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Conditioned media from mesenchymal stromal cells restore sodium transport and preserve epithelial permeability in an in vitro model of acute alveolar injury

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Goolaerts A, Pellan-Randrianarison N, Larghero J, Vanneaux V, Uzunhan Y, Gille T, Dard N, Planès C, Matthey MA, Clerici C. Conditioned media from mesenchymal stromal cells restore sodium transport and preserve epithelial permeability in an in vitro model of acute alveolar injury. Am J Physiol Lung Cell Mol Physiol 306: L975–L985, 2014. First published March 28, 2014; doi:10.1152/ajplung.00242.2013.—Mesenchymal stromal cells (MSCs) or their media (MSC-M) were reported to reverse acute lung injury (ALI)-induced decrease of alveolar fluid clearance. To determine the mechanisms by which MSC-M exert their beneficial effects, an in vitro model of alveolar epithelial injury was created by exposing primary rat alveolar epithelial cells (AECs) to hypoxia (3% O2) plus cytokinin, a combination of IL-1β, TNF-α, and IFN-γ. MSC-M were collected from human MSCs exposed for 12 h to either normoxia or hypoxia plus cytokinin (HCYT-MSC-M). This latter condition was used to model the effect of alveolar inflammation and hypoxia on paracrine secretion of MSCs in the injured lung. Comparison of paracrine soluble factors in MSC media showed that the IL-1 receptor antagonist and prostaglandin E2 were markedly increased while keratinocyte growth factor (KGF) was twofold lower in HCYT-MSC-M compared with MSC-M. In AECs, hypoxia plus cytokinin increased protein permeability, reduced amiloride-sensitive short-circuit current (AS-ENaC), and also decreased the number of α-epithelial sodium channel (α-ENaC) subunits in the apical membrane. To test the effects of MSC media, MSC-M and HCYT-MSC-M were added for an additional 12 h to AECs exposed to hypoxia plus cytokinin. MSC-M and HCYT-MSC-M completely restored epithelial permeability to normal. MSC-M, but not HCYT-MSC-M, significantly prevented the hypoxia plus cytokinin-induced decrease of ENaC activity and restored apical α-ENaC channels. Interestingly, KGF-deprived MSC-M were unable to restore amiloride-sensitive sodium transport, indicating a possible role for KGF in the beneficial effect of MSC-M. These results indicate that MSC-M may be a preferable therapeutic option for ALI.

growth factors; membrane transport; hypoxia; inflammation; mesenchymal stem cells; sodium channels

ACUTE LUNG INJURY (ALI) is a major clinical syndrome manifested by an inflammatory response with injury to the lung endothelium and the alveolar epithelium that leads to acute respiratory failure (53). Although mortality has declined with improved supportive care, especially lung protective ventilation and a conservative fluid strategy, the overall mortality of ALI remains high, ~25–40% (51). No pharmacological therapies have yet been found to improve clinical outcomes in ALI (8), thus underscoring the need for novel therapeutic strategies (40).

There has been a rapidly growing interest in the potential value of stem cell therapies as therapeutic approaches in multiple clinical disorders, including myocardial infarction, diabetes, and hepatic and acute renal failure (34). Moreover, recent studies indicate that bone marrow-derived, multipotent mesenchymal (stem) stromal cells (MSCs) have therapeutic efficacy in experimental models of ALI in adult animals. Intravenous or intra-alveolar administration of MSCs attenuates the severity of lung damage, reduces lung inflammation and fibrosis, and increases survival of rats after bleomycin, endotoxin-induced lung injury, and live bacteria or ventilator-induced injury (34, 41, 44). The potential mechanisms through which MSC therapy improves lung function include lung engraftment, anti-inflammatory, or immunomodulatory functions, and antiapoptotic effects (1, 19, 27, 28). However, although MSCs delivered by the intravenous route engraft in mice lungs after bleomycin injury (44), other reports have shown that engraftment of MSCs in the lung is very low (<5%) (9, 21). Interestingly, most studies reported striking protective effects of MSC therapy despite low engraftment rates, supporting the concept that the protective effects may be largely mediated through production of paracrine mediators that constitute the MSC secretome (29, 33, 43). This assumption has been confirmed by another recent study reporting that the cultured medium of MSC themselves can reverse hypoxia-induced lung injury in a perinatal mouse model (4).

The direct delivery of MSCs for treating acute respiratory distress syndrome (ARDS) is reasonable to test for safety and efficacy in the clinical setting, but if the cultured media of MSCs were as effective as the MSCs themselves, it would be a simpler treatment that might have fewer potential side effects that
may be associated with cell-based therapy. Moreover, in the context of ALI, these cells are exposed to acute inflammation and hypoxic conditions with unknown consequences on their secretome. Under resting conditions, MSCs secrete several soluble factors that may reduce ALI (11, 23, 33). Based on previous studies, we and others identified potential soluble factors that could be responsible for the observed beneficial effects, keratinocyte growth factor (KGF) (23, 32), IL-1 receptor antagonist (IL-1ra) (43), and prostaglandin E2 (PGE2) (42). These three paracrine products of MSCs modulate alveolar ion and fluid transport and lung barrier permeability or acute lung inflammation, important pathways of lung injury (17, 34, 42). The two objectives of this study were 1) to test the impact of the alveolar environment of acute inflammation and hypoxia on the phenotype of MSCs in terms of apoptosis and secretion of three mediators, IL-1ra, PGE2, and KGF; and 2) to assess the therapeutic efficacy of MSC-conditioned media on the injured cultured alveolar epithelial cells (AECs) and determine the mechanisms that explain how the conditioned media restored the vectorial sodium transport in an in vitro model of acute alveolar injury.

METHODS

Extraction, Culture, and Conditioning of Human MSCs

Bone marrow samples were harvested from washed filters used during bone marrow graft processing for allogeneic transplantation after healthy donor informed consent according to approved institutional guidelines (Assistance Publique–Hôpitaux de Paris, Paris, France). MSCs were cultured as previously described (3). Briefly, healthy donors bone marrow cells obtained after Ficoll (Invitrogen, Cergy-Pontoise, France) were cultured at an initial density of 5 × 10⁴ cells/cm² in minimum essential medium-α (Invitrogen), supplemented with 10% defined fetal calf serum (DFBS; HyClone, Logan, UT), 2 mM l-glutamine (Invitrogen), 1 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Lille, France), and antibiotic/antimycotic (Invitrogen). After 24–48 h, nonadherent cells were removed and the medium was changed. The media were changed every 2 or 3 days until confluence was achieved. Adherent cells were then trypsinized, harvested, and cultured by seeding 0.5 × 10⁶ cells/cm².

At confluence, the MSC medium was changed and replaced by DMEM medium containing 10% fetal bovine serum. To test the effect of inflammation on MSC secretion, MSCs were exposed to hypoxia (3% O₂) plus 20 ng/ml of cytokin. Cytokin is a mixture of IL-1β, TNF-α, and INF-γ (R&D Systems), which are major proinflammatory cytokines in the pulmonary edema fluid from patients with ARDS (31). After 12 h, the media of MSCs exposed to cytokin plus hypoxia (HCYT-MSC-M) or the media of control MSC (MSC-M) were harvested and rapidly centrifuged (10 × 10³ rpm). The aliquots were stored at −80°C.

Measurements of Soluble Mediator Concentrations in MSC Supernatants

MSCs between passages 2 and 5 were cultured on sixwell plates until 85–90% confluence was achieved. After exposure to experimental conditions, the supernatants were harvested and immediately centrifuged. The concentrations of soluble mediators, IL-1ra, PGE2, and KGF were measured by ELISA (R&D Systems). KGF (FGF-7) was measured with a twofold sample dilution factor, PGE2 was measured with a threefold sample dilution factor, and IL-1ra was measured without sample dilution as indicated by manufacturer’s instructions.

Determination of MSC Phenotype

Monoclonal antibodies conjugated with either fluorescein isothiocyanate or phycoerythrin (Becton Dickinson, Le Pont de Clai, France) and directed to CD29, CD95, CD105, and CD105a to CD105e, or matched isotype control were used for immunophenotyping, according to the manufacturer’s protocol. Data were acquired and analyzed on a five parameters flow cytometer (FACS Calibur; Becton Dickinson) with Cell Quest software.

Detection of MSC Apoptosis and Necrosis

MSC apoptosis and viability were determined at passage 4 in cells exposed to normoxia or to cytokin (20 ng/ml) plus hypoxia (3% O₂) for 12 h using the annexin V (FITC)-7AAD kit (Beckman Coulter, Roissy Charles de Gaulle, France). Briefly, cells were collected and resuspended in binding buffer. Annexin V-FITC and propidium iodide were added, and the reaction was incubated in the dark for 15 min. Cells were analyzed by flow cytometry using FACSscan flow cytometer.

Isolation and Culture of Rat AECs

The procedure of alveolar type II cell isolation from pathogen-free male Sprague-Dawley rats was performed with legislation currently in force in France and animal welfare guidelines (Ministère de la Pêche et de l’Agriculture, Agreement 5669). Alveolar type II cells were isolated from adult rats (200–250 g) by elasate digestion of lung tissue followed by sequential filtration and differential adhesion on bacteriological dishes as previously described (46). Cells (purity >90%; viability >95%) were seeded either onto Transwell/Snapwell (polycarbonate membrane with a pore size of 0.4 μm; Costar, Cambridge, MA) filters or onto 6- or 12-well plastic culture dishes and cultured in a 5% CO₂, 95% air atmosphere in DMEM containing 25 mM D-glucose, 10 mM HEPES, 23.8 mM NaHCO₃, 2 mM l-glutamine, 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml gentamycin. Transepithelial resistance (Rₑ) and transepithelial potential difference were measured on day 4 using a microvoltometer (World Precision Instruments, Astonbury, UK). Snapwell filters with Rₑ <300 Ω/cm² were discarded.

Experimental Protocols

Freshly isolated AECs were seeded on polycarbonate Transwells (Costar, Cambridge, UK) and grown for 4 days at 37°C (5% CO₂) in a liquid-liquid interface. When the AECs reached confluence, one of four conditions were applied.

Protocol 1. The media were replaced by fresh media only, and the AECs were incubated in normoxia (21% O₂-5% CO₂-74% N₂). After 6 h, the media were replaced by fresh media and AECs were incubated for an additional 12 h in normoxia.

Protocol 2. The medium was replaced by media containing 20 ng/ml cytokin, and the AECs were placed in a box flushed with a hypoxic gas (3% O₂-5% CO₂-92% N₂). After 6 h, the media of the AECs were removed and replaced by DMEM plus 20 ng/ml cytokin and AECs were incubated in hypoxia for an additional 12 h.

Protocol 3. The media were replaced by media containing 20 ng/ml cytokin, and AECs were placed in a box flushed with a hypoxic gas (3% O₂-5% CO₂-92% N₂). After 6 h, the media of the AECs were removed and replaced by MSC-M plus 20 ng/ml cytokin and cells were incubated in hypoxia for an additional 12 h.

Protocol 4. The media were replaced by media containing 20 ng/ml cytokin, and the AECs were placed in a box flushed with a hypoxic gas (3% O₂-5% CO₂-92% N₂). After 6 h, the media of the AECs were removed and replaced by HCYT-MSC-M (cytokin was not added in this protocol since HCYT-MSC-M already contained cytokin) and cells were incubated in hypoxia for additional 12 h.
Measurement of epithelial permeability to protein across AECs

Measurements of protein epithelial permeability form apical to basolateral side of AECs were done 4 days after seeding, as previously described (16). AECs were exposed to normoxia or to 20 ng/ml cytomix plus hypoxia (3% O2) for 6 h and then the media were removed and replaced by 1) DMEM alone and AECs were exposed to normoxia; 2) DMEM plus cytomix (20 ng/ml) and AECs were exposed to hypoxia (3% O2); 3) media of nonexposed MSC (MSC-M) plus cytomix (20 ng/ml) and AECs were exposed to hypoxia (3% O2); and 4) medium of MSC exposed to cytomix plus hypoxia (HCYT-MSC-M) and AECs were exposed to hypoxia (3% O2). Epithelial protein permeability was then assessed by adding labeled 0.3 μCi/ml 125I-albumin only to the upper compartment, at the time of replacement of the media, and the unidirectional flux to the lower compartment was measured over 12 h. There is no hydrostatic pressure gradient between the two compartments. Aliquots of media containing 125I-albumin were then retained as references for initial radioactivity (R0).

Twelve hours later, apical and basolateral media were collected and medium samples were placed into assay tubes for γ counting. The radioactivity expressed as counts per minute was normalized by the weight of the samples. The epithelial permeability was then calculated as follows: (Rbas/R0) × 100 = epithelial permeability to albumin over 12 h (%). AEC monolayers with baseline permeability >2% were not used for experiments.

Measurement of bioelectrical properties of AECs. Spontaneous potential difference (PD) and transepithelial resistance (Rte) across AEC monolayers were measured using an epithelial voltmeter equipped with chopstick-style electrodes (World Precision Instruments, Astonbury, UK). Equivalent short-circuit current (Isc) was calculated from Ohm’s law (Isc = PD/Rte).

Measurement of short circuit current (Isc), transepithelial potential difference, and transepithelial resistance were performed in AECs grown 5 days after seeding, as previously described (46). Snapwell inserts were mounted in vertical diffusion chambers and were bathed with Ringer solution (pH 7.4) continuously bubbled in 5% CO2, 95% air at 37°C. The apical and basolateral chambers were filled with 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, and 15 mM glucose, pH 7.4 adjusted with NaOH. The hemi-chambers were connected to a VCC MC6 voltage clamp (Physiologic Instruments, Colorado Springs, CO) and recorded in Chart software (AD Instruments). Twelve hours later, apical and basolateral media were collected and medium samples were placed into assay tubes for γ counting. The radioactivity expressed as counts per minute was normalized by the weight of the samples. The epithelial permeability was then calculated as follows: (Rbas/R0) × 100 = epithelial permeability to albumin over 12 h (%). AEC monolayers with baseline permeability >2% were not used for experiments.

Other experiments were done to measure Na-K-ATPase activity in apically permeabilized AECs. Cells were bathed in an apical and basolateral compartments containing 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, and 15 mM glucose, pH 7.4 adjusted with NaOH. After the steady-state condition was reached, the apical membrane was permeabilized by apical addition of 60 mg/ml nystatin. Ouabain (1 mM) was added to the basolateral bathing solution, and the basolateral ouabain-sensitive current (representing the fall in Isc evoked by ouabain) was measured to estimate the extrusion capacity of the basolateral Na+ pump.

Western blotting of epithelial sodium channel subunits in AECs

Biotinylation and recovery of apical membrane proteins from cultured AECs were performed as previously described (46, 48). In brief, AECs grown on Transwell filters were placed on ice and washed three times with ice-cold PBS-Ca++-Mg++ (PBS with 0.1 mM CaCl2 and 1 mM MgCl2). Apical membrane proteins were then biotinylated by a 15-min incubation at 4°C with NHS-ss-biotin 1.25 mg/ml (Pierce, Rockford, IL) freshly diluted into biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, and 150 mM NaCl, pH 7.5) with gentle agitation. Alveolar type II cells were rinsed with PBS-Ca++-Mg++-glycine (100 mM) and washed in this buffer for 20 min at 4°C to quench unreacted biotin. Cells were then rinsed twice with PBS-Ca++-Mg++, scraped in cold PBS, and pelleted at 2,000 rpm at 4°C. Pellets were solubilized for 45 min in 20 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris(hydroxymethyl) aminomethane (Tris), pH 7.5] containing protease inhibitors. The lysates were clarified by centrifugation at 14,000 g for 10 min at 4°C, and supernatants were incubated overnight with packed streptavidin-agarose beads (Pierce) to recover biotinylated proteins. The beads were then pelleted by centrifugation, and aliquots of supernatants were taken to represent the unbound, intracellular pool of proteins. Biotinylated proteins were eluted from the beads by heating to 100°C for 5 min in SDS-PAGE sample buffer [containing 10% glycerol, 12.5% 0.5 M Tris-HCl (pH 6.8), 10% 20% SDS, 5% 2-mercaptoethanol, and 2.5% of 0.05% (wt/vol) bromophenol blue]. Samples of biotinylated and nonbiotinylated proteins were resolved through 10% acrylamide gels, electroblotted, electrically transferred to nitrocellulose paper, and subsequently probed for the α- or β-rat epithelial sodium channel (ENaC) subunits. To ensure the absence of leakage of biotin into the cells, we systematically verified the absence of the intracellular protein β-actin in biotinylated extracts. Rabbit polyclonal anti-α-rat ENaC subunit and anti-β-rat ENaC subunit antibodies (14, 46) were used at the dilution 1:2,000, and mouse monoclonal anti-β-actin at the dilution 1:5,000. Quantification of rat ENaC and actin levels was obtained using National Institutes of Health Image software. The expression of γ-ENaC at the cell surface could not be investigated in this study, due to the very faint signal of biotinylated γ-ENaC in native rat AECs.

MSC media with and without KGF neutralization

To determine whether the observed effects of MSC-M were due to the presence of KGF, KGF depletion was performed using protein G/agarose beads coated with anti-KGF monoclonal antibody (R&D Systems) or with anti-IgG antibodies (as a control). MSC-conditioned media were incubated at 4°C overnight on wheel with antibodies-conjugated beads. KGF concentrations in MSC medium were measured by ELISA. AECs cultured for 96 h were exposed or not to hypoxia plus cytomix as previously described and incubated with MSC-M depleted or not from KGF. Cultures were maintained for an additional 12 h, at which point AEC monolayers were used for measurement of Isc. In another set of experiments, the preventive effect of recombinant human KGF (rhKGF) on hypoxia plus cytomix-induced injury was tested. AECs exposed or not to hypoxia plus cytomix were incubated for 12 h with rhKGF (200 and 400 pg/ml) before Isc measurements were performed.
Statistical Analysis

Data are presented as means ± SD (n = 3–5 for flow cytometry experiments, ELISA, transepithelial permeability to albumin determination, and Western blot experiments; n = 4–17 for electrophysiological measurements). Statistical analyses were done by unpaired Student’s t-test for comparison of concentrations of paracrine factors in conditioned media. One-way ANOVAs were performed for all other experiments and, when allowed by the F value, results were compared by the modified least significant difference (Fisher’s protected least significant difference). P < 0.05 was considered significant. The following software was used: StatView (SAS Institute, Cary, NC) and Prism (GraphPad Software, La Jolla, CA).

RESULTS

Exposure to Inflammatory and Hypoxic Environment Did Not Alter MSC Viability and Specific MSC Marker Expression

MSCs were exposed to cytomix (20 ng/ml) plus hypoxia (3% O₂) that is similar to the inflammatory and hypoxic environment during ALI, and they were compared with MSCs exposed to normoxia. Control MSCs as well as MSCs exposed to cytomix plus hypoxia did not express HLA-DR. The expression of the specific MSC markers CD29, CD95, CD105, CD105a, CD105b, CD105c, CD105e, and CD105f was similar in MSC exposed to cytomix plus hypoxia and in those exposed to normoxia (data not shown). Interestingly, exposure to cytomix plus hypoxia did not change the percentage of intact, apoptotic, or necrotic MSCs compared with control cells (Fig. 1). Cell morphology was also similar in both culture conditions.

Proinflammatory and Hypoxic Environment Altered Paracrine Secretion by MSCs

Previous studies have identified several potential soluble factors that could be responsible in part for the beneficial effects of MSCs on reabsorption of alveolar edema fluid in ALI, such as IL-1ra, PGE-2, and KGF (32, 42, 43). Therefore, we focused on the release of these three mediators by MSCs. The secretion was evaluated in control condition (MSC-M) or after exposure of MSCs to cytomix (20 ng/ml) plus hypoxia (HCYT-MSC-M). As shown in Fig. 2, conditioned media from MSCs exposed to cytomix plus hypoxia (HCYT-MSC-M) for 12 h altered their paracrine secretion.
compared with MSC-M. Cytomix plus hypoxia induced the release of IL-1ra from 0 to 134 ± 43 pg/ml and markedly increased the release of PGE2 from 77 ± 25 to 1,295 ± 184 pg/ml. By contrast, cytomix plus hypoxia (HCYT-MSC-M) induced a decrease of KGF secretion from 205 ± 4 to 109 ± 10 pg/ml compared with MSC-M. These results indicated that acute inflammation in an hypoxic environment modified the MSC secretion of soluble mediators. We were unable to detect IL-10 in the conditioned medium of MSC either in MSC-M or HCYT-MSC-M.

**MSC-conditioned Media Prevented the Increase in Albumin Permeability Induced by Cytomix Plus Hypoxia in AEC Monolayers**

During ALI, epithelial permeability to protein is increased and favors alveolar flooding (39). To evaluate whether media of MSCs exposed or not to cytomix plus hypoxia (HCYT-MSC-M or MSC-M) could prevent the increase in epithelial permeability (16), AECs pretreated by cytomix plus hypoxia were exposed to MSC-M or HCYT-MSC-M for 12 h before measurement of epithelial permeability to radiolabeled albumin. Exposure of AECs to hypoxia plus cytomiX increased by threefold epithelial permeability compared with the control condition. Treatment with MSC media for 12 h completely prevented the increase in transepithelial permeability to 125I-albumin (Fig. 3). MSC-M and HCYT-MSC-M had a similar protective effect on AEC permeability to protein.

**MSC-Conditioned Media Prevented the Cytomix Plus Hypoxia-Induced Decrease in Total and Amiloride-Sensitive Transepithelial Na Transport in AECs**

The transepithelial Na transport across AECs was evaluated by the amiloride-sensitive component of \( I_{sc} (A-ISc) \). In control conditions, \( A-ISc \) represented 80% of the total \( I_{sc} \) (Fig. 4A). Hypoxia plus cytomiX decreased total \( I_{sc} \) without change in amiloride-insensitive \( I_{sc} \), so that \( A-ISc \) was reduced by 60% compared with control (Fig. 4B). Addition of MSC-M completely prevented the cytomiX plus hypoxia-induced decrease in total \( I_{sc} \) and in AS-\( I_{sc} \) (Fig. 4A and B) and also increased the amiloride-insensitive component of Na current. By contrast,
HYCNYT-MSC-M had no significant protective effect on total $I_{sc}$ or AS-$I_{sc}$.

To further test the mechanisms, we evaluated both apical and basolateral Na$^+$ conductance by measuring $I_{sc}$ on permeabilized monolayers. In the first series of experiments, permeabilization of the basolateral membrane was done in the presence of an asymmetrical Na$^+$ gradient (140 mM apical vs. 10 mM basolateral). The results showed that hypoxia plus cytomix induced a 60% fall of apical AS-$I_{sc}$ and that MSC-M completely restored the apical AS-$I_{sc}$ current (Fig. 5A). In the second series of experiments, we tested the contribution of the Na$^+$-K$^+$ ATPase after permeabilization of the apical membrane. Figure 5B shows that neither hypoxia plus cytomix nor MSC-M did significantly alter the basolateral ouabain-sensitive current reflecting Na$^+$-K$^+$-ATPase activity.

**MSC-Conditioned Media Restored the Membrane Expression of $\alpha$-ENaC But Did Not Modify $\beta$-ENaC Expression After Exposure to Inflammatory and Hypoxic Conditions**

The effects of hypoxia plus cytomix in the presence or absence of MSC-M on ENaC subunit expression were evaluated (Fig. 6). First, the total protein levels of $\alpha$- and $\beta$-ENaC subunits were determined in AECs exposed to hypoxia plus cytomix in the presence or absence of MSC-M. Figure 6 shows that hypoxia plus cytomix induced no significant changes in the total protein expression of the $\alpha$-ENaC (major band at 85 kDa and minor band at 65 kDa) and $\beta$-ENaC (95 kDa band) subunits and that MSC-M had no effect on the total protein expression of $\alpha$- and $\beta$-ENaC subunits. Second, apical surface biotinylation experiments were performed to evaluate cell surface expression of $\alpha$- or $\beta$-ENaC in AECs exposed to the above experimental conditions. Exposure to hypoxia plus cytomix significantly decreased $\alpha$-ENaC protein expression in apical cell surface and had no effect on $\beta$-ENaC protein expression (Fig. 7). This result indicates that the decrease in apical AS-$I_{sc}$ was partly related to a decrease of $\alpha$-ENaC protein in apical membrane. Incubation with MSC-M completely prevented the decrease of $\alpha$-ENaC membrane expression in AECs exposed to hypoxia plus cytomix.

**KGF-Depleted MSC-M Had No Protective Effect on Hypoxia Plus Cytomix-Induced Decrease in Na Transport in AECs**

To define whether the beneficial effects of MSC-M were related in part to KGF, the MSC-M medium was depleted of KGF using a anti-KGF antibody (IgG were used as control). The concentration of KGF declined from 190 ± 63 to 0 pg/ml in control conditions and after depletion of KGF. KGF-depleted MSC-M had no protective effective on hypoxia plus cytomix-induced decrease in Na transport (Fig. 8A). To test whether KGF was sufficient per se to prevent the hypoxia plus cytomix-induced decrease in Na transport, we finally evaluated the potential effect of rhKGF. As shown in Fig. 8B, incubation of AECs with rhKGF (200 or 400 pg/ml) had no protective effect on hypoxia plus cytomix-induced decrease in Na transport.

**DISCUSSION**

The major findings of this study can be summarized as follows: 1) MSC exposure to an inflammatory and hypoxic environment induced substantial changes in the secretion of paracrine factors known to upregulate Na and fluid transport in AECs: IL-1ra, PGE2, and KGF. 2) The inflammatory and hypoxic insult to AECs induced a threefold increase in transepithelial permeability to albumin and a 60% decrease in the transepithelial sodium transport due to a decreased apical amiloride-sensitive Na uptake without a change in Na$^+$-K$^+$-ATPase activity. This decreased vectorial Na transport was

![Figure 5](https://example.com/figure5.png)  ![Figure 6](https://example.com/figure6.png)

**Fig. 5.** AECs grown on Snapwell filters were exposed to either normoxia or cytomix (20 ng/ml) plus hypoxia (3% O$_2$) for 6 h. Then, the media were replaced for additional 12 h by 1) DMEM alone and AECs were exposed to normoxia (21% O$_2$); 2) DMEM plus cytomix (20 ng/ml) and cells were exposed to hypoxia (3% O$_2$); and 3) media of nonexposed MSC (MSC-M) plus cytomix (20 ng/ml) and cells were exposed to hypoxia (3% O$_2$). A: AECs were mounted into a voltage-clamp system in the presence of a Na$^+$ concentration gradient (mucosal to serosal, 140:10 mM). Addition of nystatin into the basolateral bath increased the $I_{sc}$ to a peak value before amiloride (10 $\mu$M) was added into the apical bath. Apical amiloride-sensitive current is the difference between $I_{sc}$ peak value and $I_{sc}$ value after amiloride addition and reflects epithelial sodium channel (ENaC) activity; B: AECs were then immediately mounted into a voltage-clamp system. After stabilization, nystatin into the apical bath evoked a slowly developing rise in $I_{sc}$ and ouabain (1 mM) was added to the basolateral solution once this response had reached its peak value. Basolateral ouabain-sensitive current representing the fall in $I_{sc}$ evoked by this application of ouabain was measured to estimate the extrusion capacity of the basolateral Na$^+$ pump. The values are the means ± SD of 5–8 filters for each condition. $^*$P < 0.01, compared with control conditions (one-way ANOVA followed by Fisher’s PLSD).
MSCs may be releasing factors responsible for beneficial effect of cell therapy. Because the use of MSCs may carry some risks to the patient (2, 38), the administration of MSC-conditioned medium might provide an alternative therapeutic option for ALI/ARDS. In ALI models, MSCs delivered by either intratracheal or intravenous routes were primarily located in the lung microcirculation and alveoli and therefore are directly exposed to alveolar hypoxia and high levels of proinflammatory cytokines from existing and evolving inflammatory reactions. Therefore, we hypothesized that this inflammatory environment may induce changes in MSCs phenotype that could modify the paracrine secretion profile of MSCs and modulate their capacity to repair damaged epithelium. To evaluate the role of the inflammatory and hypoxic environment on MSCs and their conditioned medium, we exposed MSCs to hypoxia plus cytokix (HCYT-MSC-M and MSC-M) and their conditioned medium, we exposed MSCs to hypoxia plus cytokix (HCYT-MSC-M and MSC-M) and their conditioned medium, we exposed MSCs to hypoxia plus cytokix (HCYT-MSC-M and MSC-M). In contrast, HCYT-MSC-M had no significant effect associated with a decline in α-ENaC subunit expression at the apical membrane. 3) Conditioned media of MSCs exposed or not to hypoxia plus cytokix (HCYT-MSC-M and MSC-M) completely prevented the increase in transepithelial permeability to albumin. 4) MSC-M prevented cytokix-hypoxia-induced decrease of Na transport in AECs, restored amiloride-sensitive apical Na transport, and maintained α-ENaC cell surface expression. In contrast, HCYT-MSC-M had no significant effect on cytokix-hypoxia-induced decrease in Na transport. 5) secretion of KGF by MSCs was required for the protective effect of MSC-M on alveolar epithelial Na transport. This study provides the proof of concept that paracrine factors produced by MSCs in the damaged lung may have beneficial effects in vivo by preventing alveolar flooding.

In recent years, MSCs have emerged as a potential therapeutic modality for several inflammatory states, including ALI. Some studies have demonstrated benefits from intratracheal or intravenous administration of MSCs (4, 21, 41). MSCs attenuate the inflammatory response to LPS and reduce the severity of lung injury. In most ALI models, the engraftment rates of MSCs were low (26, 27, 50) and a therapeutic effect occurred within 24–48 h, suggesting that beyond cell replacement, associated with a decline in α-ENaC subunit expression at the apical membrane. 3) Conditioned media of MSCs exposed or not to hypoxia plus cytokix (HCYT-MSC-M and MSC-M) completely prevented the increase in transepithelial permeability to albumin. 4) MSC-M prevented cytokix-hypoxia-induced decrease of Na transport in AECs, restored amiloride-sensitive apical Na transport, and maintained α-ENaC cell surface expression. In contrast, HCYT-MSC-M had no significant effect on cytokix-hypoxia-induced decrease in Na transport. 5) secretion of KGF by MSCs was required for the protective effect of MSC-M on alveolar epithelial Na transport. This study provides the proof of concept that paracrine factors produced by MSCs in the damaged lung may have beneficial effects in vivo by preventing alveolar flooding.

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in control and treated cells is attested by the cell surface expression of IL-1β
experiments. *P/H11021 biotinylated surface proteins for Experimental Protocols

AECs were immediately processed for apical biotinylation experiments as described in

medium contained higher IL-1ra and PGE2 concentrations (10−
to the hypoxic and inflammatory stimuli, the conditioned
candidates in the MSC-conditioned medium. In MSCs exposed
matory and hypoxic conditions on the secretion of these key
factors including IL-1ra (43), PGE2 (42), IL-10, and KGF (23,
34). Therefore, the present study tested the influence of inflam-
atory and hypoxic conditions on the secretion of these key
candidates in the MSC-conditioned medium. In MSCs exposed
to the hypoxic and inflammatory stimuli, the conditioned
medium contained higher IL-1ra and PGE2 concentrations (10−
and 4-fold, respectively) and lower KGF concentrations com-
pared with MSCs cultured in control conditions. Hypoxia plus
cytomix probably increased IL-1ra and PGE2 secretion because
of the presence of IL-1β in the cytokin (43). Interestingly,
IL-1β is one of the major inflammatory cytokines in pulmonary
edema fluid from patients with ALI/ARDS (18, 22) and IL-1ra
competes with IL-1β for IL-1 receptor binding. In the present
study IL-10 was undetectable in control condition as well as
after hypoxic and inflammatory insults. This result was in
agreement with a prior observation that in ALI, the increase of
factors such as VEGF, IGF-1, and HGF in MSCs exposed to
LPS, TNF-α, or hypoxia (11).

The alteration of alveolar ion transport, lung inflammation,
or/and increased endothelial permeability are well-known fea-
tures of the ALI pathophysiology (17, 34, 42). Previous studies
reported a beneficial effect of MSCs in a model of ALI induced
by intratracheal instillation of Escherichia coli endotoxin with
reduced mortality and less pulmonary edema (21, 23). Similar
results were published in an ex vivo perfused human lung
model, in which MSCs reversed endotoxin-induced lung injury
by restoring alveolar fluid clearance and reducing pulmonary
edema (34). Interestingly, in a model of ventilator-induced
lung injury, a similar beneficial effect was obtained by using
either MSCs or MSC-conditioned medium (23).

In this study, we tested the efficacy of MSC-conditioned
media on the epithelial permeability and the vectorial sodium
transport in an in vitro model of ALI (16). In this in vitro model
of ALI, AECs were exposed to cytokin plus hypoxia for 6 h
before treatment with MSC-conditioned media, harvested ei-
ther from MSCs cultured in normal conditions (MSC-M) or
from MSCs exposed to inflammatory and hypoxic insults
(HCYT-MSC-M). Exposure of AECs to cytokin plus hypoxia
increased paracellular permeability by threefold as previously

Fig. 7. Effect of cytokin plus hypoxia on apical cell surface expression of α- and β-ENaC subunits in alveolar epithelial cells in the presence or absence of MSC-conditioned media. AEC grown on Transwell filters were exposed to either normoxia or cytokin (20 ng/ml) plus hypoxia (3% O2). After 6 h, the media
were replaced for additional 12 h by 1) DMEM alone and AECs were exposed to normoxia (21% O2); 2) DMEM plus cytokin (20 ng/ml) and cells were exposed to hypoxia (3% O2); and 3) the medium of nonexposed MSC (MSC-M) in the presence of cytokin (20 ng/ml) and cells were exposed to hypoxia (3% O2). Then, AECs were immediately processed for apical biotinylation experiments as described in Experimental Protocols before immunoblotting. A: representative
immunoblots of α-ENaC (left) and β-ENaC (right) cell surface, and of intracellular protein expression of β-actin. The validity of surface biotinylation approach
in control and treated cells is attested by the cell surface expression of α- and β-ENaC but not of the intracellular protein β-actin. The quantification of biotinylated surface proteins for α-ENaC (65 kDa) (left) and β-ENaC (right) is shown in B. Quantification of biotinylated α- and β-ENaC signals was obtained using Scion Image software, and the data were normalized for the actin signal in corresponding intracellular extracts. The values are the means ± SD of 4 separate experiments. *P < 0.01, compared with control conditions (one-way ANOVA followed by Fisher’s PLSD).
reported (31) and a 60% decrease in apical to basolateral vectorial Na transport. To determine whether cytomix and hypoxia altered Na entry and/or extrusion, we studied Na transport after permeabilization of AEC basolateral or apical membrane. Exposure of AECs to cytomix plus hypoxia induced a decrease in Na transport that was related to a diminished apical expression of amiloride-sensitive Na channels whereas ouabain-sensitive basolateral Na transport, reflecting Na-K-ATPase activity, remained unchanged. The cytomix plus hypoxia-induced reduction of Na current was related to a reduction of the number of Na channels in the apical membrane as demonstrated by the biotinylation experiments that showed a decrease of α-ENaC apical cell surface expression while total protein content of α-ENaC was unchanged. We and other investigators have reported that short-term exposure to moderate hypoxia decrease Na transport by internalization of ENaC subunits without a change in the total protein content (10, 12, 46). Lee et al. (32) previously showed that 50 ng/ml cytomix for 24 h decreased α-ENaC protein levels in the apical membrane of human AECs in parallel with reduced α-ENaC protein content. The discrepancy concerning the effect of cytomix on ENaC protein total cell content may be explained by the fact that we used a lower concentration of cytomix than Lee et al. In the present study, the decrease in α-ENaC subunits in the apical membrane likely results from additive effects of hypoxia plus cytomix on ENaC subunit trafficking.

The most significant finding of this study is that MSC-conditioned medium restored normal epithelial permeability to protein and prevented the decrease in transepithelial Na transport. Interestingly, HCYT-MSC-M was as potent as MSC-M for preventing the decrease in transepithelial albumin permeability, suggesting that paracrine factors released by MSC exposed to the inflammatory milieu of ALI/ARDS could protect in vivo the lungs from flooding. MSC-M (but not HCYT-MSC-M) also restored cytomix plus hypoxia-induced decrease of transepithelial Na transport. From a biochemical standpoint, MSC-M restored the cell surface expression of α-ENaC, suggesting that the beneficial effect of MSC-M on vectorial Na transport was due, at least in part, to an effect on ENaC trafficking. Another explanation would be that MSC-M somehow favored the activation of ENaC channels at the cell surface by membrane-bound serine proteases (such as channel activating proteases) (47). For instance, MSC-M may prevent the inactivation of these proteases by reactive intermediates known to be present in bronchoalveolar lavage during ALI/ARDS (30). In addition, it is possible that MSC-M also stimulated apical Na transporters distinct from ENaC [such as the cyclic nucleotide gated (CNG) cationic channels (25)], inasmuch as it increased the amiloride-insensitive component of Na transport in injured AECs. However, the increase in amiloride-insensitive currents could also be due to a decrease in the sensitivity of Na channels to amiloride, as previously reported (24, 49).

Since PGE2 and IL-1ra concentrations were low in MSC-M compared with HCYT-MSC-M, the higher potency of MSC-M to restore the Na transport under hypoxia plus cytomix seemed likely to be mediated by KGF. This result is remarkable because PGE2 is known to increase the intracellular cAMP, a key nucleotide for the stimulation of Na transport (13, 15, 46). In line with our results, prior studies in rats and sheep showed that PGE2 had no influence on alveolar liquid clearance (5).

![Graph A](http://example.com/graphA.png)

**Fig. 8.** Effect of MSC-conditioned media depleted of KGF and of recombinant human KGF (rhKGF) on the cytomix plus hypoxia-induced decrease in Na transport. A: AECs grown on Transwell filters were exposed to either normoxia or cytotoxic (20 ng/ml) plus hypoxia (3% O2). After 6 h, the media were replaced for additional 12 h by 1) DMEM alone and AECs were exposed to normoxia (21% O2); 2) DMEM plus cytomix (20 ng/ml) and cells were exposed to hypoxia (3% O2); 3) the media of nonexposed MSC (MSC-M) in the presence of cytomix (20 ng/ml) plus IgG and cells were exposed to hypoxia (3% O2); and 4) the media of nonexposed MSC (MSC-M) depleted of KGF as described in Experimental Procedures in the presence of cytotoxic (20 ng/ml) and cells were exposed to hypoxia (3% O2). \( I_{\text{en}} \) was measured before and after addition of amiloride (10 μM) was added into the apical bath. Amiloride-sensitive \( I_{\text{en}} \) represents the difference of \( I_{\text{en}} \) before and after addition of amiloride. The values are the means ± SD of 8–14 filters for each condition. **P < 0.01, compared with control conditions; §P < 0.05, compared with cytotoxic plus hypoxia; ***P < 0.005, compared with cytotoxic plus hypoxia in presence of MSC-M plus IgG (one-way ANOVA followed by Fisher’s LSD). B: after a 6 h exposure to normoxia (white bars) or cytotoxic plus hypoxia (black bars), AECs were treated with rhKGF (200 or 400 pg/ml) or vehicle for additional 12 h and amiloride-sensitive \( I_{\text{en}} \) was calculated. Values are means ± SD of 8–17 filters for each condition. ***P < 0.001, compared with normoxia corresponding value (one-way ANOVA followed by Fisher’s LSD).

<table>
<thead>
<tr>
<th>Condition</th>
<th>( I_{\text{en}} ) (μA/cm²)</th>
<th>( % ) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytomix + 3% O₂</td>
<td>9.2 ± 1.3</td>
<td>100</td>
</tr>
<tr>
<td>MSC-M + IgG</td>
<td>6.5 ± 0.8</td>
<td>71</td>
</tr>
<tr>
<td>MSC-M + anti-KGF</td>
<td>6.3 ± 1.2</td>
<td>69</td>
</tr>
</tbody>
</table>

![Graph B](http://example.com/graphB.png)

**Graph B** shows the effect of rhKGF on apical Na transport in AECs exposed to cytotoxic plus hypoxia. A: After 6 h, AECs were treated with rhKGF (200 or 400 pg/ml) or vehicle for additional 12 h and amiloride-sensitive \( I_{\text{en}} \) was calculated. Values are means ± SD of 8–17 filters for each condition. ***P < 0.001, compared with normoxia corresponding value (one-way ANOVA followed by Fisher’s LSD).

**Table.** Effects of MSC-conditioned media and recombinant human KGF (rhKGF) on apical Na transport in AECs exposed to cytotoxic plus hypoxia. A: AECs grown on Transwell filters were exposed to either normoxia or cytotoxic (20 ng/ml) plus hypoxia (3% O2). After 6 h, the media were replaced for additional 12 h by 1) DMEM alone and AECs were exposed to normoxia (21% O2); 2) DMEM plus cytomix (20 ng/ml) and cells were exposed to hypoxia (3% O2); 3) the media of nonexposed MSC (MSC-M) in the presence of cytomix (20 ng/ml) plus IgG and cells were exposed to hypoxia (3% O2); and 4) the media of nonexposed MSC (MSC-M) depleted of KGF as described in Experimental Procedures in the presence of cytotoxic (20 ng/ml) and cells were exposed to hypoxia (3% O2). \( I_{\text{en}} \) was measured before and after addition of amiloride (10 μM) was added into the apical bath. Amiloride-sensitive \( I_{\text{en}} \) represents the difference of \( I_{\text{en}} \) before and after addition of amiloride. The values are the means ± SD of 8–14 filters for each condition. **P < 0.01, compared with control conditions; §P < 0.05, compared with cytotoxic plus hypoxia; ***P < 0.005, compared with cytotoxic plus hypoxia in presence of MSC-M plus IgG (one-way ANOVA followed by Fisher’s LSD). B: after a 6 h exposure to normoxia (white bars) or cytotoxic plus hypoxia (black bars), AECs were treated with rhKGF (200 or 400 pg/ml) or vehicle for additional 12 h and amiloride-sensitive \( I_{\text{en}} \) was calculated. Values are means ± SD of 8–17 filters for each condition. ***P < 0.001, compared with normoxia corresponding value (one-way ANOVA followed by Fisher’s LSD).
The very limited effect of HCYT-MSC-M despite increased levels of IL-1ra was also surprising since cytokinin contained IL-1β, which has been reported to inhibit Na transport in AECs (22). Thus it seemed possible that the higher concentration of KGF in MSC-M compared with HCYT-MSC-M accounted for most of the beneficial effect of MSC-M. Indeed, several arguments favor this hypothesis. First, KGF has been reported to increase alveolar fluid transport in rat lung and to stimulate Na ion transport in AECs by upregulating α-ENaC protein expression and Na-K-ATPase activity (6, 20, 52). Second, Lee et al. (32) reported that in an ex vivo perfused human lung, the instillation of human MSC following endotoxin-induced lung injury restored alveolar fluid clearance and increased vectorial Na transport, in part, due to KGF release, an observation recently confirmed in a live bacterial model of lung injury in ex vivo perfused human lungs (35). Third, these authors also reported that in MSC treated with siRNA KGF the medium was unable to restore cytokinin-induced decreased transepithelial fluid transport over 24 h in AECs. Consistent with these observations, the current study shows that MSC-M completely depleted of KGF lost its beneficial effect on AEC aliloridine-sensitive Na transport. However, incubation of AECs with rhKGF at concentrations similar to what was observed in MSC-M was unable to prevent the hypoxia plus cytokinin-induced inhibition of Na transport. All together, our data indicate that secreted KGF probably plays a role in the therapeutic effect of the MSC media, although it is not sufficient per se to induce this effect.

One limitation of the present study is that our in vitro model combined cells originating from two different species, i.e., rat AECs and human MSCs. Unfortunately, human AEC lines are not really suitable for electrophysiological experiments in Ussing chamber, namely because of low endogenous expression of ENaC subunits and low transepithelial resistance. Primary rat AECs are usually considered as good surrogates for human AECs, which are very difficult to isolate. It is relevant to emphasize that previous studies have shown that human MSCs were effective in vivo in several rodent models of injury (7, 36, 37). One reason for that may be that most paracrine factors secreted by MSCs (such as KGF, IL-1-ra, or PGE2) show considerable homology across species. Indeed, it is well recognized that recombinant human proteins (rhKGF and rhIL-

One of the observations of the present study is that our in vitro model combined cells originating from two different species, i.e., rat AECs and human MSCs. Unfortunately, human AEC lines are not really suitable for electrophysiological experiments in Ussing chamber, namely because of low endogenous expression of ENaC subunits and low transepithelial resistance. Primary rat AECs are usually considered as good surrogates for human AECs, which are very difficult to isolate. It is relevant to emphasize that previous studies have shown that human MSCs were effective in vivo in several rodent models of injury (7, 36, 37). One reason for that may be that most paracrine factors secreted by MSCs (such as KGF, IL-1-ra, or PGE2) show considerable homology across species. Indeed, it is well recognized that recombinant human proteins (rhKGF and rhIL-1-ra) have a biological effect on rodent AECs (17, 45). The fact that we observed a clear beneficial effect of human MSC media on rat AECs indicates that our model, despite the species difference, still represents a valuable tool for studying in vitro the crosstalk between AECs and MSCs.

In conclusion, the conditioned media of MSCs restored epithelial permeability to normal and completely prevented the decrease in aliloridine-sensitive apical Na transport and α-ENaC cell surface expression induced by inflammatory and hypoxic insults, suggesting that the use of MSC-conditioned media may be relevant as a therapeutic in ALI. Secretion of the paracrine factor KGF by MSCs was required for the protective effect on alveolar epithelial Na transport.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


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