SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

NOME E COGNOME: Laura Micheli

UNIVERSITÀ: Università degli Studi di Firenze

DIPARTIMENTO (in caso di borsa per soggiorno all'estero specificare l'ente presso cui si è svolta la ricerca): Dipartimento di Neuroscienze, Psicologia, Area del Farmaco e salute del Bambino (NEUROFARBA)

TUTOR (in caso di borsa per soggiorno all'estero specificare il tutor dell'ente presso cui si è svolta la ricerca): Prof.ssa Carla Ghelardini

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RELAZIONE:

Introduction

Oxaliplatin is an anticancer drug primarily used in the treatment of metastatic colorectal cancer alone or in combination with other agents (De Gramont et al., 1997; Dìaz-Rubio et al., 1998). Differently from previous platinum compounds, oxaliplatin infusion is associated with mild renal-and hematological toxicity with no grade 3-4 anemia, thrombocytopenia or neutropenia (Dìaz-Rubio et al., 1998; Holmes et al., 1998). Nevertheless, oxaliplatin administration induces the development of a neuropathic syndrome in 50% of patients receiving a total cumulative dose > 1000 mg/m^2 over four cycles or more of therapy (Kannarkat et al., 2007; De Gramont et al., 2000; Souglakos et al., 2002). Neuropathic syndrome is characterized by important alteration to peripheral and central nervous system resulting in aberrant somatosensory processing (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013a). Symptoms of oxaliplatin neurotoxicity include altered pain threshold, paresthesia and dysesthesia that affect both lower and upper extremities in a characteristic "glove and stocking" distribution (Kannarkat et al., 2007; Wolf et al., 2008). Neurotoxicity is a dose-limiting side effect and a quality of life impairing factor for cancer survivors since symptoms do not remit after chemotherapy. Currently, there are no effective therapies (Hershmann, 2014).

The interest in adult mesenchymal stem cells (MSCs) for the treatment of neuropathic pain is increasing. Adult MSCs offer a pluripotent cellular source for replacing injured tissues and at the same time represent a source of neuroprotective and anti-inflammatory mediators that oppose the effect of damage. Briefly, MSCs are able to modulate the inflammatory cascade associated to immune-related diseases, such as

rheumatoid arthritis (Gonzalez-Rey et al., 2010), but also painful neuropathies consequent to nerve trauma (Sacerdote et al., 2013A; Watanabe et al., 2015) or to metabolic alterations (Shibata et al., 2008). MSCs can be isolated from fetal liver, umbelical cord blood and bone marrow; alternatively stromal vascular cell fraction (SVF) of adipose tissue is an abundant and easily accessible source of MSCs. Adipose derived stem cells (ASCs) are characterized by phenotypic characteristics and differentiating capabilities similar to bone marrow or embryonal derived MSCs (Baglioni et al., 2009; Kern et al., 2006). Indeed, several studies have demonstrated that ASCs are able to efficiently differentiate toward adipogenic- chondrogenic- neurogenic- osteogenic-like cells (Quirici et al., 2010; Choudhery et al., 2013) and to modulate biomolecular signals (Nasef et al., 2008; Yoo et al., 2009; Sacerdote et al., 2013; Gonzalez-Rey et al., 2010). The purpose of this study was to evaluate rat ASCs (rASCs) administration in a rat model of oxaliplatin induced neuropathy measuring their pain relieving effect.

Materials and methods

rASCs preparation

The sternal fat pad from Wistar rats, weighing approximately 550–600 g, was harvested in a sterile fashion, washed with phosphate-buffered saline (PBS), minced and digested with 2 mg/mL collagenase type I (from Clostidium histolyticum, Sigma-Aldrich, St. Louis, MO, USA) in PBS, for 30 min at 37°C in a shaking water bath. The sample was filtered through a 100 µm mesh filter to remove debris. Enzyme activity was neutralized with medium containing 10% fetal bovine serum (FBS; GIBCO, Life Technologies – Thermo Fisher Scientific, Waltham, MA, USA) and sample was centrifuged at 1680 r.p.m. for 10 min to obtain a high-density cell pellet. The pelleted stromal vascular fraction (SVF) containing ASCs was resuspended and plated in Nutrient mixture F-12 Ham (Sigma-Aldrich, Milan, Italy) supplemented with 20% FBS and 1% penicillin-streptomycin (P/S, Sigma-Aldrich, Milan, Italy) and incubated at 37°C in 5% CO_2 atmosphere. After 4 h, nonadherent cells were removed, and adherent cells were washed twice with medium. The medium was replaced every 2-3 days, and cells with 80-90% confluence were detached with the use of trypsin/ethylenediaminetetraacetic acid (EDTA) (0.5%) and passed. rASCs at early passages with 80-90% confluence were detached by light trypsinization and used for surface marker characterization. rASCs at passage 1 (P1) were frozen in a 10:1 ratio of FBS/dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) and cryopreserved in liquid nitrogen until required for the *in vivo* studies. In all experiments, only cells at P2 of culture were used.

Flow cytometry analysis

Cell immunophenotypical analysis of the SVF and cultured cells was performed by using the FITC-, PE-, APC or eFluor 450- conjugated monoclonal antibodies (mAbs) against CD90, CD79a, CD29, CD45 (e Biosciences, San Diego, CA, USA).

Briefly, rASCs were detached using EDTA buffer pH 7.2, washed, and resuspended in buffer EDTA+FBS 1%, for each marked sample there is a background samples. Cells were incubated with the specific mAbs at room temperature for 30 min. Cells were then washed with buffer EDTA+FBS 1%, centrifugated for 10 min at 1680 r.p.m. and resuspended in 0.8 mL of the same buffer, analyzed by flow cytometry on FACScalibur (Becton Dickinson, Sparks, Maryland) and data were analyzed on DOT-PLOT bi-parametrical diagrams using CELL QUEST software (Becton Dickinson).

rASC differentiation

For adipogenic differentiation, rASCs at 80/90% confluence were cultured with low-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.5 mmol/L isobutylmethylxanthine, 100 µmol/L indomethacin, 0.5 µmol/L dexamethasone and 10 µg/mL insulin with medium change every 3 days. After 21 days, cells were fixed with 10% formalin for 30 min, dried and stained with 0.5% Oil red O.

For osteogenic differentiation, rASCs were induced with stromal medium supplemented with 1 nmol/L dexamethasone, 2 mmol/L glycerolphosphate and 50 µmol/L ascorbate-2-phosphate, with media change every 2/3 days for 14 days. Cells were then fixed in 10% formalin and stained with 40 mmol/L alizarin red (pH 4.1).

Sections were examined using an Olympus BX40 microscope (Olympus, Milan, Italy) and photographed using a digital camera (Olympus DP50).

Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200-250 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving

animals have been reported according to ARRIVE guidelines (Kilkenny et al., 2014). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Oxaliplatin-induced neuropathic pain model

Neuropathic pain was induced by intraperitoneally (i.p.) injection of 2.4 mg kg^{-1} oxaliplatin (Sequoia Research Products, Pangbourne, UK) dissolved in a 5% glucose-water solution administered i.p. for 4 consecutive days every week for 2 weeks (Cavaletti et al., 2001 with modifications).

Stem cells administration

Rat ASCs were suspended in 400 µL of DMEM with 2.5 % of heparin 25000 U.I./5mL and injected in the tail vein in the amount of $2x10^6$ per rat; control rats received the same amount of vehicle. Administration was performed 4 times on days 6, 13, 16 and 20.

Paw-pressure test

The nociceptive threshold of rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton and collegues (1988). Briefly, before and 6, 24, 48, 72, (96) h after rASCs administration, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g.

Von Frey test

Animals were placed in 20 cm × 20 cm Plexiglas boxes equipped with a metallic mesh floor, 20 cm above the bench. Animals were allowed to habituate themselves to their environment for 15 min before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: before and 6, 24, 48, 72, (96) h after rASCs administration the withdrawal threshold was evaluated by applying forces ranging from 0 to 50 g with a 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the mesh floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum force required to elicit a robust and immediate withdrawal reflex of the paw. Measurements were performed on the anterior paw since it shows the higher sensitivity to this test (Di Cesare Mannelli et al., 2013a). Voluntary movements associated with locomotion were not considered as a withdrawal response. Stimuli were applied to each anterior paw

at 5 s intervals. Measurements were repeated 5 times and the final value was obtained by averaging the 5 measurements (Sakurai et al., 2009).

Statistical analysis

Results were expressed as means ± S.E.M. and the analysis of variance was performed by ANOVA test. A Bonferroni's significant difference procedure was used as post hoc comparison. P values less than 0.05 were considered significant. Data were analyzed using the ''Origin 8.1'' software.

Results

Different sources of rASCs were evaluated processing rat thighs, sternal and aortic fat pads. The stemness of isolated cells was assessed in terms of percentage of CD29-, CD90-, as markers for mesenchymal stem cells, and CD45-, CD79α-positive cells, as markers for hematopoietic stem cells. As shown in Table 1, all cell lines obtained from different fat pads exhibited the typical phenotype of mesenchymal stem cells ($CD90^+$ $CD29^+$ $CD45^-$ $CD79\alpha^-$). Nevertheless, sternal fat pad held the higher percentage of CD90- and CD29-positive cells (83.3% and 92.5%, respectively) compared to thighs (86.7% and 87.6% respectively) and aortic fat pads (59.9% and 88.7% respectively). The expression of CD45 and CD79α was lower in sternal fat derived cells (7%) than in thighs and aortic fat derived cells (12% and 11%). Based on these results sternal-derived rASCs were chosen to perform all experiments. Several cell lines were isolated and analyzed (P1-4) by flow cytometry. Figure 1 shows the cell phenotype of a representative rASCs preparation (P2) analyzed by FACS for surface positive CD90/CD29 and negative CD79α/CD45 markers. The CD90 positive cells which also expressed CD29 were around 91% (Figure 1a) and the CD79α negative cells which also no expressed CD45 were around 99% (Figure 1b).

Moreover, in order to highlight mesenchymal characteristics, rASCs were cultured *in vitro* and differentiated under specific conditions to mesenchymal cell lineages such are adipocytes and osteocytes (Figure 2).

Neuropathic pain was induced in rats by repeated treatments with oxaliplatin. The anticancer drug was administered (2.4 mg kg^{-1} i.p.) once a day on days 1, 2, 3 and 4 (scheme 1). On day 5, the response of oxaliplatin-treated rats to a noxious mechanical stimulus (Paw pressure test) was altered and the weight tolerated on the posterior paw significantly decreased from the control value of 71.8 ± 0.2 g to 46.5 ± 0.1 g (Figure 3A). On day 6, $2x10^6$ rASCs were injected in the caudal vein of one group of oxaliplatin treated rats. The control group and the other group of oxaliplatin treated rats received rASCs vehicle DMEM. rASCs treatment induced a significant reduction of mechanical hypersensitivity in comparison to oxaliplatin + DMEM treated rats peacking 6h after cell administration (Figure 3A). At this time the value registered by Paw pressure test increased until 60.1 ± 0.1g with respect to oxaliplatin + DMEM treated rats. The effect of rASCs on mechanical hypersensitivity lasted at least 96 h. In order to better understand the effect of rASCs

on neuropathic pain, multiple administrations of rASCs were performed (scheme 1). As shown in figure 3, rASCs determined a significant, comparable reduction of hypersensitivity induced by mechanical noxious (figure 3A) stimulus each time that cells were administered. Also the response to mechanical non-noxious stimulus was evaluated by Von Frey test. The data showed a significant reduction in withdrawal threshold starting from the 4th day of oxaliplatin treatment until day 24 (i.e. after 13 days from the last oxaliplatin administration), when the effect of oxaliplatin start to diminish. On day 6, rASCs administration was able to significantly increase the response to mechanical non-noxious stimulus in comparison to oxaliplatin + DMEM treated rats peaking 24 h after injection. This effect lasted up to 96 h vanishing 6 days after rASCs administration (Figure 3B). The subsequent rASCs treatment (repeated on days 13, 16 and 20) induced a reduction of mechanical hypersensitivity similar to that previously described.

Table 1. Immunophenotype characterization of rASCs isolated from three different anatomical regions

	Positive cells %		
Marker	**Sternal Fat**	**Thighs Fat**	**Aortic Fat**
CD90	83.35 ± 1.65	86.7 ± 2.45	59.9 ± 2.84
CD29	92.52 ± 1.90	87.6 ± 3.58	88.7 ± 4.65
CD45	7.02 ± 2.05	12.3 ± 1.52	11.2 ± 1.78
CD79a	7.65 ± 1.91	12.7 ± 1.67	11.8 ± 2.78

The expression of surface markers of sternal, thighs and aortic fat-derived ASCs was characterized by means of flow cytometry with anti-CD45, CD79a, CD90, CD29 antibodies. The results are expressed in percentage of positive cells for each antibody ± SEM.

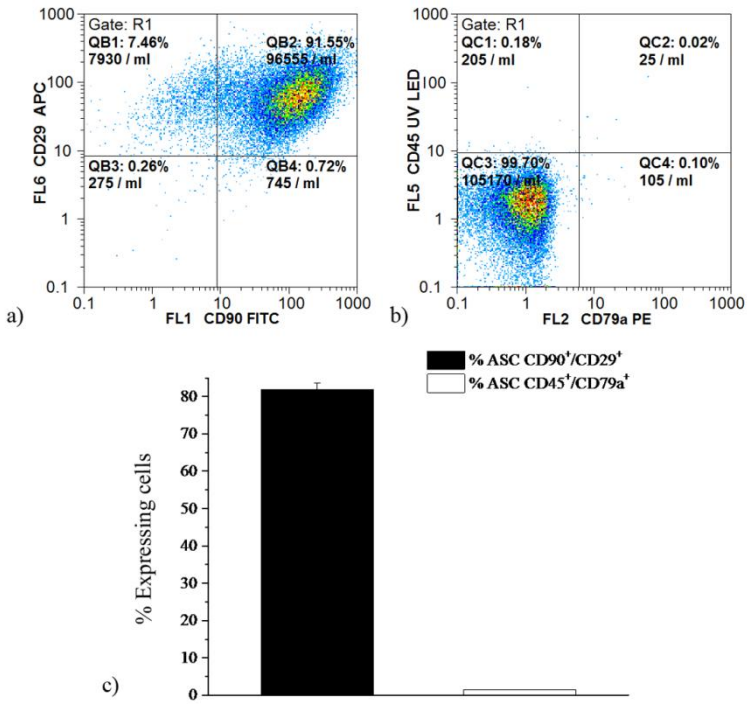


Figure 1. Immunophenotipe characterization of isolated P2 rASCs. a) and b) show the expression of rASC surface markers characterized by flow cytometry with anti-CD45, CD79a, CD90 and CD29 antibodies. C) Mean percentage of positive cells from 20 different cell lines.

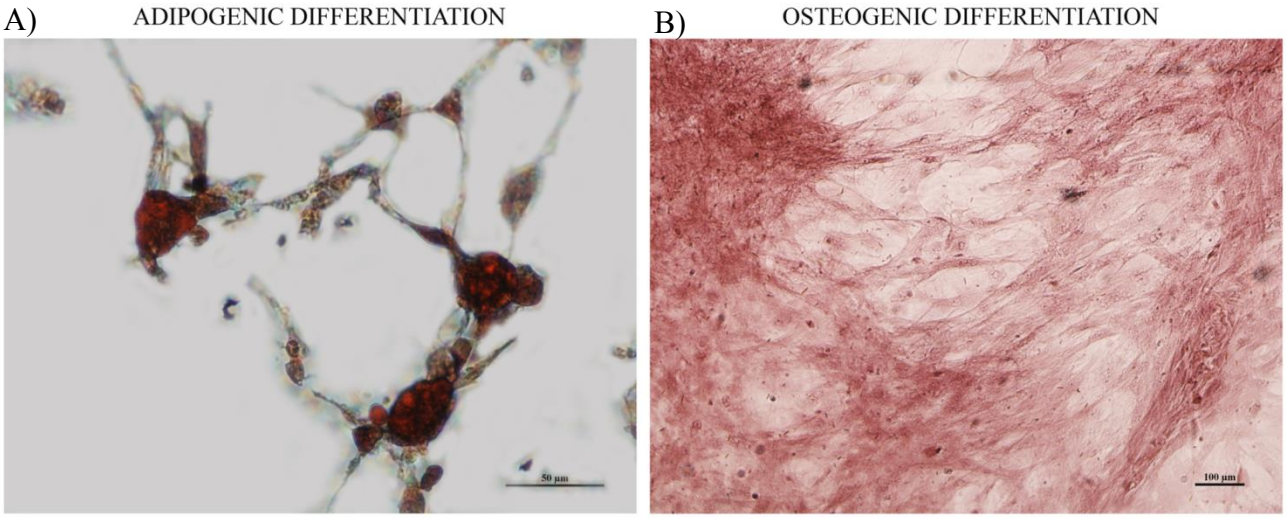
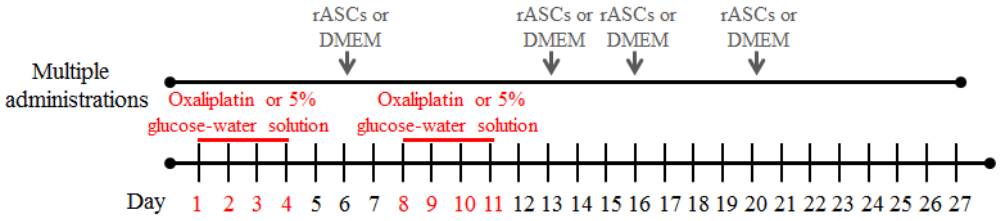


Figure 2. *In vitro* differentiation of rat mesenchymal stem cells into mesodermal lineages. P2 rASCs were used for differentiation. a): Adipogenic differentiation. Cells were cultured in adipogenesis differentiation medium for 21 days. Oil Red O staining was performed to detect an accumulation of lipid droplets in the cytoplasm of ASCs. Scale bar: 50 µm. b): Osteogenic differentiation. Cells were cultured in osteogenesis differentiation medium for 15 days. Alizarin Red S staining was performed to detect calcium accumulation. Scale bar: 100 µm.



Scheme 1. **Oxaliplatin treatment and multiple administrations of rASCs**

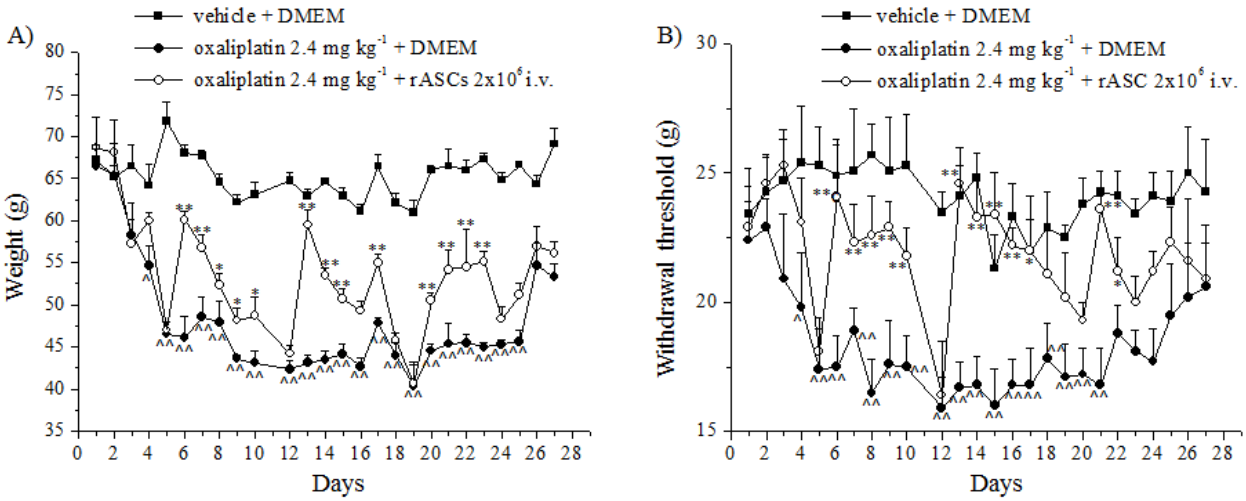


Figure 3. Cyclic pain reliever effect of rASCs administration in oxaliplatin-treated rats. A) Paw pressure and B) Von Frey tests were used to evaluate hypersensitivity to mechanical noxious and non-noxious stimuli, respectively. Oxaliplatin (2.4 mg kg^{-1} i.p.) was administered for 4 consecutive days on weeks 1 and 2. On day 6, 13, 16 and 20, $2x10^6$ rASCs, suspended in 400 µL of DMEM with 2.5 % of heparin (25000 U.I./5mL), were injected into the tail vein. Each value represents the mean ± SEM of 12 rats per group, performed in 2 different experimental sets. ^P<0.05 and ^^P<0.01 vs vehicle + DMEM treated animals; *P<0.05 and **P<0.01 vs oxaliplatin + DMEM treated animals.

Conclusions

According to the results obtained from *in vitro* experiments, sternal fat pad is a good source of mesenchymal stem cells in terms of stemness and differentiation capabilities. Moreover, the data obtained by behavioural suggest rASCs as possible approach for the treatment of oxaliplatin-induced hypersensitivity. This effect, that are characterized by a rapid onset and lasts up to 5 days from stem cells administration can be due to the secretion of several mediators. Immunomodulatory and trophic factors belonging to mesenchymal stem cells secretome promote a regenerative microenviroment that oppose the effect of nerve damage.

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