media and during lung injury, but most interesting is that the IL-10 level is not elevated in these animals, which suggests that the mechanism of protection in this model is not solely driven by IL-10, which is unlike other studies for lung IR or any type of acute lung injury.

We assess BAL differentials to assess this cellular response after pretreatment with conditioned media in lung injury. The differentials were performed on the BAL, and along the Y-axis, we have total cell counts, and along the X-axis, again, we are looking at the same 3 groups, the media IR, which is the positive control, sham, which is the negative control, and conditioned media after injury, nondistended as you can see. Total leukocyte counts for all of these groups were not different, but neutrophil counts, a measure of lung injury, are significantly reduced in the conditioned media IR group, which is the flip side. There is a substantial increase in macrophages and lymphocytes after pretreatment. To further characterize and understand the potential mechanisms, and the observed shift in the macrophage and lymphocyte populations, we

assayed for CD163, FOXP3, SOX1, SOX3, and inducible nitric oxide synthase expression in infiltrating cells. CD163 and SOX1 are markers for regulatory macrophages, SOX3 and inducible nitric oxide synthase are markers for inflammatory macrophages, and FOXP3 for this group is a transcription factor for regulatory T cells. MSC treatment was associated with a marked increase in FOXP3 expression, and CD163 and SOX1 expression were associated with reduced levels of M1 or inflammatory macrophages after treatment. This suggests that protection is driven by global polarization by immune-modulating residential and infiltrating cells to a regulatory.

The current study showed that pretreatment with MSC media confers ischemic tolerance and that this protection is characterized by a reduction in protocol-inflammatory cytokines and total infiltrating neutrophils, along with substantial increases in macrophages and lymphocytic populations. These changes are characterized by shifts toward an M2 phenotype and recruitment of regulatory T cells, and its enrichment of regulatory subsets after pretreatment has potential for significant clinical impact in the field of lung transplantation, wherein acute morbidity would be reduced in the development of donor-specific tolerance and long-term acceptance would be promoted.

Dr R. Bremner (*Phoenix, Ariz*). You've designed the experiment using this IR model in a rat to evaluate the protective effects of the supernatant from 7 different clones, not the cells themselves, but the supernatant from these clones. You've shown that at least 2 of these clones have a supernatant that can have a profound effect, and looking at the histology, it does look profound. Three of them maybe, and 2 of them not, so I'd caution you to say or to conclude that the supernatant from all of these mesenchymal stem cells has this effect, but clearly there's something exciting here. Unfortunately, you don't provide us with an answer to the begging question of what this magic stuff is, but before I get to the question of asking what you think it might be, I have a couple of other questions. When we do these IR experiments and evaluate the organ, especially in the era of ex vivo lung perfusion, we use a couple of surrogates, such as total lung weight to estimate lung water, and we also look at pulmonary venous gases. Were you able to do that in any animals to just get a functional response of what these inflammatory predictors were?

Dr Hwang. No, but there are studies looking at lung permeability or lung Evans blue dye, and correlating directly with lung weights as well as blood gases, so it's a good measure.

Dr Bremner. So you use the BAL as a surrogate like we do in clinical studies of what's actually happening in the lung, but you actually had the lung itself, so why didn't you use some of the lung to look at some of the cytokines and the cell counts rather than just the BAL?

Dr Hwang. Right now, we're trying to expedite the process and generating some clinical data, so we didn't do some of those lung assays downstream, but we will revisit and look at cytokine profiles for the lungs themselves.

Dr Bremner. You used immunohistochemistry to subtype some of these macrophages and Treg cells, but I would recommend that you do flow cytometry. This would probably give you something more definitive, but I suspect you're probably already doing it.

Dr Hwang. Correct. We have done a lot of flow studies, and we have not actually reported them yet, so they will be upcoming.

Dr Bremner. What do you think this supernatant "stuff" is and why do you think the profound effect was only in 2 of these clones? Are these cytokines or is this more an exosomal issue?

Dr Hwang. We are in the process of trying to figure out what is in the magic cocktail, but we are thinking it's more exosomal driven, and right now that's a hot topic in terms of translational medicine, but we can't discount the cytokines that could be in there, and if we even extrapolate 1 single component, it might be that we find out downstream that we need multiple components for this to work, so it's not a singular, but it's a clear tropic effect.

Discussant. Is the conditioned media better than the cells themselves?

Dr Hwang. Yes, in this case. We did experiment side by side using the MSCs from the same clones and the conditioned media, and definitely there's more of an effect with the conditioned media itself.

Dr F. (Colo). When you looked at the macrophages and other organ systems, the M2 conversion is a bad thing. Do you think this is mainly blunting the neutrophils coming in or is there a way that you can add something that blunts the macrophage well and it may accentuate what you're saying in terms of protection?

Dr Hwang. We're still early in our studies to understand the balance among the M2s, Tregs, M1s, and neutrophil infiltration, so right now we can conclude that we always see a lot of regulatory subsets from both the innate and the adaptive immune systems, but we can't allude further on down the road yet.

Dr F. What conditions are you growing the clones in? Some studies I looked at are growing them in the hypoxic conditions that enhance some of the factors that are expressed. Are you doing that?

Dr Hwang. The conditions are basic for growing these cells. There's no hypoxic conditioning or additives, things like that. The cells are almost weaned off of serum so we don't have to worry about that downstream, endotoxin-free, things like that, so no special condition.

Dr F. How about some of the advantages of using the acellular approach. For the cellular approach, did you

look at the length of how long these effects last and the cells won't be there?

Dr Hwang. That would be the next step. We would do some survival studies to see how long these effects would be, and then eventually we would like to go into more of

a larger animal model, dog studies, which we have a lot of experience in, and take those lungs that have been conditioned with the special cocktail and use those in a 1-transplant setting to see if that maintains some level of tolerance long-term.

EDITORIAL COMMENTARY

How do cells talk to each other?: Paracrine factors secreted by mesenchymal stromal cells

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Mesenchymal stromal cells (MSCs) are the subject of several clinical trials. In parallel with their major role as undifferentiated cell reserves, MSCs have immunomodulatory functions that are exerted by direct cell-to-cell contacts, by secretion of cytokines, and/or by a combination of these. Early hypotheses suggested that MSCs caused cell replacement and differentiation as their main therapeutic mechanism of action.

Evidence is mounting that secretions of MSCs are responsible for their therapeutic effects. These secretions include molecules and extracellular vesicles that have both local and distant effects—loosely referred to as “paracrine” properties. Paracrine signaling is a form of cell-to-cell communication in which a cell produces a signal to induce changes in nearby cells, altering the behavior or differentiation of those cells. Paracrine factors secreted by stem cells can do the following: induce surrounding cells to differentiate into mature cell lines; modulate inflammatory or reparative processes in surrounding tissues; and play an important role in the actions of the stem cells that secrete them, particularly MSCs. These factors allow 1-way conversations between stem cells and more-differentiated cells.

The bone marrow contains many types of cells. Among them are blood stem cells (also called hematopoietic stem cells [HSCs]) and various types of stem

cells belonging to a group called mesenchymal cells. Only about 0.01% of the cells in the bone marrow are MSCs. Obtaining a mixture of mesenchymal cell types from adult bone marrow, for research, is fairly easy. But isolating the tiny fraction of cells that are MSCs is more complicated. Some cell clones in the mixture may be able to form bone or fat tissues, for example, but still do not have all the necessary properties to be called MSCs.

The challenge is to identify and pick out the cells that can both self-renew (produce more of themselves) and differentiate into 3 cell types—bone, cartilage, and fat. Multiple studies in humans and rodents converged to identify the nonhematopoietic stem cells in the bone marrow (widely called “mesenchymal” stromal cells or “skeletal” stem cells). The term “skeletal” implies that these stem cells can differentiate into tissues that together make the skeleton (bone, cartilage, and fat). Some confusion occurred about types and origins of various stem cells.

Immunomodulation of mesenchymal stem cells.

Central Message

Paracrine factors secreted by mesenchymal stem cells have important physiologic effects on inflammation.

See Article page 841.