Stem Cell Research (2013) 10, 95–102

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/scr

Mesenchymal stem cells can improve anal pressures after anal sphincter injury☆

Levilester Salcedo [a], Maritza Mayorga [b], Margot Damaser [c,d,e], Brian Balog [c], Robert Butler [f], Marc Penn [b], Massarat Zutshi [a,*]

[a] Department of Colorectal Surgery, Cleveland Clinic, Cleveland, OH, USA
[b] Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic, Cleveland, OH, USA
[c] Department of Biomedical Engineering, Cleveland Clinic, Cleveland, OH, USA
[d] Glickman Urological and Kidney Institute, Cleveland Clinic, Cleveland, OH, USA
[e] Advanced Platform Technology Center, Louis Stokes Cleveland VA Medical Center, Cleveland, OH, USA
[f] Department of Quantitative Health Sciences, Cleveland Clinic, Cleveland, OH, USA

Received 20 February 2012; received in revised form 13 September 2012; accepted 9 October 2012
Available online 16 October 2012

Abstract Objective. Fecal incontinence reduces the quality of life of many women but has no long-term cure. Research on mesenchymal stem cell (MSC)-based therapies has shown promising results. The primary aim of this study was to evaluate functional recovery after treatment with MSCs in two animal models of anal sphincter injury.
Methods. Seventy virgin female rats received a sphincterotomy (SP) to model episiotomy, a pudendal nerve crush (PNC) to model the nerve injuries of childbirth, a sham SP, or a sham PNC. Anal sphincter pressures and electromyography (EMG) were recorded after injury but before treatment and 10 days after injury. Twenty-four hours after injury, each animal received either 0.2 ml saline or 2 million MSCs labelled with green fluorescing protein (GFP) suspended in 0.2 ml saline, either intravenously (IV) into the tail vein or intramuscularly (IM) into the anal sphincter.
Results. MSCs delivered IV after SP resulted in a significant increase in resting anal sphincter pressure and peak pressure, as well as anal sphincter EMG amplitude and frequency 10 days after injury. MSCs delivered IM after SP resulted in a significant increase in resting anal sphincter pressure and anal sphincter EMG frequency but not amplitude. There was no improvement in anal sphincter pressure or EMG with in animals receiving MSCs after PNC. GFP-labelled cells were not found near the external anal sphincter in MSC-treated animals after SP.
Conclusion. MSC treatment resulted in significant improvement in anal pressures after SP but not after PNC, suggesting that MSCs could be utilized to facilitate recovery after anal sphincter injury.

☆ Moderated Poster Presentation: 39th Annual Scientific Meeting of International Continence Society, September 29–October 3, 2009, San Francisco, CA.

* Corresponding author at: The Cleveland Clinic, Department of Colorectal Surgery, 9500 Euclid Avenue A30, Cleveland, OH 44195, USA.
E-mail address: zutshim@ccf.org (M. Zutshi).

1873-5061/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scr.2012.10.002

Introduction

Psychological and social ostracism are common issues that patients debilitated by fecal incontinence (FI) encounter (Lazarescu et al., 2009). Although the cause of anal sphincter incontinence is multi-factorial (Kouraklis and Andromanakos, 2004; Safioleas et al., 2008), the prevalence is known to be higher in women due to childbirth injuries (Pretlove et al., 2006). However, the clinical manifestations of FI may not occur at the time of injury but most often manifest years later (Halverson and Hull, 2002).

Surgical repair is one of the treatments for a damaged anal sphincter; however, sphincter function deteriorates over time and long-term outcome remains unsatisfactory (Gutirrez et al., 2003; Halverson and Hull, 2002; Karoui et al., 2000; Malouf et al., 2000; Zutshi et al., 2009b). Newer treatment options include neuromodulation (Hosker et al., 2007), the Secca procedure (Takahashi-Monroy et al., 2008), bulking agents (Chan and Tjandra, 2006; Kenefick et al., 2007) and an artificial bowel sphincter (Altomare et al., 2009). The multiple treatment options and unsatisfactory long-term outcomes point to the need for innovative treatments for FI that have long-term durability.

Several studies have investigated the role of mesenchymal stem cells (MSCs) in improving anal sphincter function after direct injection of stem cells to the anal sphincter muscles (Kajbafzadeh et al., 2010; Kang et al., 2008; Lorenzi et al., 2008; Pathi et al., 2012). The results of these studies are promising; however, only ex vivo outcomes were utilized and the in vivo effects on anal pressures were not assessed. Pathi et al. (2012) investigated the effect of IV and direct injection on neurophysiology studies and studied mRNA levels of anti-inflammatory genes, genes highly expressed after an acute and genes involved in matrix synthesis as a function of time. In addition, investigations in animal models of heart failure demonstrate a therapeutic effect of MSCs infused intravenously (IV), which may provide a less invasive delivery route for MSCs than those previously tested for treatment of FI (Shabbir et al., 2009a, 2009b).

We have developed rat models of anal sphincter dysfunction induced via sphincterotomy (SP), or pudendal nerve injury to model the nerve injuries of childbirth, and have demonstrated changes in anal sphincter pressures in vivo lasting up to 4 weeks after the injury (Salcedo et al., 2010). We have also demonstrated upregulation of MCP-3 and SDF-1 in the anal sphincter complex after injury (Salcedo et al., 2011). The goal of this project was to investigate the changes in anal sphincter pressure after IV or intramuscular (IM) injection of MSCs in our previously established animal models, with the long-term goal of developing improved therapy for patients with FI.

Material and methods

This study was approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Mesenchymal stem cell harvesting and cell culturing

Virgin female Sprague–Dawley rats were euthanized and bone marrow was harvested from the tibia and femurs by gently flushing the bone with 1 ml Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA). To separate adherent cells, bone marrow clumps were passed through 18 and 20 gauge needles. The cells were centrifuged at 2500 rpm for 5 min with three changes of PBS. The washed cells were placed in a vented cell culture T75 flask (3151 Costar, Corning Incorporated, Corning, NY) with 25 ml DMEM (Gibco, Invitrogen Corp., USA) containing 10% FBS and 1% antibiotic and anti-mycotic solution (Gibco 15240, Invitrogen Corp., USA) and were incubated at 37 °C. At this stage, the cells were identified as P0. The media was changed 3 days later to remove non-adherent cells. Succeeding media changes were made every 3–4 days according to cellular confluence. At 70–80% confluence, the adherent cells were detached after incubation with 0.05% trypsin and 2 mM EDTA for 5–10 min.

At passage P4, cultures were negatively selected for MSC. Cell sorting for MSC was performed with an EasySep pycoerythrin (PE) selection kit according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, B.C., Canada). The cultures were simultaneously depleted of $CD45^+$ and $CD34^+$ cells using 10 μl of each of the primary PE-conjugated antibodies: mouse anti-rat $CD45^+$ (BD Biosciences, San Diego, CA, USA) and mouse anti-$CD34^+$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for every 10^6 cells.

Green fluorescent protein (GFP) labelling

After sorting and MSC selection, when the cells reached 80–90% confluence, MSCs were transfected with a lentivirus vector pCCLsin.ppt.hPGK.GFP.pre (a generous gift from the Cossu Lab), which uses a human PGK promoter to constitutively express green fluorescent protein (GFP), and were processed overnight by incubation in a mixture of normal medium (6 ml), polybrene (6 μl) and 10× MOI (10 million viruses for each million cells). Transduction proceeded overnight and the medium was changed after 6–8 h incubation. MSC were checked for GFP-labelled cells under immunoflouroscopy and expanded until P12–20 when they were utilized for the study. Cultures were then trypsinized and spun at 2500 rpm for 5 min. Cells were resuspended in PBS (0.2 ml for 2 million cells) for animal experiments.

Animal models

Seventy age-matched female Sprague–Dawley rats weighing 240–260 g were randomly allocated into the following groups: sphincterotomy (SP; $n=20$), pudendal nerve crush (PNC; $n=20$), sham SP ($n=10$) and sham PNC ($n=20$). SP was performed under ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. anesthesia by incising the external and internal anal sphincters 2–3 mm deep, as we have done previously (Zutshi et al., 2009a). Anal sphincter transection was confirmed with a dissecting microscope. Since the anal sphincter in the rat is small and superficial, even a minute incision could incorporate a part of the anal sphincter. Therefore, sham SP was created by pressing a Q-tip on the anal sphincter for 5 s.

PNC was performed under the same anesthesia via a posterior incision in the sacro-coccygeal area. The pudendal nerves were isolated bilaterally in the ischiorectal fossa and crushed twice for 30 s each with a Castroviejo needle holder,