New Therapeutic Strategies in Pain Research

María Florencia Coronel[1], Norma Alejandra Chasseing[2], Marcelo José Villar[1]

[1] Facultad de Ciencias Biomédicas, Universidad Austral, Av. Pte. Perón 1500, Buenos Aires (B1629AHJ), Argentina. [2] Instituto de Biología y Medicina Experimental - CONICET, Vuelta de Obligado 2490, Buenos Aires (C1428ADN), Argentina.

Corresponding author:

Marcelo J. Villar
Facultad de Ciencias Biomédicas
Universidad Austral
Av. Pte. Perón 1500
B1629AHJ Pilar, Buenos Aires
ARGENTINA
Phone: 54 2322 482948
Fax: 54 2322 482204
e-mail: mvillar@austral.edu.ar

Abstract

In the last years, significant progress has been made in the medical treatment of pain. However, pathological pains, such us neuropathic pain, remain refractory to the currently available analgesics. Therefore, new therapeutic strategies are being evaluated. We have recently shown that both bone marrow stromal cells (MSCs) and the oligonucleotide IMT504 can prevent the development of mechanical and thermal allodynia when they are administered to rats subjected to a sciatic nerve crush. This chapter summarizes the laboratory techniques used to isolate and culture MSCs,

administer both MSCs and IMT504, perform the nerve injury and determine mechanical and thermal sensitivities.

Keywords: sciatic nerve injury, mechanical and thermal allodynia, oligodeoxinucleotides, bone marrow stromal cells

1. Introduction

Injuries of the peripheral and central nervous system often result in neuropathic pain. These injuries may result from major surgeries (amputation, thoracotomy), diabetic neuropathy, viral infection, spinal cord injury and stroke, among other insults ***(1)***. Patients with neuropathic pain report spontaneous pain, described as shooting, lancinating or burning pain, as well as pain induced by normally innocuous tactile or thermal stimuli, known as allodynia ***(2,3)***. Neuropathic pain is a chronic, pathological pain that remains refractory to the currently available analgesics ***(4,5)***. Therefore, biomedical researchers continue evaluating new therapeutic strategies. In the last years, exogenously administered bone marrow stromal cells (MSCs) have been shown to participate in the repair and regeneration of damaged tissues in a variety of animal models ***(6,7,8,9)***. Moreover, we have recently shown that MSC administration prevents the development of mechanical and thermal allodynia in animals subjected to a sciatic nerve injury ***(10,11,12)***. However, there are some limitations of this therapeutic approach, basically related to the *ex vivo* cell manipulation procedure (cell isolation, *in vitro* expansion and cell delivery) ***(13)***. IMT504, the prototype of the PyNTTTTGT class of oligodeoxinucleotides ***(14)***, is a potent stimulatory signal for MSC expansion both *in*

vitro and *in vivo* ***(15)***. Since systemic treatment with this oligonucleotide can stimulate the animals own MSCs, inducing their expansion and mobilization ***(15)***, we investigated the effect of IMT504 administration on the nociceptive behavior of rats subjected to a sciatic nerve injury. Our results show that IMT504 administration is able to reduce mechanical and thermal allodynia ***(16)***, representing a possible therapeutic approach for the treatment of neuropathic pain.

2. Materials

2.1. Animals

1. Adult Sprague-Dawley male rats (200-300 g, Fucal, Argentina) should be kept in a 12 h day/night cycle (light on 6.00 AM), with water and food *ad libitum* and controlled temperature (24°C).

2. Prepare a mixture of ketamine (50 mg/ml, Holliday-Scott SA, Buenos Aires, Argentina) and xylazine (2%, Bayer, Buenos Aires, Argentina) by mixing 4 ml of ketamine and 1 ml of xylazine. This mixture should be prepared fresh for each experiment.

2.2. Isolation of MSCs

1. Alpha minimal essential medium (α-MEM) (GIBCO/BRL, Bethesda, MD, USA) supplemented with 100 IU/ml gentamicine (Larjan, Buenos Aires, Argentina) and 2.5 μg/ml amphotericine B (PAA Laboratories, Linz, Austria).

2. Trypan blue: prepare solution A (trypan blue 0,5% in distilled water), solution B (NaCl 4,25% in distilled water) and trypan blue working solution (4 parts of A and 1 part of B).

Store at 4°C.

3. Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 100 IU/ml gentamicine, 2.5 μg/ml amphotericine, 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 20% fetal calf serum (GIBCO).

4. Hoechst 33258 (Sigma). Prepare working solution by dissolving 1 mg Hoechst in 1 ml sterile distilled water. Protected from light and stored at 4°C, working solutions are stable for at least 6 months. The Hoechst strains are known mutagens and should be handled with care. The dye must be disposed of safely and in accordance with applicable regulations.

5. Solution of trypsin 0.25% and ethylenediamine tetraacetic acid (EDTA) 1 mM (GIBCO).

6. Phosphate buffered saline (PBS). Prepare 10X stock solution with 1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO4, 18 mM KH2PO4 (adjust to pH 7.4 with HCl if necessary). Autoclave before storage at room temperature. Prepare working solution by diluting one part of stock solution with nine parts of distilled water.

7. Cell culture plasticware (pipets, flasks, conical tubes, tips, etc), all sterile.

2.3. ODN

1. IMT504 sequence is 5'-TCATCATTTTGTCATTTTGTCATT-3' (Property of Immunotech SA, Argentina).

2. The HPLC-grade single stranded ODN having phosphorothioate internucleotide linkages is purchased from Oligos ETC, Wilsonville, OR, USA.

2.4. Nerve injury model

1. Sterile surgical instruments.

2.5. IMT and MSC administration

1. Sterile 1 ml syringes and needles for injection.

2.6. Behavioral assessment

1. Von Frey hairs (Stoelting, WoodDale, IL, USA).

2. Acetone (Merck, Darmstadt, Germany). Acetone is extremely flammable with a high vapor pressure; use only with good ventilation and avoid all ignition sources.

3. Methods

3.1. Animals

1. All the experiments performed are in accordance to the policy of the Society for Neuroscience and the International Association for the Study of Pain for the use of animals in pain research. Efforts should be made in order to minimize animal discomfort and to use the fewest animals needed for statistical significance.

2. Anesthetize the animals by an intraperitoneal (i.p.) injection of the mixture of ketamine and xylazine. A 250 g rat should receive 0,3 ml of the mixture (50 mg/kg ketamine and 5 mg/kg xylazine).

3.2. Isolation of MSCs (*see* Note 1)

1. Anesthetize the animals as previously described and euthanize them for bone marrow harvesting.

2. Dissect out the femoral bones. After removing the epiphyses and gaining access to the marrow cavities, flush out whole bone marrow using a 15G needle and a 1 ml syringe

with prewarmed α-MEM supplemented with gentamicine and amphotericine. Collect the bone marrow from at least 8 femoral bones and transfer the suspension to 15 ml conical tubes. Disperse the cells by gentle pipetting up and down (Fig 1a,b).

3. Centrifuge the cell suspension at 400 x *g* for 10 min at room temperature. Discard the supernatant carefully and resuspend the pellet in fresh warm medium. Repeat this procedure twice.

4. Evaluate cell concentration by microscopic cell counting using a Neubauer hemocytometer. It is important to disperse clumps before counting the cells. To check cellular viability, mix 1 volume of the trypan blue working solution with 1 volume of the cell suspension. Wait for 5 minutes and then load 10 μl of this suspension onto the hemacytometer. View under the microscope (100 X). Trypan blue is a vital dye, viable cells remain unstained. Count the number of viable cells in each square grid, calculate the average number of cells per square grid and finally calculate the number of viable cells per ml (cells/ml= average number of cells per square grid x 2 x 10^4).

5. Centrifuge again and resuspend the cells at a concentration of 1 x 10^6 cells/ml in warmed DMEM supplemented with gentamicine, amphotericine, L-glutamine and fetal calf serum, and transfer to 25 cm^2 tissue culture flasks (Corning Inc., New York, USA).

6. Place the culture flasks in a humidified 37°C, 5% CO2 incubator.

7. After 24 hours of culture, remove media with a Pasteur pipet in order to discard the non-adherent cells (*see* **Note 2**). Add warmed culture medium and incubate the flasks until 90% confluence is reached (approx 12-14 days), renewing the medium every 3-5 days. Check the cells with an inverted microscope once every two days (Fig 1c,d). Cells

should not grow further than 90% confluence in order to avoid contact inhibition or transformation.

8. In the case you want to localize the cells in the animals´ tissues after administration, incubate MSCs with 1 μg/ml bis-benzamide (Hoechst 33258, Sigma) for 24 hs. prior to harvesting (add 5 μl of Hoechst working solution to 5 ml of culture media). *See* **Note 3** and Fig 1e-h.

9. Approx. at day 14, carefully remove media and wash adherent cells with PBS in order to remove residual fetal calf serum which may inhibit trypsin.

10. Add a small volume (0,5-1 ml) of trypsin/EDTA solution (enough to cover the monolayer). Place the culture flasks in the incubator at 37°C (to favour the enzymatic activity). Check whether the cells have detached under an inverted microscope every 2-3 min. Cells should not be exposed to trypsin for more than 15 min since prolonged exposure damages them. When cells have detached, add sufficient amount (4-5 ml) of DMEM containing 20% fetal calf serum (to inhibit trypsin) and disperse the cells by gentle pipetting up and down.

11. Check cell number and viability as previously described. Pellet cells for 5 min at 400 x *g*, remove supernatant and resuspend the cells in PBS at a concentration of 3 x 10^6 cells/ml.

3.3. ODN

1. Suspend the ODN in sterile saline (NaCl 0,9%) at a concentration of 10 mg/ml and assay for LPS contamination using the Limulus test (Pyrosate, Associates of Cape Cod,

Inc., East Falmouth, MS, USA). Only ODN preparations with undetectable LPS levels should be used.

2. Keep the ODN at -20°C until used.

3.4. Nerve injury model

1. Anesthetized the rats as previously described.

2. Expose the sciatic nerve in the right thigh and dissect it free from the surrounding tissue using sharp microscissors in a 5 to 8 mm long segment.

3. Crush the nerve for 3 s at the mid thigh level using jeweler´s forceps.

3. Mark the site of the lesion using an indelible felt-tip pen.

4. Replace the nerve under the muscle. Suture the skin wound using silk thread (3.0) (Barbour Threads, Lisburn, Ireland) and leave the animals to recover under a lamp heating.

3.5. IMT504 and MSC administration

1. Immediately after performing the nerve injury, inject a group of rats subcutaneously with 500 μl of the ODN IMT504 dissolved in saline (for 250 g rats, dose is 20 mg/kg). This injection should be repeated once daily, for the next four consecutive days.

2. Inject another group of animals with a suspension of MSCs (1.5 x 10^6/500 μl PBS). These animals should receive one intravascular administration (into the tail vein), immediately after the nerve injury is performed.

3. Include also the following control groups: 1) Animals subjected to the sciatic nerve crush alone. 2) Animals with a sciatic nerve crush receiving five subcutaneous injections of saline, once daily, starting immediately after performing the lesion. 3) Animals with a

sciatic nerve crush receiving one intravascular administration of PBS immediately after performing the lesion.

3.6. Behavioral assessment

1. Perform behavioral testing during daytime (9.00-18.00, preferably always at the same time) in a quiet room. The tests should be performed by blinded investigator. All animals should be tested before surgery (day 0) and at different time points after the sciatic nerve crush (1, 3, 7, 10, 14 and 21 days, for example). Include in the experiments only rats showing normal responses to mechanical and thermal stimulation before injury.

2. Place the animals in their acrylic testing chambers (8x8x18 cm) on a metal mesh floor (hole size of 3x3 mm), 15 min before starting the test for adaptation (Fig 2a,b).

3. In order to assess mechanical sensitivity, apply von Frey hairs in ascending order (1, 2, 4, 6, 8, 10, 15, 26 g) from below the mesh floor to the center of the plantar surface of both ipsilateral and contralateral hindpaws ***(17)***. *See* **Note 4** and Fig 2b,c.

4. Deliver each hair three times with 5 s intervals and register whether the stimulation induces a brisk foot withdrawal. The lowest force at which application elicits a paw withdrawal is taken as the mechanical response threshold. A paw withdrawal reflex obtained with 6 g or less is considered an allodynic response. See Fig 3a.

5. In order to determine cold sensitivity, apply 100 µl of acetone to the plantar surface of the paw using a plastic tubule connected to a 1 ml syringe (Choi test) ***(18)***. Apply the bubble of acetone five times to each paw with an interval of at least 5 min and record the number of brisk foot withdrawals (from 0 to 5). See Fig 3b.

4. Notes

1. Sterile conditions must be maintained at all times and all culture work should be performed under a laminar flow hood. Media, trypsin/EDTA solutions and PBS should be warmed to 37°C in a water bath before use.

2. MSCs grow in a monolayer. Cells with different morphologies are obtained: fibroblast-like spindled-shaped cells, rounded cells and large flat cells (Fig 1c,d).

3. Hoechst 33258 is a cell permeable nucleic acid stain that emits blue fluorescence when bound to double strand DNA (Fig 1e-h). This fluorophore is retained for long periods by viable cells.

4. Von Frey hairs are plastic monofilaments that exert an increasing pressure on the skin as they are pressed harder and harder, up to the point where they begin to bend. Pressure on the skin then remains constant over a considerable range of bending. Therefore, filaments should be pressed onto the animal's hindpaws until they begin to bend (Fig 3).

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Figure legends

Fig 1: Microphotographs illustrating different steps in the process of MSC isolation. In (a,b) whole bone marrow smears obtained from the femoral bones of Sprague-Dawley rats and stained with Giemsa are shown. In culture, MSCs grow in a monolayer as fibroblast-like spindled-shaped cells (c,d). In (e-h) a suspension of MSCs stained with Hoechst is shown. Micrographs e and f were taken under visible and UV illumination, while g and h were taken under UV illumination only. As it can be observed, Hoechst-positive cells show nuclear blue fluorescence.

Fig 2: Photographs showing animals placed in their individual acrylic chambers (a) located onto a metal mesh floor (b), in order to determine mechanical and thermal sensitivities. In (c) three of the von Frey filaments used, corresponding to 1, 6 and 26 g are shown. Note that the filaments have increasing diameters which results in an increasing applied force. A series of ten filaments is applied in ascending order to both hindpaws. Each hair is applied three times until it begins to bend, as shown in (b).

Fig 3: Effect of either IMT504 or MSC treatment on the development of mechanical (a) and thermal (b) allodynia in the ipsilateral hindpaw of animals subjected to a sciatic nerve crush. The nociceptive behavior of lesioned animals without any treatment is also shown. These control rats showed a behavioral pattern similar to that of animals receiving either saline or PBS after the sciatic nerve lesion (not shown). (a) The sciatic nerve crush induced a significant decrease in paw withdrawal threshold to the von Frey filaments in

control animals. Nociceptive responses in the allodynic range were detected three, seven and ten days after the lesion. It is noticeable that the administration of either IMT504 or MSCs prevented the development of mechanical allodynia. Animals receiving either treatment showed similar behavioral responses at all the evaluated time points. (b) A significant increase in the number of allodynic responses to cold stimuli was induced in the ipsilateral hindpaw footpad after the sciatic nerve crush. Note that the administration of IMT504 significantly reduces the number of nociceptive responses to cold stimulation, three and seven days after the lesion. MSC administration also results in fewer painful responses when compared to control animals. Values show mean +/- S.E.M. Only statistically significant differences between treated and control animals are stated in the graphs, using the following symbols to represent p values: * $0.05>p>0.01$; ** $0.01>p>0.001$ and *** $p<0.001$.