Abstract. Background/Aim: Peripheral neurotoxicity is a dose-limiting factor of many chemotherapeutic agents, including cisplatin. Mesenchymal stem cells are promising for the treatment of several neurological disorders, and our aim was to verify the neuroprotective potential of human mesenchymal stem cells (hMSCs) on dorsal root ganglia (DRG) exposed to cisplatin. Materials and Methods: DRG were exposed to different cisplatin concentrations and then co-cultured with hMSCs or with hMSC-conditioned medium. Results: hMSCs showed a neuroprotective effect on cisplatin-induced death of DRG, mediated by direct contact. Moreover, DRG exhibited an MSC-dependent promotion of neurite outgrowth, in particular at early time points. For this effect, the expression of Neurite Outgrowth Inhibitor (NOGO) and Myelin Associated Glycoprotein (MAG) by hMSCs was pivotal. Conclusion: hMSCs are a promising tool for reducing the neurotoxic effect of cisplatin.

An important problem regarding the administration of chemotherapeutic drugs is the induction of sensory peripheral neuropathy, which often makes it necessary to stop the treatment or to reduce the administered dose of the drug (1); neuropathy may even persist after the therapy is interrupted. This important and serious side-effect is due to the accumulation of the drugs in the sensory dorsal root ganglia (DRG) which are not protected by the blood–brain barrier and, therefore, represent the main target of the neurotoxic action of the drug (1). Among the different neurotoxic anticancer drugs, cisplatin is one of the most extensively studied, together with the sensory peripheral neuropathy that it induces. In particular, cisplatin acts on the DRG in a two-fold way: by damaging the neuritic processes of DRG, and by inducing the neuronal death of sensory neurons which, together with satellite cells, constitute the DRG (2). Among the various therapies suggested to reduce cisplatin neurotoxicity, mesenchymal stem cells (MSCs) represent suitable candidates for supporting protection of DRG because of their biological properties and their possible autologous use (3). It has already been demonstrated that rat-derived MSCs (rMSCs) have a positive effect on DRG, through multiple mechanisms (4-6), but since rat and human MSCs (hMSCs) have different biological behavior (7, 8), it is also essential to investigate the effect of hMSCs, with the purpose of autologous clinical use. Aim of this study is, therefore, to verify the effect of hMSCs on cisplatin-treated DRG, also focusing our attention on the putative molecules involved in their action.

Materials and Methods

All procedures on animals were performed under anesthesia and in accordance with the European Communities Council Directive 86/609/EEC.

Drug. Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in sterile water to make a stock solution of 1 mg/ml, which was diluted with medium to obtain the different working concentrations.

DRG explants. DRG from 15-day-old embryonic Sprague-Dawley rats were aseptically removed and cultured onto a single layer of rat-tail collagen surface in 35-mm dishes (4 ganglia/dish). DRG were incubated in AN2 medium (Minimum Essential Medium plus...
10% calf bovine serum, 50 μg/ml ascorbic acid, 1.4 mM L-glutamine, 0.6% glucose) plus 5 ng/ml Nerve Growth Factor (NGF) for 2 hours in a 5% CO₂ humidified incubator at 37°C. After 2 h, the DRG were treated with different concentrations of cisplatin (3.5 and 7.5 μg/ml) for 24 h and the medium was then replaced with fresh medium. The AN2 medium was changed every 3 days. At different time points, DRG were photographed (48 h, and 1, 2, 3 and 4 weeks). For each DRG, the longest neurite and the area of death were measured using ImageJ (NIH, Bethesda, MD, USA). These measurements were compared with a calibration grating photographed under identical conditions. The percentage area of death was determined as: (dead area/total DRG area)×100.

**hMSC Culture.** After obtaining informed consent, hMSCs were isolated from bone marrow harvested from the iliac crest of six healthy donors as described previously (13) and cultured in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Lonza, Verviers, Belgium) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 250 mg/ml fungizone (Lonza), and 10% defined fetal bovine serum (FBS; HyClone, Logan, UT, USA). Cells were plated in culture flasks at a high density and were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The cells were used from the 4th to the 8th passages.

**Direct co-culture.** hMSCs were added to DRG explants at a density of 80,000 cells/dish. Co-cultures were treated with AN2 medium supplemented with 5 ng/ml NGF and then photographed at different time points to analyze the progressive neurite elongation (48 hours and 1, 2, 3 and 4 weeks). The AN2 medium was changed every 3 days. Each experiment was performed three times to validate the results.

**hMSC-conditioned medium (CM).** A total of 80,000 hMSCs were seeded onto collagen-coated dishes and cultured in AN2 medium 10% Calf Bovine Serum plus NGF. After 24 h, the medium was collected and used as CM at a 1:1 ratio with fresh AN2 medium plus NGF. The medium was changed every 3 days. Each experiment was performed three times.

**Sham CM.** In a parallel experiment, to verify whether the effect of hMSCs on neurite elongation was specific, the culture medium from untreated DRG was used at a 1:1 ratio with fresh AN2 medium plus NGF as sham CM.

**Immunofluorescence experiments.** hMSCs were added to DRG explants as described above and a fluorescence study was performed using anti-Neurite Outgrowth Inhibitor (NOGO)-A/B/C (1:50; Millipore, Billerica, MA, USA), and anti-Myelin Associated Glycoprotein (MAG) (1:2000; Abcam, Cambridge, UK). Phalloidin (1:20; Invitrogen, Carlsbad, CA, USA) was used to visualize actin filaments of hMSCs. For the fluorescence studies, the cultures were washed with Phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde. Non-specific binding was blocked with 3% Bovine Serum Albumine in PBS for 1 h, then cells were incubated overnight at 4°C with the primary antibodies. After washing with PBS, the cultures were incubated with secondary antibodies for 1 hour at room temperature. Then cells were washed and coverslips were mounted using mounting medium with DABCO (Sigma-Aldrich, St. Louis, MO, USA). To study the expression of NOGO and MAG, cells were examined using confocal laser microscopy, carried out with a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA, USA) equipped with a krypton/argon laser. Noise reduction was achieved by Kalman filtering during acquisition.

**Western blot analysis.** Cells were washed twice with PBS, and solubilized in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 5 mM EGTA] containing protease and phosphatase inhibitors (4 mM phenylmethlysulfonyl fluoride, 1% aprotinin, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate) (Sigma Chemical Co.). Total proteins were measured with a Coomassie® Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Protein aliquots (15 μg) were solubilized in Laemmli buffer 5X, boiled for 5 min and run on 10% SDS-polyacrylamide gels.

Immunoblotting analysis was performed according to the manufacturer’s instructions [anti-NOGO-A/B/C, 1:1000 (Millipore); anti-MAG, 1:500 (Abcam); anti-ß-actin, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)]. The immunoreactive proteins were visualized using ECL Prime (GE Healthcare Piscataway, NJ, USA). All data were normalized by actin immunoblotting.

**siRNA.** NOGO and MAG siRNA were prepared following the manufacturer’s protocol (Santa Cruz Biotechnology). hMSCs were cultured with NOGO and MAG siRNA for 2 days and every day the medium was collected and used as siRNA hMSC CM. Downregulation by NOGO and MAG siRNA was assessed by western blot analysis.

A statistical analysis was carried-out with the one-way ANOVA and Tukey post hoc test with the statistical package GraphPad Prism (GraphPad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered statistically significant.

**Results**

**Effect of hMSCs on the survival of cisplatin-treated DRG.** In order to investigate the protective effect of hMSCs, DRG were treated for 24 h with 5 μg/ml cisplatin. At the end of treatment, the cisplatin was removed and 80,000 hMSCs were added. The percentage area of death in untreated and treated DRG cultured with and without hMSCs at different time points (1, 2, 3 and 4 weeks) was measured. The central dark DRG area was considered as the area of death (6), and represented the degenerating cellular bodies of neurons and satellite cells (Figure 1a). After 2 weeks of culture, cisplatin-treated DRG had a percentage of death of about 90-100% (Figure 1b). On the contrary, cisplatin-treated DRG directly co-cultured with hMSCs had a percentage area of death of 50% after 2 weeks and of 70% after 4 weeks. This difference in percentage death between the cisplatin-treated DRG with and without hMSCs was statistically significant (p<0.05) starting from 2 weeks of culture (Figure 1b). In order to verify whether the positive effect of hMSCs was due to the secretion of soluble factors, DRG were also cultured in the presence of hMSC CM. CM of hMSC did not improve
survival of cisplatin-treated DRG, suggesting that direct contact rather than release of soluble molecules by hMSCs was involved in the hMSC promotion of DRG survival throughout the observation period.

The effect of hMSCs on neurite length. The neurite length of untreated and cisplatin-treated DRG cultured with and without hMSCs was measured after 48 h of culture. Moreover, to verify whether hMSCs affected neurite length
by releasing soluble factors, we also used CM derived from hMSCs. The treatment of DRG with different cisplatin concentrations (3.5, 5 and 7.5 μg/ml) for 24 h reduced the elongation of neurite length by about 30, 50 and 75%, respectively, at an early time point (48 h). At the same time, an increase in neurite length was observed both for DRG directly co-cultured with hMSCs, and for DRG cultured with hMSC CM compared to DRG cultured alone (Figure 2a and b), thus suggesting that treatment with hMSCs improves neurite length as an early event, and that this effect may be mediated by soluble factors.

At longer time points (4 weeks), cisplatin-treated DRG cultured with or without hMSCs presented similar DRG neurite length, as expected, since cisplatin has a reversible effect, while DRG cultured with hMSC CM exhibited a reduction in neurite length (Figure 2 c).

Figure 2. Effect of human mesenchymal stem cells (hMSCs) on neurite elongation of dorsal root ganglia (DRG). a: DRG when untreated (Ctrl), treated with cisplatin (CDDP; 5 μg/ml) alone, with hMSC conditioned medium (CM), and in direct co-culture with hMSCs. Bar=500 μm. b: Neurite length of DRG when untreated, treated with cisplatin (3.5 μg/ml, 5 μg/ml and 7.5 μg/ml) cultured alone, in culture with hMSC CM and in direct co-culture with hMSCs. *p<0.05 with respect to DRG treated with 5 μg/ml cisplatin. c: Neurite length of DRG when untreated, treated with 5 μg/ml cisplatin cultured alone and cultured with hMSC CM or direct co-culture with hMSC after 1 month of culture.
Figure 3. Analysis of the molecules involved in neurite elongation. a: Immunofluorescence on human mesenchymal stem cells (hMSCs). Neurite Outgrowth Inhibitor (NOGO) and Myelin Associated Glycoprotein (MAG) in red, phalloidin in blue. Scale bar=50 μm. b: Western blot analysis of NOGO and MAG on hMSCs (Ctrl) and hMSCs treated with siRNA specific for NOGO and MAG. Actin was used as internal loading control. c and d: Neurite length of untreated DRG and cisplatin (CDDP)-treated DRG cultured alone, in co-culture with hMSCs, and cultured with conditioned medium derived from hMSCs (hMSC CM), from DRG (sham CM), or from hMSC treated with NOGO siRNA or MAG siRNA, after 48 h (c) and 4 weeks of culture (d). *p<0.05 with respect to cisplatin-treated DRG when cultured alone.
**NOGO and MAG involvement in neurite elongation.** In order to clarify the effect of soluble factors released by hMSCs on DRG neurite elongation, some molecules that play a pivotal role in neurite sprouting were examined, and we focused our attention on NOGO and MAG. After 4 weeks, NOGO and MAG expression in hMSCs was demonstrated both by immunofluorescence and by western blot experiments (Figure 3a and b).

To investigate in greater depth the role of these molecules on neurite elongation induced by hMSCs, down-regulation experiments with siRNA were performed on hMSCs, and the CM was then used to culture DRG. Moreover, in order to verify whether the effect of hMSCs on neurite reduction at longer time points was specific, experiments with sham CM (see Materials and Methods) were also performed. After NOGO or MAG down-regulation (verified by western blot analysis, Figure 3b), after 48 hours of culture hMSC CM was no longer able to increase the neurite length, which was found to be similar to that of cisplatin-treated DRG cultured alone and cisplatin-treated DRG cultured with sham CM (Figure 3c).

At longer time points (4 weeks), cisplatin-treated DRG cultured with hMSCs had the same neurite length as cisplatin-treated DRG cultured alone. Cisplatin-treated DRG cultured with hMSC CM or with sham CM exhibited a reduction in neurite length with respect to the neurite length of cisplatin-treated DRG cultured alone, suggesting that the reduction was due to the use of CM (therefore with fewer nutrients), rather than to the specific action of hMSC CM. Shorter neurites were also present after siRNA down-regulation of NOGO and MAG, suggesting that NOGO and MAG were not involved in the reduction in neurite length induced by CM (Figure 3d).

**Discussion**

Chemotherapeutic-induced neurotoxicity is a very severe problem that limits the use and, consequently, the therapeutic effect of anticancer drugs. The current therapies used to limit this side-effect have mainly been ineffective and, as an alternative, MSCs have been proposed for the treatment of peripheral neuropathies (14). In a previous study, it was demonstrated that rMSCs promoted the survival of cisplatin-treated DRG only if co-cultured in direct contact, while they had no effect on neurite elongation. Since it has been demonstrated that hMSCs and rMSCs have different properties (7, 8), and in order to make clinical use of MSCs, it was necessary to study the effect of hMSCs on cisplatin-treated DRG.

Herein we demonstrated that hMSCs also had a protective effect on survival of cisplatin-treated DRG and this effect was mediated by direct contact rather than due to the release of soluble molecules by hMSCs. Some authors have demonstrated a positive effect of CM derived from hMSCs on the survival of different cell populations (5, 15), suggesting the importance of the release of soluble factors to improve neuronal survival. This difference with respect to our results may be explained by the different cellular models used (organotypic vs. primary cultures or cell line) and time points for evaluation of survival. The evaluation of long-lasting survival of DRG is important with regard to the possible clinical application, making it necessary to observe the effect of hMSCs over the course of time.

Concerning the effect of hMSCs on rat DRG neurite length, we observed an early positive effect of both direct culture with hMSCs and that with hMSC CM on cisplatin-treated DRG, suggesting that the secretion of neurotrophic factors by hMSCs could be responsible for the modulation of neurite length. At this time point of culture, many researchers observed a positive effect of hMSC CM on neurites in different kinds of cellular models (5, 15-17), suggesting that at early time points, hMSCs release some trophic factors that mediate neurite elongation.

To investigate the positive effect of hMSCs on neurite length in greater depth, we studied some molecules that are involved in neurite sprouting, namely NOGO and MAG. These molecules mediate the development and the plasticity of the nervous system (10-12) and, moreover, are axonal elongation inhibitory molecules in vivo if present in the glial scar after nerve damage (18-20). hMSCs expressed these molecules and, after their siRNA-specific down-regulation, we observed that the positive effect of hMSC CM was no longer present, suggesting that NOGO and MAG were involved in neurite elongation after cisplatin treatment. The positive effect of molecules that usually inhibit nerve regeneration might be explained by the fact that we used embryonic DRG in which MAG could promote neurite elongation instead of inhibiting it, as previously reported by others (12). Moreover, in the developing nervous system, NOGO and MAG are involved in neurite sprouting and regulate axonal growth and branching (9, 12). At longer time points, hMSCs were no longer able to rescue cisplatin-treated DRG neurite elongation, thus behaving like rMSCs, despite the structural and behavioral differences existing between these kinds of cells (7, 8). The same neurite length of cisplatin-treated DRG cultured alone and directly co-cultured with hMSCs after 4 weeks can be explained, as for rMSCs, by the fact that the effect of cisplatin is reversible, and at longer time points the neurites did not need hMSC to grow, as occurred at early time points. After 4 weeks, the neurites of cisplatin-treated DRG cultured with siRNA hMSC CM had the same length as those cultured with hMSC CM and sham CM, demonstrating that MAG and NOGO were not involved in the long-lasting blocking of neurite elongation caused by the use of CM (with a lower amount of nutrients) and a lack of fresh medium.
Rat and human MSC, despite their different in vitro properties, had the same effect on cisplatin-treated DRG, promoting the long-lasting survival of cisplatin-treated DRG if co-cultured in direct contact and not effecting neurite elongation at longer time points. This information could be important regarding the use of rMSCs as a cellular model for studying MSCs. Moreover, the fact that MSCs from two different species have the same biological effect makes it possible to hypothesize a general mechanism of MSC action: a supportive role mediated both by contact and by release of soluble factor(s). We also demonstrated a possible molecular mechanism that could explain the positive effect of hMSCs on neurite length, involving NOGO and MAG expression in hMSCs.

Acknowledgements

The Authors are grateful to Dr. E. Genton for her language assistance. This work was supported by MIUR – FIRB Futuro in Ricerca 2008 Prot. N° RBFR08VSV1_001.

References