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Paracrine Interaction between Bone Marrow-derived Stem Cells and Renal Epithelial Cells

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Key Words

Bone marrow-derived cells • Mesenchymal stem cells • Metabolic stress • Paracrine interactions

Abstract

Background/Aims: Renal tubular cells are the main target of ischemic insult associated with acute renal injury. Low oxygen and nutrient supplies result in ATP depletion, leading to cell death and loss of renal function. A possible mechanism by which bone marrow-derived cells support renal tissue regeneration relies on the capacity of mononuclear cells (BMMC), particularly mesenchymal stem cells (MSC), to secrete paracrine factors that mediate support for kidney regeneration. Methods: BMMC/MSC and renal cells (LLC-PK_1 from pig and IRPTC from rat) were co-cultured under stressful conditions (ATP depletion and/or serum free starvation), physically separated by a microporous membrane (0.4 μm), was used to determine whether bone marrow-derived cells can interact with renal cells in a paracrine manner. Results: This interaction resulted in stimulation of renal cell proliferation and the arrest of cell death. MSC elicit effective responses in renal cells in terms of stimulating proliferation and protection. Such effects are observed in renal cells co-cultured with rat BMMC/MSC, an indication that paracrine mechanisms are not entirely species-specific. Conclusion: The paracrine action of BMMC/MSC was influenced by a renal cell stimulus released during stress, indicating that cross-talk with injured cells is required for renal regeneration supported by bone marrow-derived cells.

Introduction

Adult stem cells play a key role in kidney tissue regeneration, replacing cells lost through injury [1]. Among several other events, cytokines and growth factors secreted into the blood by injured renal tissue mobilize bone marrow stem cells to the affected regions, thereby modulating kidney repair and regenerative responses [2-4]. These processes aid the restoration of tissue architecture and the reestablishment of renal function [5]. Although a role for bone marrow mononuclear cells (BMMC) in renal injuries is well established, the

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mechanism whereby bone marrow-derived cells support the recovery of renal tissue remains to be elucidated. Several studies have suggested that the differentiation of bone marrow stem cells is involved in kidney regeneration [6-8]. However, these results have yet to be confirmed, and subsequent findings suggest that the probability of such an event occurring is too low to account for the improvement in renal function observed after the infusion of BMMC or mesenchymal (MSC) stem cells [9, 10]. In this regard, there are considerable data supporting the idea of a paracrine effect mediated by soluble factors that would account for the recovery of damaged structures and improvement of renal function [11-13]. Therefore, BMMC/MSC could act via a differentiation-independent mechanism, supporting renal recovery through the secretion of factors that stimulate the surviving renal cell population to proliferate and replace cells lost through injury [1].

Understanding the mechanisms that mediate the interactions between renal cells and BMMC/MSC is important for the development of cell therapies to treat kidney diseases. Therefore, the potential of BMMC and MSC to act upon two important events during renal recovery, *viz.* cell death and the proliferation of renal tubule epithelial cells was the focus of this study. To ascertain whether paracrine factors are involved in the stem cell-mediated protective and proliferating events, LLC-PK_1 cells and immortalized renal proximal tubular cells (IRPTC) were co-cultured with BMMC or MSC in distinct compartments. However, the cell types tested shared the same medium despite their being no direct contact between them. These experiments provided evidence for a bidirectional interaction between renal cells and bone marrow-derived cells through secreted factors, and this may be the key event in the re-establishment of renal structure and function, and constitute a viable target for cell therapy.

Materials and Methods

Materials

A Neubauer chamber (Boeco) was used for cell counting in all experiments. Millicell™ inserts were used to separate different cell lineages in co-culture experiments. An Olympus light microscope and Axiophot immunofluorescence microscope were used for analyses. Low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) with (for LLC-PK_1 cells) or without (for IRPTC) amphotericin B (25 µg/mL); high-glucose DMEM with penicillin (100 U/mL) and streptomycin (100 µg/mL) fetal bovine serum (FBS); and trypsin solution (0.25% trypsin + 0.04% EDTA-tetrasodium salt) were obtained from GIBCO. Ficoll-Paque™ Plus was obtained from GE Healthcare Bio-Science AB. Trypan blue solution 1% (w/v), propidium iodide solution (1.0 mg/mL in water), paraformaldehyde 4% (w/v) in 0.1 M phosphate buffer (pH 7.4) and neutral red (100 µg/mL) were obtained from Sigma-Aldrich. Blocking solution (BSA 5% in phosphate-buffered saline – PBS) and acetate buffer (5% w/v) neutralized with Tris (pH 3.3) were freshly prepared. Mouse anti-proliferative cell nuclear antigen (PCNA) antibody was purchased from DakoCytomation. Sytox® Green Alexa 555-conjugated anti-mouse antibody, Alexa 555-conjugated anti-rabbit antibody and Annexin V-Alexa Fluor® 488 were purchased from Invitrogen. Anti-activated caspase-3 antibody was obtained from Cell Signalling. 4'6-diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich. MSC markers were immunodetected using a Mesenchymal Stem Cell Characterization Kit (Rat) (Chemicon). Environmental conditions for cell culture were set at 5% CO_2 at 37°C in a humidified atmosphere. Murine embryonic fibroblast (MEF) and rat embryonic fibroblast (REF) cultures were a kind gift from Dr. Stevens K. Rehen (Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Brazil).

Animal care

The procedures involving animals (isolation of BMMC and MSC) were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Rio de Janeiro (protocol Nº IBCCF 086), and were performed in accordance with the Committee's guidelines.

Renal epithelial cell cultures

The LLC-PK_1 line and a lineage of renal proximal tubule cells from male Wistar rats (IRPTC) were used as models of proximal tubule cells. LLC-PK_1 cells represent a widely-used model for the study of tubular processes [14]; IRPTC were chosen as a species-matched control for the actions of BMMC and MSC as they preserve the normal transport and signaling properties of primary cultured cells [15]. The cells were initially grown for four days in low-glucose DMEM (10% FBS) with differential antibiotic supplementation as described. The cultures were maintained under a humidified atmosphere of 5% CO_2 at 37°C. During assays, the cells were cultured with DMEM in the absence of FBS.

Extraction of BMMC, and isolation and expansion of MSC

Bone marrow was collected from the femurs and tibias of male Wistar rats. The nucleated cells were isolated using a Ficoll-paque density gradient and resuspended in DMEM (low-glucose and serum-free). BMMC were counted in a Neubauer chamber, checked for viability using trypan blue exclusion and characterized by flow cytometry using a FACS Aria apparatus (BD Biosciences) as previously described [16]. MSC were isolated as in [17]. Briefly, nucleated bone marrow cells were plated in 75 cm^2 plastic culture flasks containing DMEM with 10% FBS, and maintained in culture until 80-90% confluence was reached. Adherent cells were detached using trypsin and

cultured in DMEM with 20% FBS. This procedure was repeated between six and seven times to ensure a homogeneous population of MSC. Cells were suspended, counted and used immediately. MSC were characterized following the criteria defined by the International Society for Cellular Therapy [18]. Moreover, immunofluorescent detection of surface antigens was routinely performed as controls. Briefly, confluent MSC cells were fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, washed three times with PBS and incubated with blocking buffer for 2 h prior to be probed with the selected primary antibodies. These cells were positive for integrin β1, fibronectin and CD54, while there was no detection of CD45 antigen (data not shown).

Murine embryonic fibroblast (MEF) and rat embryonic fibroblast (REF) cell cultures

MEF and REF were used as controls for testing the specificity of BMMC and MSC trophic factors. These cells were plated in 75 cm^2 flasks with high-glucose DMEM supplemented with 200 mM L-glutamine and 10% FBS, and grown in the humidified environment described above. Adherent viable cells were harvested just before use.

Preparation and assay of BMMC and MSC conditioned media

After BMMC and MSC had been obtained they were incubated in six well culture plates (10^7 cells per well for BMMC; 10^6 cells per well for MSC) containing serum-free DMEM. After 72 h, the conditioned medium was carefully removed, centrifuged at 200 g in the cold, filtered through nitrocellulose filters (0.4 μm pore diameter) and used immediately.

LLC-PK_1 cells (5×10^4) were suspended in 2 mL of conditioned medium and cultured in wells for 72 h at 37°C in the humidified atmosphere. Proliferation was evaluated by counting viable cells, and the protective effect was determined by observing pyknotic nuclei (see below).

Preparation and assay of MSC and LLC-PK_1 co-cultured conditioned media

LLC-PK_1 were cultured in DMEM containing low glucose and supplemented with 10% FBS. After confluence, the renal cells were washed with PBS and co-cultured in serum-free DMEM for 72 h with MSC using the Millicell™ system. The porous transwell membranes separated the two cell populations, thus allowing later recovery of soluble factors that were secreted without cell-to cell contact [19]. The medium was then collected and filtered in a 0.4 μm porous membrane. New renal cell cultures were then assayed with this co-cultured medium for evaluation of proliferation and cell death.

Evaluation of renal cell proliferation

To evaluate the influence of BMMC, without contact, on the proliferation of LLC-PK_1 cells, BMMC and MSC were layered on the surfaces of porous membranes in the upper compartment of a Millicell™ system. The renal cell population (10^5 per well) was plated beneath the membrane and the system was supplied with low-glucose DMEM. After the times indicated in the legends, the Millicell™ inserts were withdrawn, the cells were washed with PBS and harvested with trypsin-EDTA solution. Proliferation was evaluated by counting the number of viable cells in the presence of trypan blue. The influences of MSC and IRPTC on the proliferation of LLC-PK_1 cells were compared in parallel 72-h experiments.

To measure proliferation using the PCNA assay, renal cells were plated on to sterile glass coverslips for 24 h in DMEM containing low glucose and supplemented with 10% FBS, and maintained in serum-free DMEM for an additional period of 48 h. The renal cells were co-cultured with various amounts of BMMC or MSC for 24 h, washed in PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min before being incubated with mouse PCNA antibody (1:100) overnight in a 37°C humidified incubator. The cells were washed three times with PBS, incubated at 37°C for 1 h with secondary antibody (goat Alexa 555-conjugated anti-mouse; 1:200) suspended in blocking solution, and washed again with PBS. Nuclei were stained with DAPI; PCNA and cell nuclei were located using an immunofluorescence microscope (Axiophot).

Analysis of cell death

LLC-PK_1 cells were plated at a concentration of 10^5 cells per well and co-cultured with BMMC, MSC, MEF or REF. At various time points, LLC-PK_1 cells were washed with PBS and fixed for 10 min with 4% (w/v) paraformaldehyde. In experiments aimed at comparing the influence of MSC on the death of IRPTC, they were co-cultured for 72 h. After incubation and fixation, the renal cells were washed with acetate buffer and stained with neutral red to identify nuclear chromatin. The cells were washed with acetate buffer and left to dry at room temperature for 24 h. Cells undergoing apoptosis presented with condensed chromatin (pyknotic nuclei) and more intense neutral red staining. The cell death rate was determined by the number of cells with pyknotic nuclei. Estimations were made by counting a total of 500 cells in random fields using light microscopy at a magnification of 20x.

A large fraction of apoptotic cells detached from the chamber. Therefore, the influence of BMMC was investigated in the supernatant of the lower chamber of the Millicell™ system. After removing the inserts, aliquots of the respective supernatants (LLC-PK_1 cells co-cultured with BMMC or grown alone) were carefully removed in order to count the number of detached cells.

Analysis of apoptosis through activated caspase-3 levels

LLC-PK_1 cells were grown on sterile glass coverslips in DMEM containing low-glucose supplemented with 10% FBS for 24 h, washed with PBS and cultured with serum-free DMEM until confluent. They were co-cultured for 24 h with various concentrations of BMMC or MSC in Millicell™ inserts, washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. After two washes with PBS, the cells were permeabilized for 1 h in blocking solution before being incubated with rabbit anti-caspase-3 (active) antibody (1:200) overnight at 4°C. After three washes in PBS, cells were incubated at 37°C for 1 h with the secondary antibody (1:200 in a blocking solution) and washed with PBS. Nuclei were stained with Sytox® Green nuclei acid stain and the cells were washed twice with PBS. The active

caspase-3 and changes in nuclear morphology were located using immunofluorescent microscopy.

Analysis of cell viability after ATP depletion

Although incubation in SFM represents an important stress imposed on LLC-PK_1 cells, a condition that mimics the relevant injurious stress of tubule cells during acute kidney ischemia was also analyzed: that represented by ATP depletion. Confluent LLC-PK_1 cells were incubated in SF low-glucose DMEM in the presence of 1 μM antimycin A to block the mitochondrial respiratory chain at the level of complex III thus avoiding oxidation of any substrate. In LLC-PK_1 cells antimycin A leads to almost complete exhaustion of ATP stores after 45 min with slow and partial spontaneous recovery of ATP levels after removal of the inhibitor [20]. After 2 h the medium was removed, the cells were washed three times with PBS and incubated for 6 h at 37°C with low-glucose DMEM supplemented with 1 μg/mL propidium iodide (PI) either alone or in co-culture with MSC. Cell death was evaluated by counting the PI-positive cells per field.

Renal cell death analysis was also performed by FACS analysis. After antimycin A treatment, renal cells were washed with PBS and harvested using trypsin solution at 37°C. After centrifugation, the cells (10^5) were resuspended with binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) and incubated in darkness with Annexin V-Alexa Fluor® (5 μL of the stock commercial solution in 100 μL buffer) for 15 min at room temperature. Propidium iodide (1.5 μM final concentration) was then added, and the samples were placed on ice and analyzed immediately. Cell death analysis was carried out with the use of a flow cell based bench top FACS Calibur Cytometer (BD Biosciences).

Statistical analysis

Statistical analyses were carried out using the One-way ANOVA test and Newman-Keuls post-test. Statistical significance was set at $p < 0.05$. Data were analyzed using the GraphPad Prism 5.0 program.

Results

BMMC and MSC stimulate renal epithelial cell proliferation

LLC-PK_1 cells cultured in a growth factor-deprived medium enter quiescence within 48 h [21], as shown in Fig. 1A (empty columns). Cultures were examined at 12, 24, 48 and 72 h to test whether BMMC or MSC co-cultured with LLC-PK_1 cells in the Millicell™ system stimulated the proliferation of the latter. After a 12 h lag phase, BMMC and MSC induced LLC-PK_1 cells to re-enter the cell cycle, reaching a two or three-fold increase in cell numbers at 72 h (Fig. 1A). BMMC- or MSC-conditioned media recovered after 72 h incubation without LLC-PK_1 cells failed to elicit proliferative responses in contrast with media recovered after co-culture of MSC

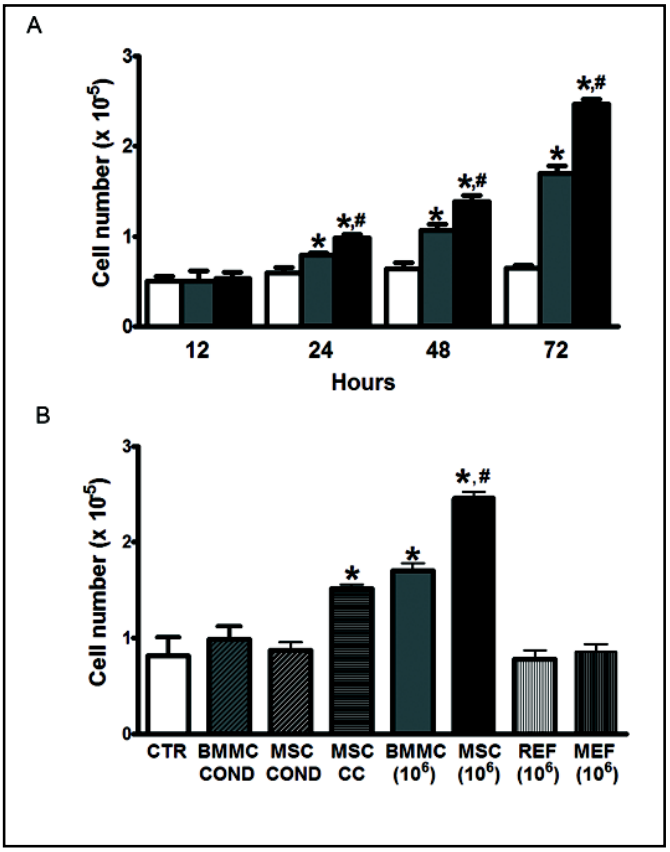


Fig. 1. (A) BMMC and MSC stimulate proliferation of LLC-PK_1 cells in a physically separated co-culture system. LLC-PK_1 cells were co-cultured with BMMC or MSC in a Millicell™ system for the times shown on the abscissa, as described in the Materials and Methods section. Proliferation was evaluated by counting viable cells in the culture. Empty bars represent the control group (renal cells cultured without bone marrow-derived cells in the upper compartment); gray bars, renal cells co-cultured with BMMC; black bars, renal cells co-cultured with MSC. (B) BMMC and MSC require stimulation from renal cells to secrete proliferative factors. LLC-PK_1 cells were cultured for 72 h with filtered conditioned medium (COND), previously obtained by incubating BMMC or MSC for 72 h in serum-free DMEM without exposure to renal cells, as indicated on the abscissa. Renal cells were also cultured for 72 h with co-culture conditioned medium (CC) recovered after co-culture of MSC and LLC-PK_1 separated by the porous membrane of the transwell. In parallel experiments, LLC-PK_1 cells were co-cultured in a Millicell™ system with BMMC, MSC or embryonic fibroblasts from rat (REF) or mouse (MEF), as indicated on the abscissa (number of cells per well in parenthesis). CTR: renal cells cultured without any exposure. Renal cell proliferation was evaluated by counting viable cells. Data are mean ± SEM of renal cells cultured in triplicate with BMMC- or MSC-conditioned media from three rats, assayed with MSC co-culture conditioned medium, co-cultured in triplicate with BMMC or MSC from three rats or co-cultured in triplicate with MEF or REF from a single lineage, as shown on the abscissa. *: statistical difference with respect to CTR; #: statistical difference between BMMC and MSC.

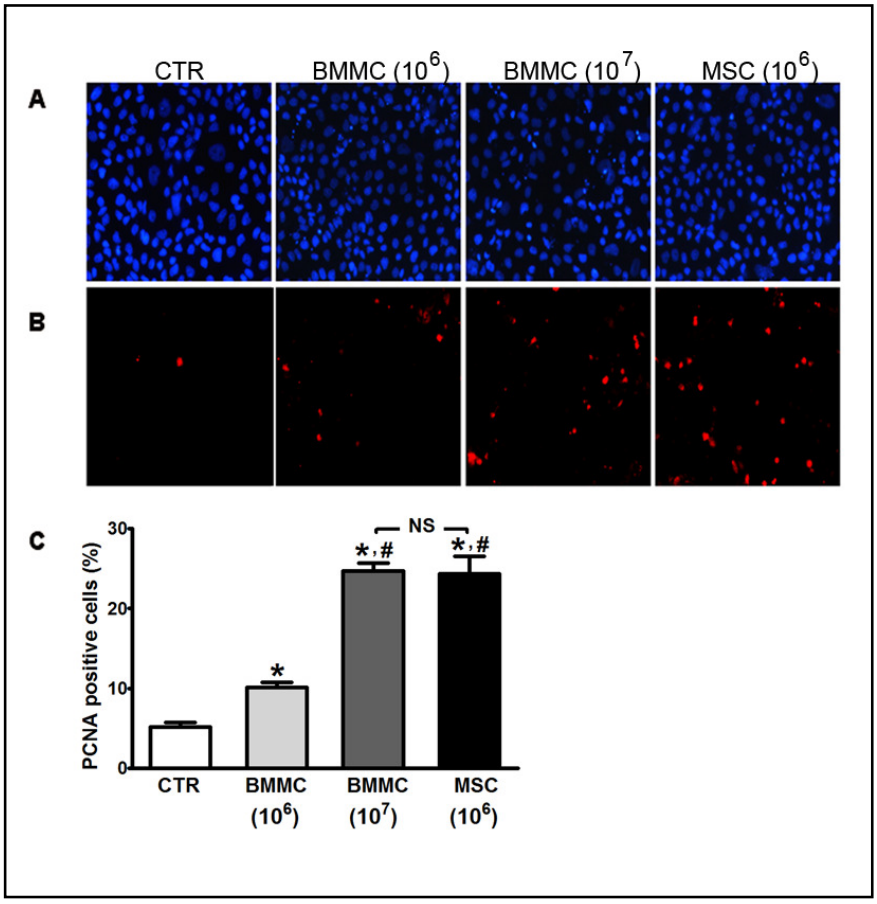


Fig. 2. Bone marrow-derived cells stimulate proliferation of serum deprived LLC-PK$_1$ cells. Renal cells were cultured for 48 h in serum-free DMEM, co-cultured with BMMC or MSC in a Millicell™ system for an additional period of 24 h (or without other cells; control) and then assayed for PCNA-positive cells as described in the Materials and Methods section. The type and number (in parenthesis) of bone marrow-derived cells plated in the upper compartment of the wells are indicated above the panels or on the abscissa of the graphic; CTR: control (renal cells without BMMC or MSC). (A) Representative nuclear staining of LLC-PK$_1$ with DAPI. (B) PCNA immunofluorescent labeling of LLC-PK$_1$ cells in the same field shown in (A). (C) Quantification of PCNA-positive cells (% values with respect to the total number of cells in the same field) counted at random as described in the Materials and Methods. *: statistical difference with respect to CTR; #: statistical difference with respect to the 10^6 BMMC group. NS: no significant difference between the 10^7 BMMC and 10^6 MSC groups. Results in (C) are mean ± SEM of the PCNA-positive cell fraction in separated cultures with bone marrow-derived cells from five rats.

and LLC-PK$_1$ cells that were separated by the porous transwell membrane to avoid cell-to-cell contact [19] (Fig. 1B; compare 3rd and 4th columns). The proliferative response mediated by these co-cultured medium was similar to that obtained in the presence of 10^6 BMMC. The effect was specific for BMMC and MSC since there was no effect using embryonic fibroblasts (REF or MEF) co-cultured with LLC-PK$_1$ cells.

Once the increased number of viable cells was the result of a balance between proliferation and cell death, the cell proliferation was determined by the content of PCNA in LLC-PK$_1$ cells co-cultured with BMMC/MSC (Fig. 2). Increased numbers of PCNA-positive cells depended on the number of BMMC, but a 10-fold increase in the number of BMMC raised the number of PCNA-positive cells 2.5 times. This indicates that the response to BMMC declines as the number of bone marrow-derived cells increases, suggesting a possible maximal proliferation rate that is also reached with 10^6 MSC.

The proliferative effect in LLC-PK$_1$ cells was also observed when MSC cells were co-cultured with renal tubular cells (IRPTC) of the same species (Fig. 3). The increase in IRPTC number after 72 h of co-culture with MSC was similar to that observed in LLC-PK$_1$ cells,

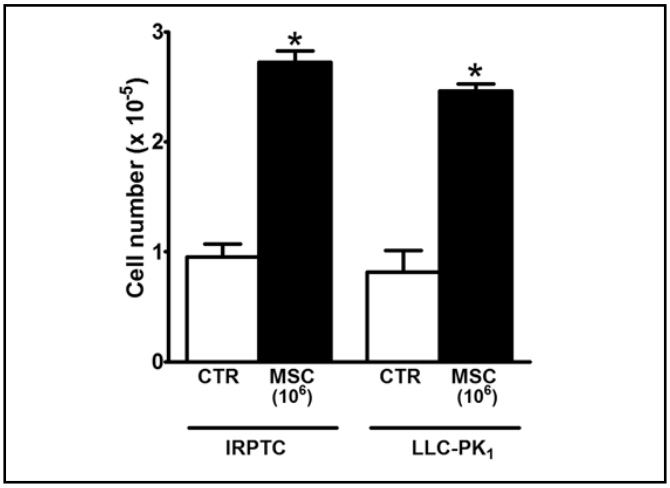
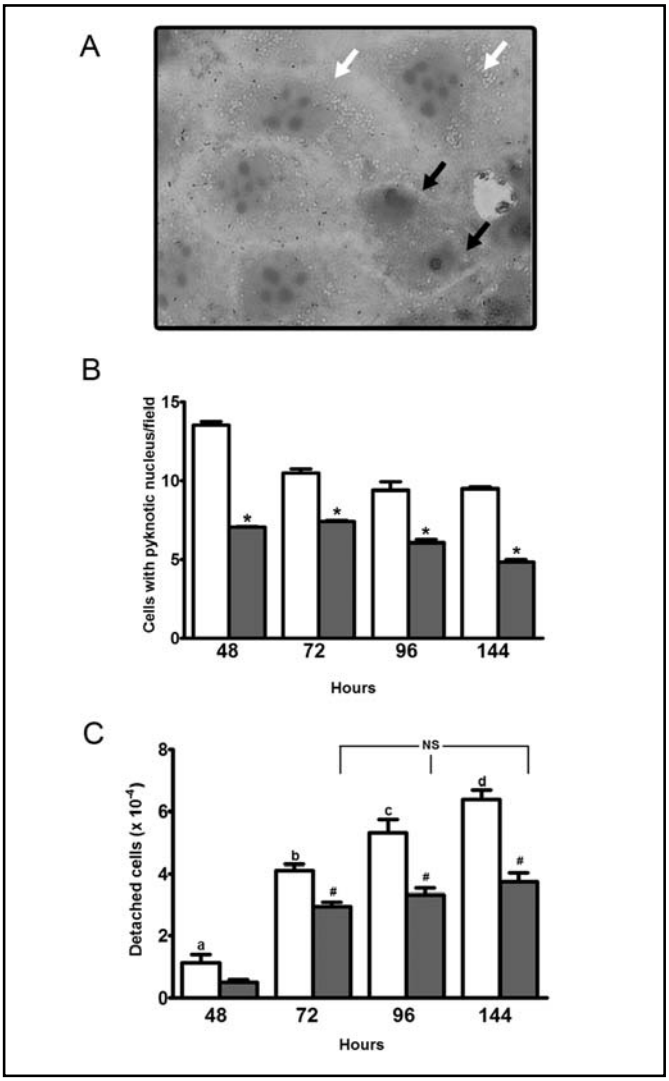


Fig. 3. Rat MSC promote similar proliferative responses in renal tubular cells from different species. IRPTC and LLC-PK$_1$ cells were cultured under the same conditions in serum-free DMEM for 72 h, with or without 10^6 MSC per well. Proliferation was evaluated by counting the number of viable cells after trypsin-EDTA addition. Empty bars represent the control group where renal cells (IRPTC or LLC-PK$_1$) were cultured without MSC in the upper compartment. Black bars represent the group of renal cells co-cultured with MSC. Results are mean ± SEM of renal cells co-cultured in triplicate with various samples of MSC originating from three rats. *: statistical difference with respect to the corresponding CTR group.

Fig. 4. BMMC arrest progressive death of LLC-PK_1 cells. LLC-PK_1 cells were co-cultured with BMMC in serum-free DMEM. (A) Light microscopy of a representative LLC-PK_1 culture stained with neutral red. Black arrows indicate renal cells with pyknotic nuclei; empty arrows demonstrate normal cells. (B) Cell death was determined by the quantification of attached cells with pyknotic nuclei. At the times of culture indicated on the abscissa, renal cells were washed, fixed, stained with neutral red and counted as described in the Materials and Methods section to determine the percentage of cells with pyknotic nuclei. (C) Cell death was evaluated by counting the number of spontaneously detached cells. At the indicated times, the medium containing the detached cells was collected and the number of cells was counted in a Neubauer Chamber. In (B) and (C) empty bars represent the control group (no co-culture with BMMC) and gray bars represent LLC-PK_1 cells co-cultured with BMMC. Results are mean ± SEM of renal cells with pyknotic nuclei (B) or detached (C) after co-culture in triplicate with different BMMC samples originating from five rats. In (B) the asterisks indicate differences from the corresponding time-matched control without co-culture. In (C) different lowercase letters indicate statistical difference in the control group as long as the incubation time increases; *: difference to the corresponding time-matched control without co-culture. NS: no difference in the number of detached LLC-PK_1 cells co-cultured with BMMC as long as the incubation time increases from 72 to 144 h.



indicating that the effects mediated by paracrine secretion of MSC are not species-specific.

BMMC and MSC prevent cell death

We tested whether co-culture with BMMC or MSC prevented LLC-PK_1 cell death. The number of cells with a pyknotic nucleus was counted at various times during culture in serum-free medium (Fig. 4A and B). The presence of BMMC in the co-culture led to a reduction in the number of renal cells with pyknotic nuclei at all times (Fig. 4B). Pyknotic nuclei are an initial characteristic of the cell death process that eventually results in cell detachment, explaining the apparent stabilization of cell death in the control groups (empty bars in Fig. 4B). Therefore, the number of detached renal cells was also determined (Fig. 4C). The continuous increase in the number of detached cells was prevented by co-culturing with BMMC, demonstrating that cell death was arrested. As with proliferation, a 10-fold increase in BMMC was not proportional to the reduction of the number of cells with pyknotic nuclei, and the same effect was promoted by 10^7 BMMC and 10^6 MSC (Fig. 5).

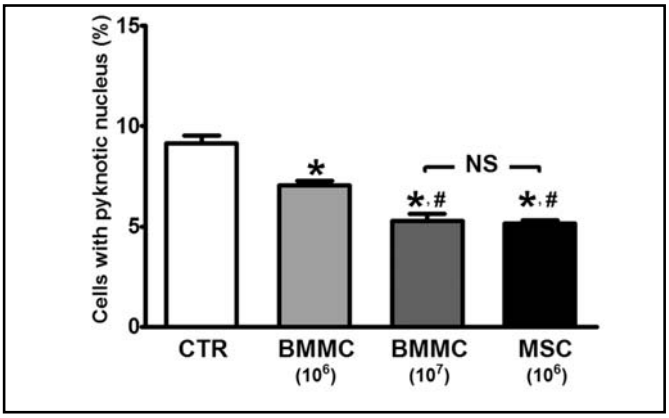


Fig. 5. The reduction in pyknotic nucleus depends on the type and number of bone marrow-derived cells. LLC-PK_1 cells were co-cultured and assayed for pyknotic nuclei as described in the legend to Fig. 4, with the exception that two different amounts of BMMC were compared with MSC, as indicated on the abscissa during a period of 24 h; CTR, renal cells cultured without bone marrow-derived cells. *: different from CTR; #: different from 10^6 BMMC; NS: no difference between the 10^7 BMMC and 10^6 MSC groups.

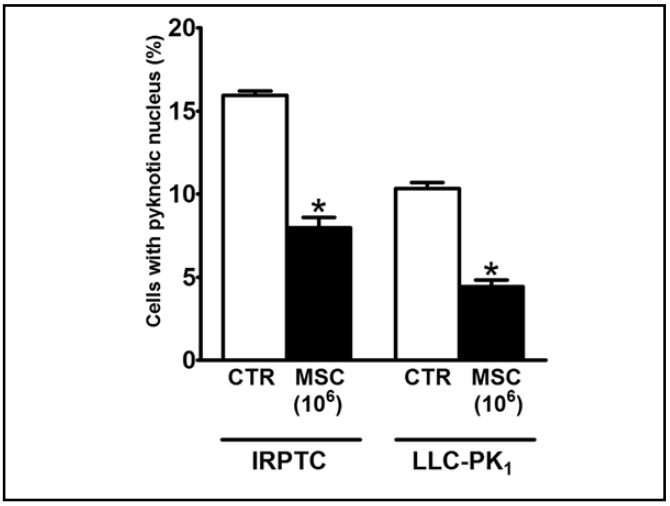


Fig. 6. Similar reduction in pyknotic nucleus is evident in LLC-PK_1 cells and IRPTC using rat MSC. LLC-PK_1 and IRPTC were cultured under the same conditions. Both cell types were cultured in the presence of serum-free DMEM for 48 h. Confluent cells (LLC-PK_1 or IRPTC) were cultured in serum-free DMEM for 24 h in the absence (CTR) or presence of MSC (10^6 cells per well). Cell death was determined by quantification of attached cells with pyknotic nuclei. Results are mean ± SEM of renal cells co-cultured in triplicate with various samples of MSC originating from three rats. *: statistical difference with respect to the corresponding CTR group.

The beneficial effects of MSC-secreted factors presented the same pattern in terms of the reduction in the number of cells with pyknotic nuclei in IRPTC and LLC-PK_1 cells (Fig. 6). As observed with the proliferation data (Fig. 3), the effects of factors secreted by MSC against cell death were not species-specific.

MSC promote renal tubular cell recovery after ATP depletion injury

ATP depletion is one of the components of ischemic insult. To mimic this condition *in vitro*, LLC-PK_1 cells were incubated with antimycin A for 2 h, and for a further 6 h period after removal of the inhibitor. ATP depletion insult led to an increase in the cell death rate as demonstrated by the large number of propidium iodide-positive cells (ATP dp, Fig. 7). This was completely prevented when MSC were co-cultured with ATP-depleted renal cells (ATP dp/MSC, Fig. 7). FACS analysis shows a reduction in cells death by apoptosis and necrosis when renal cells, after ATP depletion, were incubated with MSC. ATP depletion increases apoptotic cells in more than 100% 3 h after removal of antimycin A, whereas the co-culture of ATP-depleted cells with MSC during this period dropped the number

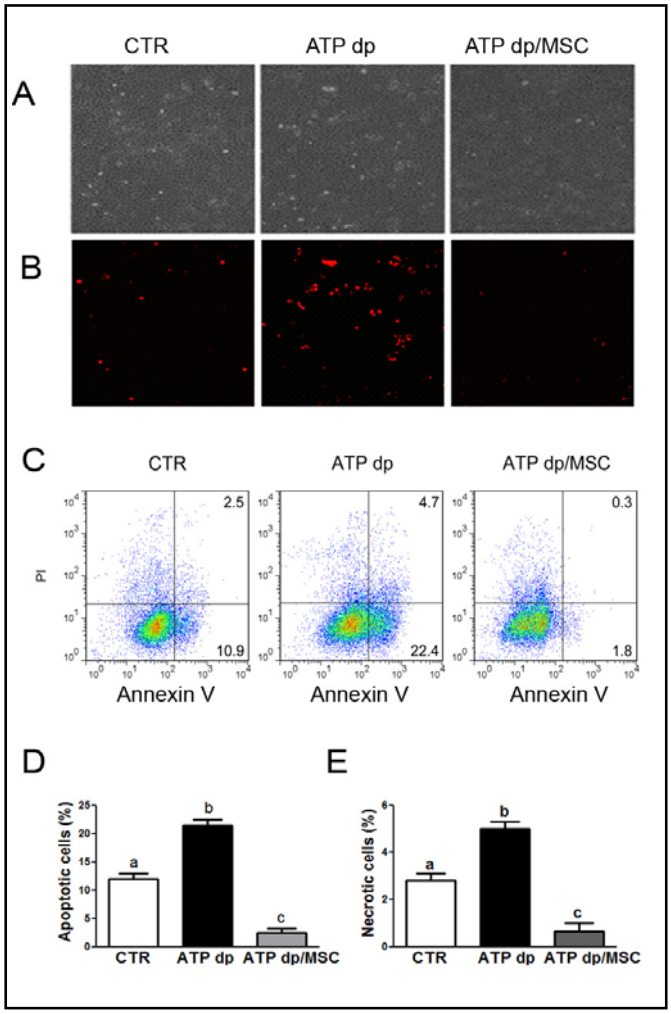


Fig. 7. MSC maintain normal cell death levels after *in vitro* ischemic injury caused by ATP depletion (ATP dp). LLC-PK_1 cells were incubated with 1 μM antimycin A for 2 h to block mitochondrial electron transport and oxidative phosphorylation. The cells were washed with PBS and incubated in serum-free DMEM for 6 h in the absence (CTR and ATP dp) or presence (ATP dp/MSC) of 10^6 MSC per well. Cell death was determined by quantification of propidium iodide (PI) positive cells. (A) Light microcopy of representative fields of LLC-PK_1 cultures under the indicated conditions. (B) Immunofluorescent detection of PI-positive cells in the same corresponding fields. (C) Cells were labeled with Annexin V-Alexa Fluor® 488 and propidium iodide (PI) and analyzed as described under Material and Methods. (D) and (E) Graphic representation of the percentages of apoptotic and necrotic cells, respectively. Bars represent the control group not subjected to ATP depletion (CTR), renal cells subjected to ATP depletion (ATP dp) and renal cells subjected to ATP depletion and co-cultured with MSC as indicated on the abscissae. Notice the differences in scales on the ordinates. The results are mean ± SEM of three determinations performed in triplicate using different renal cells cultures and different samples of MSC originated from three rats. The different lowercase letters above the bars indicate statistical differences among the groups.

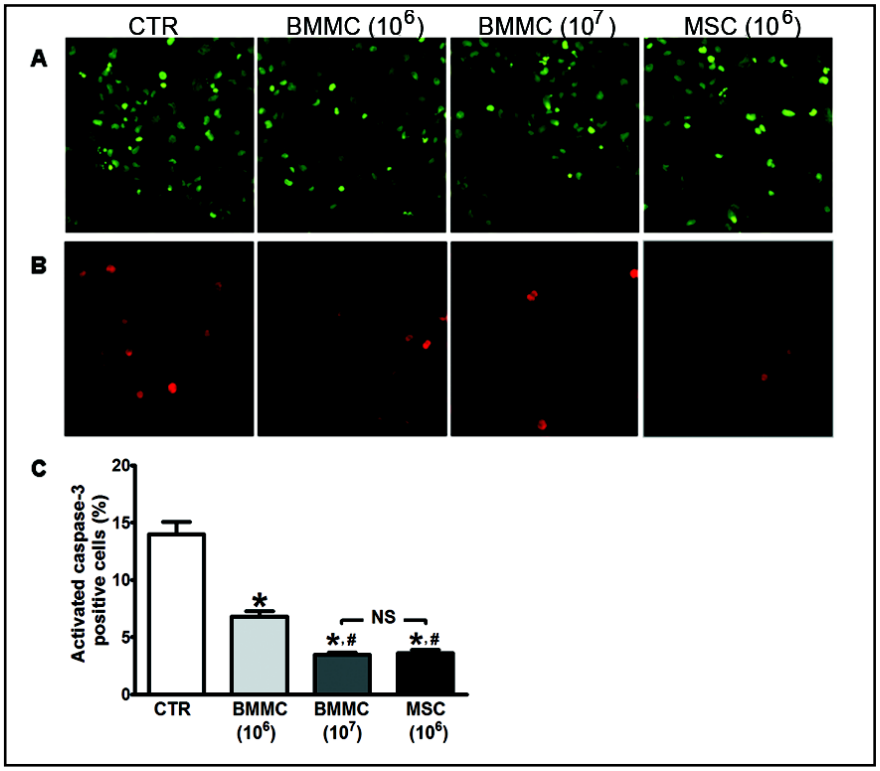


Fig. 8. Bone marrow-derived cells reduce apoptosis in LLC-PK$_1$ cells by inhibiting caspase-3 activation. LLC-PK$_1$ cells were cultured for 48 h in serum-free DMEM and co-cultured for an additional period of 24 h with the type and number of bone marrow-derived cells indicated above the panels or on the abscissa of the graphic; CTR: renal cells without BMMC or MSC. (A) Representative nuclear staining of LLC-PK$_1$ cells with Sytox® Green. (B) Immunofluorescent detection of activated caspase-3 in LLC-PK$_1$ cells in the corresponding fields shown in (A). (C) Quantification of activated caspase-3 positive cells (% values with respect to the total number of cells in the same field) counted at random as described in the Materials and Methods. *indicates statistical difference with respect to CTR; #: statistical difference with respect to 10^6 BMMC. Results in (C) are mean ± SEM of activated caspase-3 positive cell fractions in separated co-cultures with bone marrow-derived cells from five rats.

of Annexin-positive cells even below the control levels (Fig. 7C and D). A similar ratio was also observed when necrosis (Annexin plus PI-marked cells) was evaluated (Fig. 7C and E).

Co-culture with BMMC and MSC prevents caspase-3 activation

Immunodetection of activated caspase-3 was used to test whether co-culture with BMMC prevents LLC-PK$_1$ cells from undergoing apoptosis (Fig. 8). After 24 h of co-culture, renal cells were immunolabeled with an antibody against the activated or cleaved form of caspase-3. Increasing numbers of BMMC (10^6, 10^7) reproduced a correspondingly greater reduction in the number of LLC-PK$_1$ cells positive for activated caspase-3. As in the proliferation and pyknotic nuclei assays, both 10^7 BMMC and 10^6 MSC induced similar responses. BMMC- or MSC-conditioned media that had not previously had humoral interaction with serum-deprived LLC-PK$_1$ cells had no influence in the level of pyknotic nucleus found in renal cells that were challenged by serum deprivation, as was the case with co-cultured embryonic fibroblasts (REF or MEF) (Fig. 9). Differently, when renal cells were cultured with MSC/LLC-PK$_1$ co-culture conditioned media reduced the number of cells with pyknotic nucleus to a level that was similar to that observed in the 10^6 BMMC group (Fig. 9).

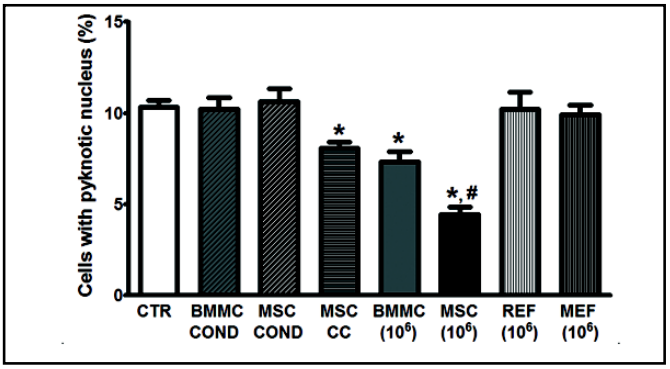


Fig. 9. BMMC and MSC require stimulation from renal cells to prevent the increase in pyknotic nuclei in serum-deprived LLC-PK$_1$ cells. LLC-PK$_1$ cells were cultured for 48 h in serum-free DMEM and cultured for an additional period of 24 h with filtered conditioned medium (COND) previously obtained by incubating BMMC or MSC for 72 h in serum-free DMEM (without exposure to renal cells), as indicated on the abscissa. Pyknotic nucleus were also evaluated in renal cells exposed to conditioned medium (CC) obtained after co-culture of MSC with LLC-PK$_1$ for 72 h, as described in the Materials and Methods section. In parallel, LLC-PK$_1$ cells were co-cultured in a Millicell™ system with BMMC, MSC, REF or MEF as indicated on the abscissa. CTR: renal cells cultured without exposure. Pyknotic nuclei were evaluated as described in the legend to Fig. 4. Data are mean ± SEM of renal cells cultured in triplicate with BMMC- or MSC-conditioned media from three rats, assayed with MSC co-culture conditioned medium, co-cultured in triplicate with BMMC or MSC from three rats or co-cultured in triplicate with MEF or REF from a single lineage, as shown on the abscissa. *: statistical difference with respect to CTR; #: statistical difference between BMMC and MSC.

Discussion

The present study demonstrates that paracrine factors secreted by BMMC and MSC populations in response to injured renal cells can promote renal epithelial cell proliferation (Fig. 1-3) and protect against cell death (Fig. 4-9). These effects may help to explain why bone marrow-derived cells ameliorate renal structure and function after various insults. Kidney regeneration depends on the nature of the injury, which can be caused by ischemia [22], ureteral obstruction [23] and endogenous or exogenous toxins [24, 25]. The primary target for these insults is the renal tubular epithelial cells. Depending on the severity of injury it may cause the loss of renal tubular epithelial cell polarity, impairing transepithelial transport, and trigger cell death [26]. The ability of injured tissues to regenerate is related to the action of adult stem cells; however, despite the presence of tissue-specific adult stem cells, the repair process appears to require the recruitment of bone marrow-derived adult stem cells [9], particularly to achieve specific actions. For example, the presence of MSC results in an improvement in repair owing to their immunomodulatory actions [27].

We demonstrate here that bone marrow-derived cells interact with renal epithelial cells despite being separated by a porous membrane. We conclude that a paracrine mechanism leads to proliferation and protection of renal cells. This may explain how BMMC administration promotes significant renoprotection despite a relatively modest recruitment after unilateral obstruction [28], and why MSC infusion improves renal tissue architecture and function despite the low level of differentiation [29-31]. Several studies have shown that MSC support renal recovery through a paracrine mechanism. Some growth factors such as VEGF, IGF-1 and HGF, which are important for renal recovery, have been identified as molecules secreted by MSC [32-36]. Data presented by Villegas and coworkers [13] demonstrated that VEGF act directly on renal epithelial cells via a VEGFR-2 receptor stimulating proliferation and promoting survival. These responses are similar to those obtained in this study by co-culturing renal cells and stem cells that were physically separated by a membrane in the same well, giving support to the idea that VEGF could be one paracrine effector that mediates the beneficial influence of BMMC and MSC. Thus, VEGF and the other aforementioned factors are possible candidates for explaining the benefit of indirect interaction between MSC and renal cells demonstrated in the present work.

Interestingly, renal tubular epithelial cells cultured with the conditioned medium from BMMC or MSC alone (without previous interaction with LLC-PK_1cells) had no beneficial effect in co-culture assays. However, the medium recovered after co-culture of MSC and LLC-PK_1 induced both cell death arrest and proliferative response (Fig. 1 and 9). These results indicate that BMMC and MSC require a previous stimulus in order to secret their bioactive molecules. Therefore, the BMMC/MSC beneficial effects in renal recovery are a result of mutual crosstalk between bone marrow and renal cells through a paracrine mechanism. In other words, bioactive factors that stimulate renal cell proliferation are secreted by bone marrow derived cells *only* when they have been primed with a stimulus originated from renal cells threatened by serum deprivation. During starvation, as was the case in all cell cultures carried out without nutrients or serum or following other injury that markedly decreased cellular ATP [20], several chemokines and growth factors were released, mobilizing bone marrow cells to the injured areas [37, 38]. These results indicate that the beneficial effects of BMMC and MSC on LLC-PK_1 and IRPCT are a result of crosstalk between the co-cultured cells that promotes proliferation and protection of renal cells. Recent studies have demonstrated that unstimulated MSC have no immunomodulatory actions [27, 39], indicating that a previous stimulus such as local growth factors and cytokines is essential for triggering MSC properties. Despite originating from different species, the proliferative and antiapoptotic responses observed in LLC-PK_1 cells (pig origin) when co-cultured with rat MSC are similar to those obtained with IRPTC (rat origin) (Figs. 3 and 6). Therefore, the secreted factors that mediate these interactions are not species-specific, at least for the processes investigated here. Several other studies have demonstrated beneficial effects of infused MSC originating from a species different from the receptor [40-42]. At this point it is interesting to mention that paracrine interactions involving immunomodulatory responses appear to be relevant for the cell cycle in kidney tissue beyond unidirectional actions of repair. Soluble factors secreted by activated macrophages direct apoptosis in proximal tubules [19], an effect that could be relevant for the slow physiological renewal of renal cells.

Under normal conditions adult kidneys have a low cellular turnover [43] that increases after injury [44]. The fact that renal cells are stimulated to proliferate even in the absence of physical contact with bone marrow cells supports the view that repopulation of injured tubular regions is predominantly due to paracrine-induced

proliferation of intrinsic epithelial cells [29, 45], rather than transdifferentiation. However, the results presented herein are consistent with the view that *extrinsic* adult stem cells (BMMC or MSC) may play an important role in stimulating this process.

Kidney regeneration is closely related to the capacity of the remaining cells to proliferate, and the success of the recovery is closely related to the survival of renal cells after cessation of the injury. To test for a renoprotective effect of either BMMC or MSC in the renal cultures, a serum-deprivation model [46] and an ATP-depletion model [20] were used as inducers of stress that triggers cell death. Such experiments simulate conditions in which insufficient provision of oxygen, metabolic substrates and growth factors correlate with acute and/or chronic tubular injuries [47-51]. Co-cultures of BMMC or MSC with renal cells were effective in preventing both apoptotic and necrotic cell death (Figs. 4-9) through a paracrine effect. This effect, however, was not evident when co-cultures were carried out with renal epithelial cells and embryonic fibroblasts (REF or MEF), which are known to secrete bioactive factors [52] (Fig. 1B and 9), thereby establishing the specificity of the paracrine action.

The data suggest that MSC are more effective than BMMC, as a 10-fold increase in BMMC cell inoculum was required to achieve the same end-result as MSC (Figs. 1, 2, 5, 8 and 9). However, MSC represent <0.1% of the total BMMC population [53] and a 10-fold increase in the number of BMMC contains approximately 1% of the number of MSC in a purified MSC culture. This suggests that other cells among the heterogeneous BMMC population synergize with MSC in their paracrine action on renal cells. This observation may impact eventual clinical trials concerning cell therapy as the isolation of MSC requires more steps than BMMC isolation.

In conclusion, BMMC and MSC could act on renal epithelial tubular cells through a differentiation-independent mechanism, promoting protection and stimulating proliferation. The beneficial effects depend on crosstalk between renal and bone marrow-derived cells, mediated by mutual paracrine secretions. This is consistent with the view that BMMC and MSC act in response to environmental changes in order to lead to the growth and survival of the renal epithelium.

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