Intercellular communication

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Improving the Post-Stroke Therapeutic Potency of Mesenchymal Multipotent Stromal Cells by Cocultivation With Cortical Neurons: The Role of Crosstalk Between Cells

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ABSTRACT

The goal of the present study was to maximally alleviate the negative impact of stroke by increasing the therapeutic potency of injected mesenchymal multipotent stromal cells (MMSCs). To pursue this goal, the intercellular communications of MMSCs and neuronal cells were studied in vitro. As a result of cocultivation of MMSCs and rat cortical neurons, we proved the existence of intercellular contacts providing transfer of cellular contents from one cell to another. We present evidence of intercellular exchange with fluorescent probes specifically occupied by cytosol with preferential transfer from neurons toward MMSCs. In contrast, we observed a reversed transfer of mitochondria (from MMSCs to neural cells). Intravenous injection of MMSCs in a postischemic period alleviated the pathological indexes of a stroke, expressed as a lower infarct volume in the brain and partial restoration of neurological status. Also, MMSCs after cocultivation with neurons demonstrated more profound neuroprotective effects than did unprimed MMSCs. The production of the brain-derived neurotrophic factor was slightly increased in MMSCs, and the factor itself was redistributed in these cells after cocultivation. The level of Miro1 responsible for intercellular traffic of mitochondria was increased in MMSCs after co-cultivation. We present evidence of intercellular exchange with fluorescent probes specifically occupied by cytosol with preferential transfer from neurons toward MMSCs. In contrast, we observed a reversed transfer of mitochondria (from MMSCs to neural cells). Intravenous injection of MMSCs in a postischemic period alleviated the pathological indexes of a stroke, expressed as a lower infarct volume in the brain and partial restoration of neurological status. Also, MMSCs after cocultivation with neurons demonstrated more profound neuroprotective effects than did unprimed MMSCs. The production of the brain-derived neurotrophic factor was slightly increased in MMSCs, and the factor itself was redistributed in these cells after cocultivation. The level of Miro1 responsible for intercellular traffic of mitochondria was increased in MMSCs after co-cultivation. We conclude that the exchange by cellular compartments between neural and stem cells improves MMSCs’ protective abilities for better rehabilitation after stroke. This could be used as an approach to enhance the therapeutic benefits of stem cell therapy to the damaged brain.

SIGNIFICANCE

The idea of priming stem cells before practical use for clinical purposes was applied. Thus, cells were pre-conditioned by coculturing them with the targeted cells (i.e., neurons for the treatment of brain pathological features) before the transfusion of stem cells to the organism. Such priming improved the capacity of stem cells to treat stroke. Some additional minimal study will be required to develop a detailed protocol for coculturing followed by cell separation.

INTRODUCTION

It has generally been recognized that stroke is a global health burden affecting both developed and developing countries. Transient focal cerebral ischemia results in severe irreversible loss of neuronal cells with persistent neurological deficits in individuals. To date, most neuroprotective drugs that have passed through clinical trials have shown limited benefit [1]. Cell therapy is a promising and highly potent approach for the treatment and prophylaxis of neurodegenerative pathological entities. This has placed this technology at the focus of a specialized medicobiological antistroke strategy. One of the modern trends in the neuroscience specialized in neuroprotection is the development of regenerative cell technology. This includes the transplantation of different stem and progenitor cells to improve brain function and ameliorate the effects of brain damage caused by ischemic stroke.

In particular, attractive candidates for cytotherapy are multipotent mesenchymal stromal cells (MMSCs) owing to their neuroprotective potentials and low immunogenicity [2–4]. Several studies conducted in rats have demonstrated that the administration of MMSCs after experimental stroke results in smaller infarct volumes and improved functional recovery [5–9]. However,
owing to the unknown nature of such recovery, insight into the mechanisms of cell therapy for stroke by MMSCs and exploration of the essence of neuron-MMSC interactions are still needed. Furthermore, the regenerative potential of MMSCs has usually been temporally restricted, because the introduced MMSCs tend to disappear quickly after transplantation—usually within a few days [10]. Thus, a vital need exists to magnify the neuroprotective potency of MMSCs. Much evidence has suggested that the therapeutic effects of cell therapy might not be fully attributable to the replacement of damaged neuronal cells by stem cells through differentiation [11]. This suggests the existence of some paracrine mechanisms resulting from cell-to-cell interactions. Recently, the possibility of cell-to-cell crosstalk with transfer of cytoplasm [12–14] or mitochondria [14–17] was demonstrated for different types of stem and differentiated cells. For neurons and MMSCs, such interactions have been poorly explored, although the cell-to-cell transfer of cell contents could have a critical role for the physiology of both neurons and MMSCs.

In the present study, we examined the transient exchange of cytoplasm between bone marrow MMSCs and cultivated rat cortical neurons (RCNs) during coculturing and the influence of such cocultivation on the neuroprotective properties of MMSCs in the treatment of experimental stroke.

**Materials and Methods**

### Use of Animals

Experimental procedures were conducted in accordance with the European Community Council 2010/63/EU directive, and the local institutional animal ethics committee approved the present study. The experiments were performed on outbred white male rats (weight, 320–350 g). The rats had unlimited access to food and water and were kept in cages with a temperature-controlled environment (20°C ± 1°C) with the light on from 9 a.m. to 9 p.m. For all surgical procedures, the rats were anesthetized with an i.p. injection of 300 mg/kg (12%) chloral hydrate. A feedback-controlled heating pad maintained the rats’ core temperature (37.0°C ± 0.5°C) during ischemia supplemented with an IR lamp until awake.

### Cell Culture

#### Preparation of Primary Culture of Neuronal Cells

Primary neuronal cultures of cerebral cortex were obtained from embryos (16–18 days of gestation) of outbred white rats. Cultures were prepared according to Brewer [18], with modifications. The cerebral cortex was dissected, the meninges were removed, and the tissue was incubated for 15 minutes in trypsin/EDTA (0.05%/0.02% wt/vol in phosphate-buffered saline [PBS]) at 37°C. The cultures were rinsed twice with PBS and once with Neurobasal medium as described previously.

#### Preparation of Astroglial Cells

Astroglial cultures were prepared from cerebral cortical tissue of 1–2-day-old outbred white rats according to McCarthy and de Vellis [19]. After removal of the meninges, the cerebral cortices were dissected, and tissue was incubated for 30 minutes in trypsin/EDTA (0.05%/0.02% wt/vol in PBS) at 37°C. The cortex tissue pieces were rinsed with PBS and complete medium (Dulbecco’s modified Eagle’s medium [DMEM]/F12 supplemented with 10% fetal bovine serum [FBS]) (PAA Laboratories GmbH, Pasching, Austria) and 0.5 mM l-glutamine and dissociated by pipetting. Cell suspension was applied to poly-l-lysine-coated flasks. Cultures were kept at 37°C (5% CO₂). Every 3 days, one half of the medium was changed. After the astrocytes became confluent, the culture flasks were shaken for 15–18 hours (37°C, 250 rpm) to remove the overlaying microglia and oligodendrocyte precursor cells from the astrocyte layer. The supernatant was discarded, and the astrocytes were passed into a new flask. At 12–14 days after the split, the astrocytes were ready to use in experiments.

#### Cocultivation of RCNs and MMSCs

The MMSCs used for coculture experiments were detached and dissociated with 0.25% trypsin/EDTA, and the suspension was added to cultured adhesive neural cells. The coculture was incubated for 24 hours in NBM supplemented with 2% FBS for different time intervals.

#### Staining With Fluorescent Probes

The transport of cytoplasmic contents was tracked using Calcein-AM (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) cell staining. The cells were incubated with 2.5 μM Calcein-AM for 30 minutes at 37°C, followed by a wash with the DMEM/F12 medium as described previously.

### MMSCs

Human bone-marrow MMSCs were received from the Research Center of Obstetrics, Gynecology and Perinatology. Their use was approved by the Board of Research Ethics (according to Ministry of Public Health order no. 302 of 28.12.1993). The research was performed in accordance with the World Health Organization Declaration of Helsinki, and all subjects provided informed consent. The cells were cultivated in DMEM/F12 (1:1) containing 10% FBS.
Immunophenotyping of MMSCs

For immunophenotyping, MMSCs were detached and dissociated using 0.05% trypsin/EDTA, washed in PBS/1% BSA, and pelleted by spinning at 740g for 5 minutes. Cells at a concentration of 10^6 cells per milliliter were incubated for 30 minutes with anti-rat antibodies to CD44, CD90, CD45, CD34, and CD31 (each conjugated with fluorescein isothiocyanate [FITC]) and CD105 and CD31, both phycoerythrin (PE)-conjugated in a dilution of 1:100 at room temperature. Murine IgG1, labeled with FITC or PE, was used as an isotypic control. After incubation, the cells were washed in PBS, centrifuged at 740g for 5 minutes, and analyzed by flow cytometry.

Confocal Microscopy

The cell cultures were studied using an LSM510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com). Fluorescence analysis was performed in glass-bottom dishes with excitation at 488 nm and 543 nm and emission collected at 500–530 nm and >560 nm, respectively.

Flow Cytometry Analysis

Comparative analysis of the Calcein content in the cells at different stages of cocultivation was undertaken using a CyFlow cyometric analyzer (Sysmex Partec GmbH, Muenster, Germany, http://www.sysmex-partec.com). A 488-nm laser was used for excitation of Calcein Green, and emission beyond 530 nm was collected.

Immunocytochemistry

The cells were washed in PBS, fixed for 15 minutes in 4% formaldehyde with PBS at 4°C, and permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, followed by blocking in PBS with 1% BSA for 60 minutes. Specific primary antibodies (neurospecific β-III-tubulin and glial fibrillar acidic protein; Abcam, Cambridge, U.K., http://www.abcam.com) were diluted 1:500 in PBS-BSA and incubated with cells overnight at 4°C. After three 15-minute rinses in PBS-BSA, the cells were incubated for 1 hour with secondary antibodies (FITC-conjugated goat anti-rabbit, CY3-conjugated donkey anti-rabbit, and CY5-labeled anti-mouse IgG; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com) diluted 1:100.

Middle Cerebral Artery Occlusion Model of Focal Ischemia

Middle cerebral artery occlusion (MCAO) or sham surgery was performed as previously described [21]. In brief, the right common carotid artery was exposed through a midline cervical incision, and a heparinized intraluminal silicon-coated monofilament with Ø 0.25 mm was introduced via the external carotid artery into the internal carotid artery to occlude the blood supply to the middle cerebral artery territory. After 60 minutes of occlusion, the filament was gently removed, and the external carotid artery was permanently closed by cautery. In the sham-operated rats, the right common carotid artery was exposed, and the external carotid artery was electrocoagulated without introducing the filament into the internal carotid artery. The rats were injected i.v. with the cell suspension (3 × 10^6 per kilogram MMSCs, neurons, or MMSCs cocultivated with neurons) and randomly divided into the following groups: (a) sham plus saline (n = 6), (b) MCAO plus saline (n = 8), (c) MCAO plus neurons (n = 6), (d) MCAO plus MMSCs (n = 9), and (e) MCAO plus MMSC coculture (n = 7). The infarct volume was quantified by analyzing brain magnetic resonance images obtained 8 days after MCAO, as described previously [22]. The ischemic infarct volume for each group was normalized to the mean for the MCAO plus saline group.

Limb-Placing Test

A modified version of the limb-placing test, consisting of seven tasks, was used to assess the forelimb and hindlimb responses to tactile and proprioceptive stimulation [23]. The rats were habituated for handling and tested before surgery and at the first, second, fourth, and eighth postischemic days. For each task, the following scores were used: 2 points, normal response; 1 point, delayed and/or incomplete response; 0 points, no response. The test involved the following seven tasks. First, the rat’s head was kept upward at a 45° angle, and the rat was not allowed to visualize the table or contact it with its vibrissae. The third and fourth tests tested the lateral placement of the forelimb and hindlimb, respectively, by placing the rat along the edge of the table. Fifth, the rat was placed on the table, setting its hindlimbs again on the edge of the table. Each hindlimb was pulled down and gently pushed outside the table. Sixth, the rat was turned by 180° to set its forelimbs against the edge of the table and gently pushed outside the table. A normal rat would resist the push, but an ischemic rat cannot resist fully. Finally, the rat was slowly lowered with its head down. A normal rat would stretch both its forelimbs in an attempt to touch the table when the distance to the surface is approximately 10 cm. The total score for all 7 tests for healthy rats was 14.

Statistical Analysis

Statistical analyses were performed using STATISTICA, version 7.0, for Windows (StatSoft, Tulsa, OK, http://www.statsoft.com). Data are presented as the mean ± SEM. Variance homogeneity was assessed using Levene’s test. Statistical differences in infarct volume and brain swelling among the groups were analyzed using one-way analysis of variance with Tukey’s post hoc test. Statistical differences in the limb-placing test scores among the groups were analyzed using the Kruskal-Wallis test, with the Mann-Whitney U test (the Bonferroni post hoc correction was applied). Differences were considered significant at p ≤ .05.

RESULTS

Phenotyping of MMSC and Neuronal Cell Cultures

Fluorescence-activated cell sorting analysis was performed to confirm the identity of bone marrow-derived MMSCs. Cells from the fourth cultures were collected after the third passage and tested for CD44, CD90, CD73, and CD105, markers specific for mesenchymal stem cells. We found that in this culture, 96% of the cells were positive for these markers. Most tested cells were negative for CD45 and CD34, which are specific for hematopoietic stem cells, and CD31, which is a marker of endothelial cells (less than 2% of cells in culture were positive for this marker). Thus, we confirmed that the sample of cells consisted of MMSCs with no hematopoietic cells (Fig. 1A).
Characterization of Cultured Cells Derived From Embryonic Rat Brain

Phenotypic analysis of the cultured cells derived from the brain of rat embryos (Fig. 1B) was performed after 7 days in vitro using immunocytochemical staining with specific antibodies to neuronal marker β-III-tubulin and glial fibrillar acidic protein (GFAP), a marker for astrocytes. The results demonstrated that the culture was heterogeneous, containing 85 ± 3% neurons and 4% ± 0.8% astrocytes (Fig. 1C).

Cell-to-Cell Cytosol Transfer

The possibility of cytosolic content exchange between cells in coculture was explored using the fluorescent probes Calcein Green AM and Calcein Red-Orange AM. Calcein is a low-molecular-weight fluorescent dye that freely permeates lipid membranes, accumulates in the cytosol and organelles of the cell, and, after removal of the acetoxymethyl group by intracellular esterases, becomes trapped in the cell, unable to cross the membranes. Thus, one can monitor the traffic of cytosol and cellular organelles by these fluorescent probes (Calcein Green emits green fluorescence and Calcein Red-Orange emits red fluorescence).

The exchange by cytosolic components between MMSCs and RCNs in coculture was analyzed using flow cytometry. Two series of experiments with different types of cell staining before cocultivation were conducted. In one series, neural cells were stained with Calcein Green AM followed by cocultivation with unstained MMSCs in culture flasks. The cells were dissociated and analyzed using a flow cytometer after 25 minutes and 24 hours of cultivation. After 25 minutes, the mixed cell culture was clearly divided into two subpopulations by the distinct fluorescence of Calcein Green (Fig. 2A–2C). Unstained cells formed a peak in the area of low fluorescence, as confirmed by previous analysis of the control, unstained cell culture. The high-fluorescence population corresponded to the Calcein-stained cells. After 24 hours of cocultivation of MMSCs and stained neural cells, the division into two subpopulations disappeared, and a uniform distribution with a single peak was observed (Fig. 2B). If this distribution was compared with that of cells after 25 minutes of cocultivation (Fig. 2A), an obvious decrease in the number of cells with high fluorescence intensity could be seen. In the second series of experiments, MMSCs were stained with Calcein Green AM and added to unstained neural cells. After 25 minutes of coculturing, two subpopulations were detected (Fig. 2D). In contrast, after 24 hours of cocultivation, the division into two subpopulations remained, but some cells demonstrated intermediate fluorescence intensity (Fig. 2E). When the cocultivation conditions excluded direct contact between the prestained neural cells and MMSCs (achieved by cocultivation on separate coverslips), these cells exhibited a bimodal distribution of fluorescence intensity even after 24 hours of cocultivation (Fig. 2C). This observation minimized the possibility of dye transfer without intercellular contact, although some
The intercellular transfer of cytosolic probe Calcein Green between neural cells and mesenchymal multipotent stromal cells (MMSCs) or astrocytes and MMSCs was explored by monitoring the ratio of stained and unstained cells in coculture by flow cytometry. (A–C): Stained neural culture. (D, E): Stained culture of MMSCs. Two subpopulations of cells were clearly observable after 25 minutes of cocultivation (A, D), with significant coalescence or even convergence of peaks evident after 24 hours of coculture. (B, E): A lack of convergence of populations under cocultivating on separate slides for 24 hours was seen. (F, G): MMSCs stained with Calcein were cocultivated with astrocytes. (H–J): Astrocytes stained with Calcein were cocultivated with human MMSCs under conditions providing direct contact (H, I) or separation of cocultivated cells by a semipermeable membrane (J). The cells were analyzed on a flow cytometer after 25 minutes (F, H) and 24 hours (G, I, J) of cocultivation.

The transport of cytoplasm was further analyzed by confocal microscopy. MMSCs were stained with Calcein Red-Orange AM, dissociated by trypsin-EDTA, washed, and cocultured with neural cells prestained with Calcein Green AM. The cells were analyzed after 1.5 and 24 hours of cocultivation. We found that after 1.5 hours of cocultivation, the fluorescence of Calcein Red-Orange was observed only in MMSCs and the fluorescence of Calcein Green was presented only in neural cells (Fig. 3A). After 24 hours of cocultivation, some cells harboring both fluorescent probes were observed (Fig. 3B, 3D, 3E). Based on the cell morphology (Fig. 3C), it was concluded that both probes were predominantly observed in the MMSCs, and the neurons remained stained with a single dye.

Data on the preferential transport of cytoplasmic content from neurons to MMSCs was confirmed by cocultivation of MMSCs with rat neurons and subsequent immunocytochemistry. Neurons were stained with Calcein Green and cocultured with unstained MMSCs for 24 hours, followed by staining with antibodies to human nuclei to identify human MMSCs and discriminate them from rat cells. In cells positive for human nuclei, the green fluorescence of Calcein was observed only in MMSCs and the fluorescence of Calcein Red-Orange was visible, suggesting that MMSCs received cytoplasm from Calcein from neurons (Fig. 4A).

In another set of experiments, Calcein-stained MMSCs were added to unstained neurons. Staining for specific neural markers (GFAP for astrocytes, b-III-tubulin for neurons) revealed that the fluorescence of Calcein was observed only in MMSCs even after 24 hours of cocultivation (Fig. 4B). Neurons positively stained for neuronspecific tubulin (red fluorescence) did not contain Calcein (green fluorescence). Astrocytes, present in a small number in the culture and positively stained for the specific marker GFAP also did not contain Calcein (Fig. 4C).

To prove that the transfer of cytoplasm is not solely based on observations using the dye (Calcein) of relatively low molecular weight (~800 Da), we performed experiments with neurons that
were transfected with the lentiviral vector carrying GFP and then cocultivated with MMSCs. Neurons expressing GFP effectively transferred it to MMSCs (Fig. 4D). Thus, we confirmed the unidirectional transport (from neurons to MMSC) of not only cytosolic dye but also cytosolic proteins.

Mitochondrial Transfer

After cocultivation, we observed a transfer of mitochondria from MMSCs to neural cells. To track mitochondria, we cocultivated MMSCs transfected with a lentiviral construct carrying mitoDsRed and neurons transfected with a lentiviral construct carrying mitoGFP. After 2 days of cocultivation, mitochondria fluorescing in red were observed in neurons harboring mitochondria carrying green fluorescence (Fig. 5A). In contrast, MMSCs did not possess neuronal mitochondria carrying green fluorescence after cocultivation. Similar experiments were conducted by cocultivation of MMSCs with astroglial, and the transfer of mitochondria from MMSCs into astrocytes was observed, but the reverse transfer was not detected (Fig. 5B). In addition, the amount of important intracellular factor associated with mitochondria transport, specifically, Rho GTPase Miro1, was evaluated. This ubiquitous GTPase is known to be responsible for intercellular traffic of mitochondria [24]. The level of this protein was detectible in native MMSCs but was more than doubled in MMSCs exposed to the cocultivation procedure with neural cells (Fig. 5C).

These data suggest that at least in vitro, mitochondria are transferred from MMSCs to neural cells. To prove the relevance of such transfers to the situation in vivo, we used MMSCs expressing mitoGFP for injection into the rat brain. In slices of the proper region of the rat brain with injected MMSCs, we observed GFP-positive particles in cellular bodies of neurons (visualized by β-III-tubulin-positive immunostaining) adjacent to the MMSCs (Fig. 5E, 5F).

Cocultivation of MMSCs With Neuronal Culture Did not Induce Differentiation

Because we previously demonstrated that cocultivation of MMSCs with cardiac cells or epitheliocytes associates with proper differentiation [14, 16], we explored the possibility of directed differentiation of MMSCs toward neurons or astrocytes after cocultivation. However, after 24 hours of cocultivation of MMSCs with neural culture, we could not detect the expression of neuronspecific β-III-tubulin (supplemental online Fig. 1A) or astroglial-specific GFAP (supplemental online Fig. 1B) in MMSCs positive for human nuclei. Thus, we concluded that after 24 hours of cocultivation, the differentiation of MMSCs along a neuronal or glial pathway does not occur.

The Therapeutic Effect of MMSCs Under Experimental Stroke in Rats

Finally, we tested the effect of MMSCs, both with and without cocultivation with neurons, to ameliorate ischemic brain damage in rats that had undergone experimental stroke. We found that native MMSCs reduced the brain lesion volume and severity of neurological deficits when administered intravenously 1 day after...
stroke (Fig. 6). A similar introduction of MMSCs cocultivated with neurons for 24 hours demonstrated a more pronounced reduction in neurological deficits compared with introduction of native MMSCs starting from the fourth day of treatment, although the volumes of the ischemic lesion in rats treated with either cocultured MMSCs or unprimed MMSCs were the same (Fig. 6). Because MMSCs introduced after cocultivation could contain neurons, in the control experiments, primary cultures of neurons were intravenously injected into the rats. However, no improvement in neurological status or reduction in the ischemic lesion volume in the brain was observed.

Admitting a role of exosomes in the communication between neural cells and MMSCs, which can contribute to the beneficial effect of MMSCs, we primed MMSCs with conditioned medium from neurons, which presumably could contain exosomes. For this purpose, neurobasal medium, collected after cultivation of approximately $5 \times 10^6$ neurons in a 175-cm² culture flask with 25 ml of medium, was put on the same number of MMSCs ($5 \times 10^6$) cultivated in a separate flask. After 24 hours of cultivation in this medium, MMSCs were dissociated and used for administration to rats, which underwent MCAO. The second group of rats after MCAO received untreated MMSCs. Analysis of the neurological deficit and extent of the ischemic lesion in the brain did not reveal differences in the effect of MMSCs and MMSCs treated with exosome-containing medium (i.e., the improvements in neurological deficit were identical in both groups for up to 8 days of observation; data not shown). Thus, assuming a potential

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**Figure 5.** Transfer of mitochondria from MMSCs to neural cells in vitro and in vivo. MMSCs were transfected with Discosoma species red fluorescent protein fused with the mitochondrial localization signal of cytochrome c oxidase subunit VIII construct (red fluorescence in mitochondria), and neurons were transfected with green fluorescent protein (GFP) fused with the mitochondrial localization signal of cytochrome c oxidase subunit VIII (mitoGFP) construct (green fluorescence). Red mitochondria in neurons (A, arrowhead) and astrocytes (B, arrowheads) according to cell morphology are shown. (C): Miro1 levels, the protein responsible for mitochondria transfer, were increased in MMSCs after coculturing with neurons. (D): The densitometry results represent an average over three bands obtained from the three different MMSC cultures and three cocultures. Band densities for Miro1 were normalized to the density of the total actin bands. (E, F): Slices of the rat brain with noticeable injected mitoGFP-expressing MMSCs. Neuronal cells are discriminated by staining for β-III-tubulin specific for neurons (red fluorescence). Note that to observe the green fluorescent particles in neurons, the detector gain for green fluorescence was maximally increased, such that the fluorescence of MMSCs is in saturation and individual mitochondria in MMSCs cannot be resolved. Nuclei were stained with To-Pro-3 (magenta). Scale bars = 10 μm (A, B), 100 μm (E), and 20 μm (F). Abbreviations: MMSC, mesenchymal multipotent stromal cell; MMSCcocult, middle cerebral artery occlusion plus MMSCs previously cocultivated with neurons.

**Figure 6.** Beneficial effects of MMSC injection after experimental insult. (A): Representative T2-weighted magnetic resonance (MR) images from coronal brain sections obtained at 8 days after reperfusion. Hyperintensive regions refer to ischemic areas. (B): The volume of the ischemic lesion in the brain on day 8 after MCAO as determined from MR images. Intravenous injection with either native MMSCs or MMSCs previously cocultivated with neurons caused a significant decrease in the volume of brain damage. The injection of neurons had no effect on the size of the lesion. (C): Effect of MMSC transplantation on neurological status at different periods after insult. *p < .05 compared with ischemic saline controls; †, p < .05 compared with ischemic MMSC-treated rats. Abbreviations: MCAO, middle cerebral artery occlusion; MMSC, mesenchymal multipotent stromal cell; MMSCcocult, MCAO plus MMSCs previously cocultivated with neurons.

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exchange with exosomes, it did not have any noticeable effect on the neuroprotective properties of MMSCs.

Production of Growth Factors

In order to understand the superior therapeutic effect of MMSCs cocultivated with neurons, we analyzed these MMSCs for the production of some of the most important neurotrophic factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β) before and after cocultivation. In cocultured MMSCs, the production of BDNF slightly increased (supplemental online Fig. 2A–2C), but that of TGF-β and VEGF did not change after 1 day of cocultivation (data not shown). However, although the BDNF-positive material was distributed uniformly in the cytoplasm of untreated MMSCs, after cocultivation with neurons, this material was redistributed in the cell, becoming localized in the vesicular structures adjacent to the plasma membrane (supplemental online Fig. 2B).

DISCUSSION

MMSCs have recently been considered as particularly attractive candidates for cell therapy for stroke because of their proven neuroprotective properties and low immunogenicity. To date, stem cell scientists have been clearly divided into two camps—some believe that the introduction of the stem and progenitor cells into the damaged organ leads to differentiation of the latter into the cells lost or damaged in the body and others suggest a positive paracrine effect achieved by the synthesis of certain signaling molecules essential for survival [25, 26]. Both groups acknowledge the positive therapeutic effect of the introduction of stem and progenitor cells for the treatment of organ pathological entities. Regarding the neurological benefits, several studies conducted on rats have demonstrated that the introduction of MMSCs after experimental stroke reduces the volume of infarction and improves functional recovery of the brain [5–9]. Among the arguments supporting a paracrine effect of stem cells, one is the observation of the rapid disappearance of MMSCs from the organism after transplantation, usually within a few days. In addition, very often the positive effects of MMSCs are observed early after their introduction, at which time it is very unlikely that they have been integrated into the nervous tissue in the form of functional neurons. Although markers of differentiation of MMSCs into neural cells types are often observed, to date, complete differentiation into cells possessing a neuronal action potential and forming vital junctions with neurons of a recipient has not been shown [27, 28]. This paradox redirects the researcher’s attention toward the paracrine mechanisms by which MMSCs can protect neural tissue. Today, it is widely accepted that MMSCs enhance tissue regeneration by secreting growth factors and cytokines, including BDNF, nerve growth factor, VEGF, and some others [29, 30]. In addition, MMSCs might modulate the inflammatory response, which plays an important role in postischemic brain damage, because they secrete a number of immunomodulatory factors, such as prostaglandin E2, tumor necrosis factor-α, TGF-β, interleukin 6, and others [31–34]. However, the mechanisms underlying the full regenerative potential of MMSCs remain to be elucidated.

One way to distinguish between alternative routes of impact of stem cells on organ function is to explore the cellular communications between native and introduced cells. This leads to the search for chemical compounds that stem cells might form or secrete into the external milieu after their cocultivation with differentiated cells. This is also applicable to the host cells, which can respond to the presence of alien cells by the synthesis of some biological compounds. This “crosstalk” between “roommate” cells or even remote organs within one organism has been suggested, and a large amount of data supporting this phenomenon has been published [35–39]. However, intercellular communication by secretion of some biologically active compounds for reception by neighboring cells is somewhat limited by diffusion. This limitation can be minimized by direct and sealed transfer of cell components from cell to cell by newly formed intercellular contacts. One example of such a contact is the gap junctions, which provide intercellular communication with the involvement of connexin 43 [40] and result in electrical coupling of neighboring cells and transmission of depolarization signals. Several types of intercellular communications that could be relevant to the data in the present study involve directed migration of cytosolic components between contacting cells along cellular extensions, in particular, tunneling nanotubes [41, 42]. For a number of differentiated cells in direct contact with progenitor cells, evidence of a cytoplasmic exchange has been presented [43, 44], including intercellular transport of mitochondria.

Direct intercellular contact probably provides the most low-cost method for the exchange of chemical information between cells; however, it is limited by the very small ratio of stem cells to differentiated cells and, accordingly, the small number of intercellular contacts. The more costly method is the secretion by the cells of biologically active molecules [45] into the external milieu (the cultivation medium of contacting cells in vitro and the extracellular volume when the cells are contacting in vivo). On a higher level, chemical communication between cells can use interorgan crosstalk, which involves the secretion of some factors into the circulating blood, which then delivers these factors to distant organs. The second and, in particular, third mechanisms of crosstalk undermine the extremely high efficiency of biologically active molecules, which are strongly diluted in the extracellular milieu or the blood.

Attempts to track the mechanisms of such interorgan signal transmission have been made using parabiosis, an experimental model in which the circulatory systems of animals of the same or different age (homochronic or heterochronic systems, respectively) were united [46]. In particular, it was found that heterochronic parabiosis enhances the proliferation of neural stem cells in older animals and that these neural stem cells differentiated into neuroblasts and migrated to the olfactory bulb, resulting in a significant increase in the discrimination of smell [47]. However, the genesis of this effect is obscure and the question of whether these changes result from enhanced neurogenesis or a general response to parabiosis remains unclear. For a biologically active molecule, the fundamental understanding of global or partial mechanisms of such transfer (which could be beneficial or deleterious) is of great importance.

In the present study, we explored the role of intercellular contacts and exchange of cellular components in providing paracrine effects in the implementation of the neuroprotective potential of MMSCs in stroke. We demonstrated that neural cells, both neurons and astrocytes, are capable of forming intercellular contacts with MMSCs and sharing their cytosolic contents. In this case, the transfer of cytosol, evaluated by the transport of Calcein, occurred primarily in the direction from neurons to MMSCs. Alternatively, we showed that the transport of mitochondria was from MMSCs to neurons or glial cells. Such mitochondrial transfer between MMSCs and cardiomyocytes or renal epithelial cells was
previously reported by us [14, 16], and others [15, 48, 49]. Moreover, it has been shown that such a mitochondria “donation” can result in recovery of lost mitochondrial functions in recipient cells [50]. Moreover, in a number of studies, it has been shown that mitochondrial transport from MMSCs to damaged cells is responsible for the therapeutic effect of MMSCs. This emphasizes the significance of our findings on the induced elevation in MMSCs of Miro1, which is responsible for mitochondrial transport. Earlier it was demonstrated that a genetically induced increase of Miro1 in MMSCs results in greater therapeutic efficiency of MMSCs [24]. Accordingly, a higher level of Miro1 can be reached, not only by introduction of a genetic construct, but also by a cocultivation procedure.

Our cocultivation system containing MMSCs and neurons can be considered a model for studying the intercellular interactions of MMSCs with brain tissue when MMSCs are transplanted after stroke. Intravenously introduced MMSCs migrate to the brain [5, 51, 52] and possibly can communicate with neurons and astrocytes, sharing cytoplasmic content and mitochondria. This could lead to changes in the properties of MMSCs that enhance their neuroprotective effect, which we observed after the administration of MMSCs previously cocultured with neurons compared with untreated MMSC.

To gain insight into the nature of the enhanced therapeutic effect of MMSCs cocultured with neurons, we analyzed MMSCs for the production of one of the most important neurotrophic factors, BDNF, before and after cocultivation. In intact MMSCs, BDNF is produced to some extent but will be distributed uniformly in the cytoplasm of the MMSCs. However, after cocultivation with neurons, only a slight increase in the BDNF levels was observed, but the BDNF had been redistributed in the cell and was localized in the vesicular structures adjacent to the plasma membrane. Thus, it is possible that after cocultivation, MMSCs more actively secrete BDNF. Perhaps it is the BDNF produced by MMSCs that is responsible for the improvement in the neuroplasticity of the infarcted brain observed after injection of native MMSCs and, in particular, MMSCs cocultivated with neurons.

In contrast, the observed in vitro transport of mitochondria from MMSCs to neurons and glial cells can occur in vivo. It is known that mitochondrial damage and the associated oxidative stress are two of the main causes of the brain dysfunction after a stroke [53–55]. In this case, the transport of healthy mitochondria from MMSCs to neurons, where the mitochondria are damaged, can provide better survival and function of nerve cells, as was shown for other cell types [50].

Ultimately, we can propose a tentative scheme highlighting the role of intercellular crosstalk in the implementation of the neuroprotective effects of the MMSCs in stroke (Fig. 7). According to this scheme, the cytosol of neural cells is transferred to MMSCs, inducing a more efficient production of neurotrophic factors that increases the neuroplasticity of the brain and results in more effective recovery after a stroke. For transplanted cells primed by an in vitro cocultivation, the recovery of neurological functions after ischemic damage will occur sooner (Fig. 6C). In contrast, the beneficial outcomes of intercellular contacts with MMSCs might result from the transfer of their mitochondria to neuronal cells, where mitochondria have been damaged during ischemia. The increased population of healthy mitochondria in neurons might provide better survival and maintenance of energy balance, thereby reducing or delaying nerve cell death and postischemic neurodegeneration.

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AUTHOR CONTRIBUTIONS

V.A.B., L.D.Z., and I.B.P.: data analysis and interpretation, collection and/or assembly of data; D.N.S.: data analysis and interpretation, collection and/or assembly of data, manuscript writing, conception and design; A.A.K.: collection and/or assembly of data; E.Y.P.: data analysis and interpretation, manuscript writing, conception and design; G.T.S.: conception and design, provision of study material or patients; D.B.Z.: conception and design, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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