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Therapeutic assessment of mesenchymal stem cells delivered within a PEGylated fibrin gel following an ischemic injury

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ABSTRACT

The intent of the current study was to investigate the therapeutic contribution of MSCs to vascular regeneration and functional recovery of ischemic tissue. We used a rodent hind limb ischemia model and intramuscularly delivered MSCs within a PEGylated fibrin gel matrix. Within this model, we demonstrated that MSC therapy, when delivered in PEGylated fibrin, results in significantly higher mature blood vessel formation, which allows for greater functional recovery of skeletal muscle tissue as assessed using force production measurements. We observed initial signs of vascular repair at early time points when MSCs were delivered without PEGylated fibrin, but this did not persist or lead to recovery of the tissue in the long-term. Furthermore, animals which were treated with PEGylated fibrin alone exhibited a greater number of mature blood vessels, but they did not arterialize and did not show improvements in force production. These results demonstrate that revascularization of ischemic tissue may be a necessary but not sufficient step to complete functional repair of the injured tissue. This work has implications on stem cell therapies for ischemic diseases and also potentially on how such therapies are evaluated.

1. Introduction

Peripheral artery disease (PAD) is a growing health concern in the United States, with 8–10 million people being affected by the disease [1]. Intermittent claudication is an early, moderate manifestation of the disease and is characterized by leg pain and muscle weakness [1,2]. However, PAD can quickly develop into critical limb ischemia, which is a more chronic and severe problem [1,2]. Critical limb ischemia often results in tissue infection, death, and potentially amputation because the resting metabolic needs of the tissue are not met by the available blood supply [1,2]. Although current revascularization therapies (including bypass surgery and balloon angioplasty) may provide some benefit to patients, not all patients are eligible for such procedures [1–4]. Major limb amputation is often necessary if revascularization therapy is not possible, or was not successful [3,4].

Many have investigated the use of angiogenic gene and protein therapy as an alternative revascularization strategy [2,5,6]. Although gene and protein strategies have shown some success in preclinical animal models, there have not been significant improvements in clinical studies [5,6]. This could potentially be attributed to the fact that the ischemic wound healing response is very complex, and thus delivering the correct genes/proteins is very difficult [5,6], especially when taking into account necessary temporal release kinetics and concentration gradients. As a result, cell-based approaches are an attractive therapy option because the cells can supply the necessary cytokines and growth factors necessary to promote, as well as support, vascular regeneration [2]. In addition, the use of multipotent stem cells, such as bone marrow-derived mesenchymal stem cells (MSCs), could provide other therapeutic effects in addition to blood vessel growth [2].

Mesenchymal stem cells (MSCs) are an adult stem cell population found within multiple regions of the body [7,8] which can terminally differentiate into mesodermal lineages. MSCs have been shown to secrete factors which are pro-angiogenic and promote wound healing, and thus are an ideal candidate cell type for

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therapeutic revascularization [7,9–11]. Many research groups have demonstrated blood vessel formation and increased reperfusion of ischemic areas following delivery of MSCs or other stem cells [2,11,12], either through direct cell differentiation or paracrine effects [7,10,11]. A gap exists in the literature between demonstrating increased perfusion as a result of stem cell treatment and whether or not that leads to functional recovery.

This study evaluated the use of bone marrow-derived mesenchymal stem cells as a therapy for peripheral arterial disease. A rat model of acute hind limb ischemia was used in which the femoral artery was ligated and excised. MSCs were intramuscularly delivered within a PEGylated fibrin gel following ischemia. The PEGylated fibrin delivery system offers several advantages, including retaining MSCs at the delivery site. In addition, fibrin is a natural component of the wound healing response and has been demonstrated to have angiogenic properties [13,14]. To evaluate the therapeutic contribution of treatment, restoration of blood flow and muscle function were evaluated. Histological analysis was also performed to evaluate tissue architecture and blood vessel formation. The results of this study have important implications pertaining to alternative cell-based therapies for ischemic and cardiovascular diseases.

2. Materials and methods

2.1. Rat MSC isolation and culture

Bone marrow MSCs were isolated from Lewis rats (8–10 weeks old). The femoral marrow cavity was flushed and adherent cells were collected and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% glutamax, and 1% penicillin-streptomycin. After 24 h, non-adherent cells were removed by replacing the medium. The adherent cells underwent medium changes every 2 days and were passaged once they reached approximately 80% confluency. Passage 4 cells were used in the current study.

2.2. Ischemic injury

Animal handling and care followed the recommendations of the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All protocols were approved by the Animal Care Committee at the University of Texas at Austin (AUP-2011-00162). Lewis rats (11 weeks) weighing 250–300 g were used. To induce hind limb ischemia, femoral artery ligation in Lewis rats (11 weeks, male) was performed. Rats were anesthetized using isoflurane (0.5–2%) infused with oxygen (2 L/min). Through a small incision on the medial side of the thigh, the femoral artery of a single hind limb was separated from the nearby nerve and vein and ligated immediately distal to the inferior epigastric artery and proximal to the branch point of the popliteal and saphenous arteries using Prolene 5-0 sutures (Fig. 1). The ligated segment (~0.5 cm) was then excised and the skin incision closed with interrupted sutures. The animal was allowed to recover overnight and the following day (about 24 h later) therapy was delivered.

2.3. PEGylated fibrin gel delivery of MSCs

MSCs were injected intramuscularly into the gastrocnemius muscle of the ligated limb. PEGylated fibrin injections were prepared by combining difunctional succinimidyl glutarate polyethylene glycol (PEG) (4 mg/mL in PBS without calcium; NOF America) with human fibrinogen (40 mg/mL in PBS without calcium; Sigma) in a 1:1 vol ratio. An equal volume of rat MSCs was mixed with the PEGylated fibrin solution in a 1:1 vol ratio at a concentration of 13×10^6 cells/mL. The solution was then loaded into a 23G needle syringe, followed by an equal volume of thrombin (25 U/mL in 40 mM $CaCl_2$). The solution was mixed thoroughly within the syringe and the gel solution (300 μL) was injected into the gastrocnemius of the rat. The final concentrations in the gel were 5 mg/mL of fibrinogen; 0.5 mg/mL of SG-PEG-SG; 3.33×10^6 cells/mL; and 12.5 U/mL of thrombin. Other treatment groups consisted of no treatment, PEGylated fibrin gel, MSCs, and 10% FBS containing DMEM (serum). For the PEGylated fibrin gel treatment, 300 μL of PEGylated fibrin gel was injected into the lateral gastrocnemius muscle, with the cell portion replaced by 10% FBS containing DMEM. For the MSC treatment, 300 μL of MSCs (3.33×10^6 cells/mL) suspended in 10% FBS containing DMEM was injected into the lateral gastrocnemius muscle. Prior to delivery, MSCs were fluorescently labeled with CellTracker™ CM-DiI (Invitrogen). The cells were incubated with CM-DiI (15 μM) at 37 °C for 8 min and then 4 °C for 15 min, washed with PBS, and resuspended in DMEM. For the serum group, 300 μL of 10% FBS containing DMEM was injected into the lateral gastrocnemius. The serum group (sham treatment) was included to evaluate if the injection procedure itself lead to a response (e.g. inflammatory response) that could contribute to vascular and/or tissue repair.

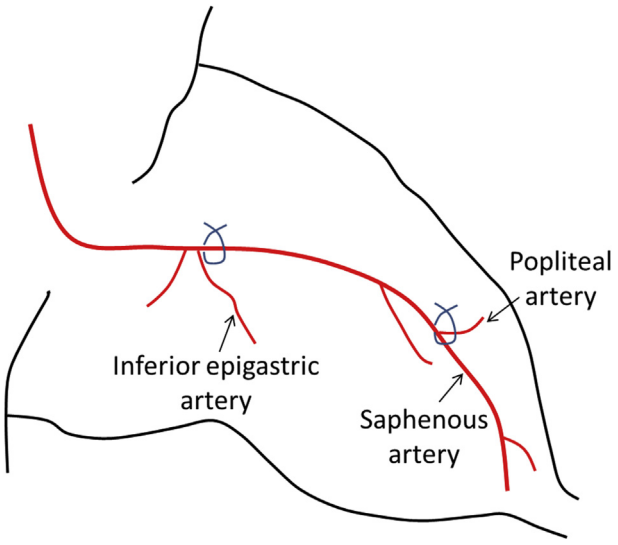

Fig. 1. Schematic illustrating the sites of ligation immediately distal to the inferior epigastric artery and proximal to the branch point of the popliteal and saphenous arteries. The femoral artery was ligated and excised (~0.5 cm portion) in order to induce hind limb ischemia.

2.4. Blood flow measurements

The blood flow to the ischemic hind limbs was imaged using laser speckle imaging. Rats were anesthetized using isoflurane (0.5–2%) infused with oxygen (2 L/min). The speckle imaging system consisted of a diode laser (785 nm, 50 mW; Thor Labs), Basler 1920 × 1080 monochrome CCD with a zoom lens (Zoom7000; Navitar) mounted on a microscope boom stand and used to record speckle images of blood perfusion. The raw speckle images were converted into speckle contrast images and analyzed using Matlab code to quantify the blood flow to the ischemic hind limb as a percentage of the contralateral control.

2.5. Force production measurements

Force production measurements of the lateral gastrocnemius