Cytotherapy, 2017; ■■: ■■–■■

International Society for Cellular Therapy
ISCT®

Placenta-derived PLX-PAD mesenchymal-like stromal cells are efficacious in rescuing blood flow in hind limb ischemia mouse model by a dose- and site-dependent mechanism of action

EFRAT ZAHAVI-GOLDSTEIN, MICHAL BLUMENFELD, DANA FUCHS-TELEM, LENA PINZUR, SHY RUBIN, ZAMI ABERMAN, NOA SHER & RACHELI OFIR

Pluristem Ltd., Haifa, Israel

Abstract

Background. In peripheral artery disease (PAD), blockage of the blood supply to the limbs, most frequently the legs, leads to impaired blood flow and tissue ischemia. Pluristem's PLX-PAD cells are placenta-derived mesenchymal stromal-like cells currently in clinical trials for the treatment of peripheral artery diseases. *Methods.* In this work, the hind limb ischemia (HLI) mouse model was utilized to study the efficacy and mechanism of action of PLX-PAD cells. ELISA assays were performed to characterize and quantitate PLX-PAD secretions in vitro. *Results.* PLX-PAD cells administered intramuscularly rescued blood flow to the lower limb after HLI induction in a dose-dependent manner. While rescue of blood flow was site-dependent, numerous administration regimes enabled rescue of blood flow, indicating a systemic effect mediated by PLX-PAD secretions. Live PLX-PAD cells were more efficacious than cell lysate in rescuing blood flow, indicating the importance of prolonged cytokine secretion for maximal blood flow recovery. In vitro studies showed a multifactorial secretion profile including numerous pro-angiogenic proteins; these are likely involved in the PLX-PAD mechanism of action. *Discussion.* Live PLX-PAD cells were efficacious in rescuing blood flow after the induction of HLI in the mouse model in a dose- and site-dependent manner. The fact that various administration routes of PLX-PAD rescued blood flow indicates that the mechanism of action likely involves one of systemic secretions which promote angiogenesis. Taken together, the data support the further clinical testing of PLX-PAD cells for PAD indications.

Key Words: *hind limb ischemia, mesenchymal stromal cells, placenta, PLX-PAD*

Introduction

Peripheral artery disease (PAD) is a vascular disease in which there is a partial or total blockage of the blood supply to a limb, usually the leg, leading to impaired blood flow and hypoxia in the tissue. The disease affects more than 200 million people worldwide, including 18.6% of the 85- to 90-year-old population [1] and results in more than 40 000 deaths per year [2]. The atherosclerotic changes in the peripheral arteries cause intermittent claudication (IC) and critical limb ischemia (CLI), which lead to pain, tissue damage and ulceration. Mortality at 1 year in patients with CLI is roughly 25%, with an additional 30% of patients requiring amputation [3]. Although early PAD is sometimes treatable by lifestyle changes and medication, more advanced disease stages necessitate revascularization procedures. Unfortunately, as many as 30% of patients are unable to undergo surgical or endovascular procedures due to the extent of disease and comorbidities, and therefore ultimately require amputation [4,5].

In all PAD management regimes, revascularization of the limb plays a central role. Current treatment options for PAD fall into several broad categories: pharmacological [6], endovascular [7], surgical [8] and advanced regenerative treatments. The latter include protein, gene and cell therapies [9], all of which aim to promote collateral vessel formation. Cell therapy has several advantages over the other regenerative methodologies aiming to treat PAD disorders. First, the secretome of cells is multifactorial in nature, as opposed to protein- or gene-based therapies. Second, cells act as a slow-release drug delivery system and are therefore expected to be more efficient and long-lasting than protein-based therapies [10] (Supplementary Figure S1). This solves issues of the short half-lives of some angiogenesis-producing proteins (e.g.,

Correspondence: **Racheli Ofir**, PhD, Pluristem Ltd., MATAM Park, Building 05, Haifa 31905, Israel. E-mail: racheli@Pluristem.com

(Received 29 May 2017; accepted 15 September 2017)

https://doi.org/10.1016/j.jcyt.2017.09.010

hepatocyte growth factor [HGF], the half-life of which is 3–5 minutes) [11]. Finally, cells sense their environment (e.g., hypoxia, stress signals) and react to it by secreting different combinations of cytokines and growth factors.

Numerous clinical cell therapy studies with the aim of restoring circulation and thereby oxygen supply in the ischemic areas of IC and CLI patients are currently underway. These include trials using bone marrow mononuclear cells, granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells, endothelial progenitor cells and mesenchymal stromal cells (MSCs) [12,13], all with the purpose of inducing therapeutic angiogenesis. Pluristem's PLX-PAD cells are derived from human full-term placentae and are grown under Good Manufacturing Practice conditions using the three-dimensional (3D) micro-environmental technology. They are an "off-the-shelf" product that requires no tissue matching before administration [10,14]. The profile of membrane markers expressed by the cells is compatible with the typical expression patterns of MSCs—high expression of CD105, CD73 and CD29 with undetectable CD14, CD19, CD31, CD45 and HLA-DR. Recent studies have indicated that the mechanism of action of many cell therapies is paracrine in nature and does not necessarily involve cellular differentiation [15–17], and indeed, as opposed to bone marrow–derived MSCs, PLX-PAD cells do not proliferate or differentiate into adipocytes or osteoblasts. Therefore, PLX-PAD cells are not considered classical MSCs and are rather defined as mesenchymal-like adherent stromal cells.

Among the many uncertainties in therapeutic angiogenesis are the clinical questions regarding optimal cell dose and site of administration. Several murine hind limb ischemia (HLI) models have been used to test a variety of therapeutic approaches to treat PAD [18], including many that probe cell-based products and their capacity to enhance angiogenesis [19,20]. The studies described herein use an accepted HLI mouse model of ischemia to examine the effect of different doses of PLX-PAD cells and to compare efficacy at different injections sites and method of administration of PLX-PAD cells. We identify the minimal cell dose required for blood flow recovery and use results of comparative injection sites and methods to provide clues for the PLX-PAD mechanism of action. We show that cell-based therapy is more potent than treatment with cell lysate alone, indicating that the injection of intact cells is critical for full potency of treatment. Finally, we identify cytokines secreted by PLX-PAD, which may be involved in the *in vivo* rescue of blood flow. Taken together, this study illustrates that treatment with live PLX-PAD cells ameliorates experimentally induced HLI symptoms, attributable largely to pro-angiogenic mechanisms of action.

Methods

PLX-PAD cell and cell lysate preparation

The PLX-PAD cell production process is composed of two primary steps: (i) isolation of cells from the placenta and culturing the adherent stromal cells in tissue culture flasks and (ii) a 3D growth phase on nonwoven fiber carriers in controlled bioreactors as previously described by Prather *et al.* [14]. After this stage, the cells are harvested and cryopreserved in liquid nitrogen in PlasmaLyte A solution (Baxter) containing 5% human serum albumin (w/v), and 10% dimethyl sulfoxide. Cells are an "off-the-shelf" allogeneic adult cell source product, mainly maternally derived [10,21], ready for injection after thawing.

PLX-PAD cell lysate was prepared by passing cold (on ice) PLX-PAD cells (1×10^6 cells/mL) in PlasmaLyte A 30 times through a 29-gauge needle. A small sample of cells were stained with trypan blue and observed under the microscope to ensure complete lysis, and the lysate was re-passed through the needle as required. PAD cells are injected as is (in cryopreservation solution). In the experiment with cell lysate because cells were washed to create lysate, all cells and cell lysate were injected in PlasmaLyte A only.

Animal care

The animals selected for the present study were healthy adult male C57BL/6 mice aged 8–9 weeks (Harlan Laboratories) weighing about 24 g at study initiation.

The protocol including surgery and all follow-up examinations was approved by the Israel Board for Animal Experiments and was in compliance with the Israel Animal Welfare Act IL-13-10-195. Animals were housed under standard laboratory conditions, air-conditioned and filtered (HEPA F6/6) with adequate fresh air supply (minimum 15 air changes/h). Animals were kept in a climate-controlled environment. Temperature range was 20–24°C, and relative humidity was 30–70% with a 12-h light–dark cycle. Animals had free access to food and water.

Induction of HLI

The HLI mouse model has been used historically for the assessment and evaluation of the potential of implanted cells to reduce ischemic damage [18,22]. On the day of surgery, anesthesia was induced by 1.5–3.0% isoflurane, 1.5% N_2O and 0.5% O_2. Under anesthesia, the mouse was placed ventral side up and an incision (0.5–1.0 cm) was made in the skin in the inguinal area. The femoral artery was ligated with 6-0 silk thread, proximally just after the distal part of the