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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

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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

iliac artery and distally after its bifurcation with the profound femoral artery and then transected and excised between the two ligatures. The wound was then closed with 4-0 silk thread, and the mouse was allowed to recover [18,23].

Treatment of animals

PLX-PAD or placebo vials were thawed in a 37°C water bath, and cells were counted on a hemocytometer with trypan blue to assess cell number and viability. The cells were adjusted to various concentrations from $0.02–10 \times 10^6$ cells/mL in placebo solution (PlasmaLyte A containing 5% human serum albumin [w/v] and 10% dimethyl sulfoxide).

PLX-PAD or placebo solution was injected via intramuscular (IM) route into the operated or contralateral limb or via subcutaneous (SC) route using a 1-mL syringe connected to a 27-gauge needle. Two injections were given, 50 µL/injection site, for a total of 100 µL per animal.

Blood flow measurement

Blood flow in both hind limbs was measured with a noncontact FlowR (Pulse-Or Hemodynamics Imaging), a technique based on laser speckle contrast analysis, an imaging method that visualizes tissue blood perfusion in the microcirculation instantaneously. The FlowR System laser wavelengths (red and near infrared) help to reduce the skin's color influence and increase the examined skin depth to 3 mm. The FlowR System has a range of detection of 15- to 20-fold, and a resolution enabling visualization of blood vessels of 20–25 µm in diameter. Blood flow measurements, before surgery and during the study, were performed blind and expressed as the ratio of the flow in the ischemic limb compared with that of the normal limb.

In vivo assessment of limb function and ischemic damage

A semiquantitative assessment of the level of impairment of the ischemic limb was performed once a week after surgery using the scale [24] described in Table I.

Table I. Assessment of limb function.

Grade	Description
0	Flexing the toes to resist gentle traction of the tail
1	Plantar flexion
2	No dragging but no plantar flexion
3	Dragging of foot

Conditioned medium production and enzyme-linked immunosorbent assays

PLX-PAD were thawed and plated on six-well culture plates, 1×10^6 cells per well in full Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 2 mmol/L L-glutamine and 50 µg/mL gentamycin) and incubated for 24 h at 37°C, 21% O_2 and 5% CO_2 in a humidified incubator. After 24 h, each well was rinsed with 1 mL phosphate-buffered saline, replenished with 1 mL endothelial basal medium (EBM-2, Lonza) and incubated for an additional 24 h in hypoxic conditions at 37°C, 1% O_2 and 5% CO_2 in a humidified incubator. Supernatant was collected, centrifuged once and kept at –80°C until further analysis. Enzyme-linked immunosorbent assays (ELISAs; RayBio Human HGF ELISA kit, cat. no. ELH-HGF; Quantikine ELISA Human Angiogenin Immunoassay, R&D Systems, cat. no. DAN00; Quantikine ELISA Human Angiopoietin-1 Immunoassay, R&D Systems, cat. no. DANG10; Quantikine ELISA Human VEGF Immunoassay, R&D Systems, cat. no. SVE00; Quantikine ELISA Human Osteopontin (OPN) Immunoassay, R&D Systems, cat. no. DOST00; Quantikine ELISA Total MMP-2 Immunoassay, R&D Systems, cat. no. MMP200) were performed as per manufacturer's instructions.

Statistical analysis

Numerical results are given as the mean ± SD of the mean. Statistical significance was determined by two-way analysis of variance for repeated measures, followed by the Bonferroni post hoc test.

Results

PLX-PAD efficiently rescues blood flow and limb function in a HLI murine model

To assess the efficacy of PLX-PAD cells in the HLI murine model, 1×10^6 PLX-PAD cells or vehicle were administered 1 day after induction of ischemia by two IM injections, one proximal and one distal to the femoral dissection site. Blood flow in both limbs was monitored once a week in a blinded study for 21 days. Blood flow of the operated limb was measured and the perfusion rates were normalized to the blood flow in the unoperated contralateral limb. Higher blood flow perfusion rates were observed in mice administered IM PLX-PAD cells compared with the placebo-treated control group (Figure 1). A trend toward improvement was already visible from day 7, and a significant improvement in blood flow was demonstrated from day 14 until the end of the study.

To test whether PAD-PLX cells rescued not only blood flow but also enabled better functionality of the operated limbs, limb function was tested in a

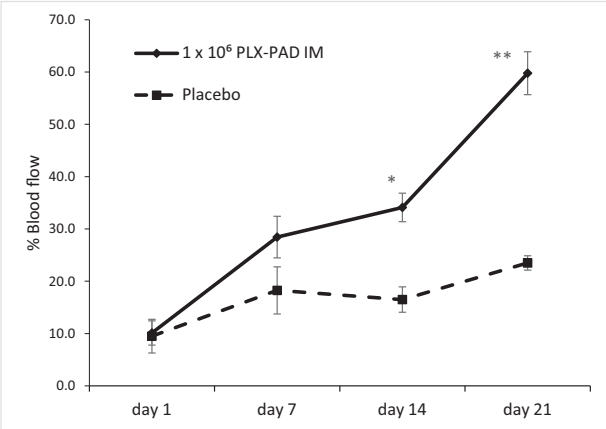

Figure 1. PLX-PAD cells rescue blood flow rates in the HLI murine model. Blood flow rates of the operated limb versus the contralateral limb were measured at various time points after administration (on day 1) of 1×10^6 PLX-PAD cells (solid line, n = 9) or placebo (dashed line, n = 4). $F_{6,72} = 4.318$; $P < 0.001$; $^*P < 0.05$; $^{**}P < 0.0001$.

separate experiment in which function was followed up to 35 days post-operation. Mice administered 1×10^6 PLX-PAD cells IM in the operated leg had improved scores of limb function (see Methods) compared with the placebo treated mice on day 35 (Table II). An additional control included animals that received PLX-PAD cell injections but in which HLI was not induced (unoperated).

The effect of PLX-PAD cells on HLI is systemic

Previously performed preclinical studies have shown that IM-injected PLX-PAD cells do not migrate from the injection site to other tissues or organs, suggesting that the mode of action of PLX-PAD cells is through secretion of proteins, rather than through differentiation of PLX-PAD cells [10]. The therapeutic potential of PLX-PAD has been evaluated both *in vitro* and *in vivo* and suggests an endocrine mechanism of action that includes angiogenesis, modulation of the inflammatory response and promotion of muscle regeneration [10,25–28].

To test whether the effect of the PLX-PAD cells in the HLI model also involves an endocrine mechanism, different routes of administration were used to investigate the possibility of a systemic effect of the cells. One day and 21 days post-ligation, cells (1×10^6 per animal) were administered either IM to the ischemic or to the contralateral limb or subcutaneously (SC) to the dorsal torso, and the effect on blood flow up to day 35 was monitored.

PLX-PAD treatment significantly increased the blood flow to the ischemic limb of HLI mice compared with the relevant placebo through all the administration routes tested (Figure 2), indicating systemic activity of PLX-PAD cells. A lower recovery of blood flow was obtained after SC administration compared with IM administration (on day 35, there was a statistically significant difference between SC and either IM administered to operated limb or to contralateral limb, $P < 0.001$).

PLX-PAD administered to the operated limb continued to provide a rescue in blood flow throughout the time course of the study, whereas the efficacy of IM injection to the contralateral limb started to drop after day 14 and that of SC injection after day 7. These drops in efficacy were rescued by the second administration, when once again the blood-flow-promoting effect afforded by the SC route seemed to only last 1 week and that of the contralateral intramuscular administration lasted 2 weeks. This may be due to more rapid clearance of the cells in the SC locale or to a reduced efficacy due to reduced interaction with immune cells. Interestingly, the second administration of PLX-PAD on day 21 had little to no effect on efficacy. The lack of added benefit from the second administration may stem from the fact that the PLX-PAD cells injected close to the region of HLI induction are already at their maximal efficacy because factors are secreted extremely near the source of injury. Importantly, however, no harmful effect on efficacy was noted by the second administration at the intramuscular site in the operated leg. Taken together, the fact that all methods and locations of administration of PLX-PAD cells were able to rescue blood flow to some extent indicates that at least part of the mechanism of action of the cells is endocrine in nature.

Dose dependency of PLX-PAD cell treatment in the HLI murine model

Given the rescue of limb blood flow and function upon injection of 1×10^6 PLX-PAD cells per mouse, we decided to examine the dose dependency of the effect.

Table II. Incidence of mice with limb function scores 0, 1, 2 and 3 on day 35.

	Score			
Group	0 (%)	1 (%)	2 (%)	3 (%)
A (placebo, HLI-induced)	81.8	18.2	0.0	0.0
B (PLX-PAD treated, HLI-induced)	92.3	7.7	0.0	0.0
C (PLX-PAD treated, unoperated)	100.0	0.0	0.0	0.0

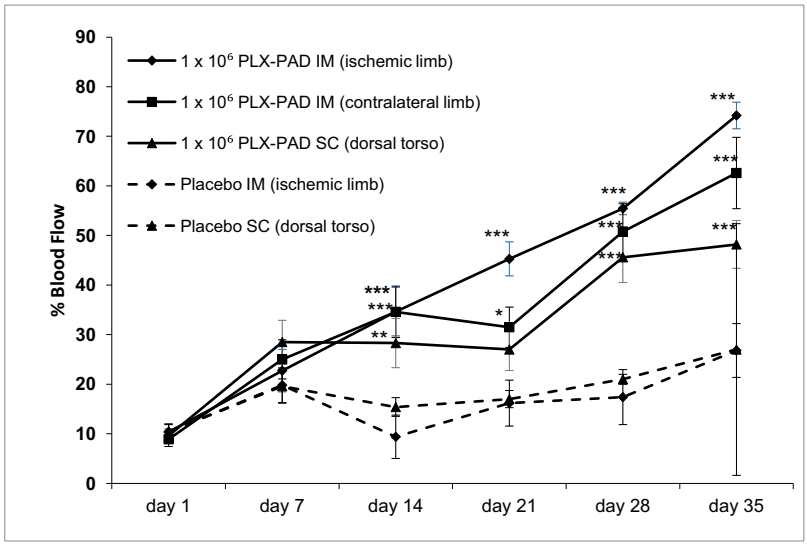

Figure 2. PLX-PAD cells are effective in rescuing blood flow in the HLI mouse model via various routes of administration. IM administration to the ischemic or contralateral limb or SC administration to the dorsal torso were more effective than their relevant controls in rescuing blood flow to the ischemic limb. n = 10 for each PLX-PAD-treated group, and n = 5 for each control group. $F_{39,70} = 30.82$, $P < 0.0001$. Blood flow is measured as percent of flow relative to the contralateral limb. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$, compared with the relevant placebo control.

Preliminary experiments using three concentrations of PLX-PAD cells (0.05, 0.1 and 0.2×10^6 cells per animal) gave similar improvements in the rate of blood flow to that in Figure 1 (data not shown). Therefore, we performed experiments with even fewer cells to identify the minimal effective dose. To this end, PLX-PAD cells (0.05, 0.01 and 0.002×10^6 per animal) were injected IM in two injections to the ischemic limb, one proximal and one distal to the femoral dissection site, and blood flow rescue was followed weekly up to day 21.

IM administration of PLX-PAD cells at the two highest doses tested to significantly improve blood perfusion compared with control (Figure 3), with the highest dose (50 000 cells per animal) providing a response similar to that seen using 1×10^6 cells/mouse (compare with Figure 1). In animals receiving 0.05×10^6 cells, there was a statistically significant effect starting at 70 days post-treatment, reaching a 2.5-fold improvement compared with control on day 21. Overall, the dose-dependence studies indicate that a near-maximal blood flow rescue by PLX-PAD cells

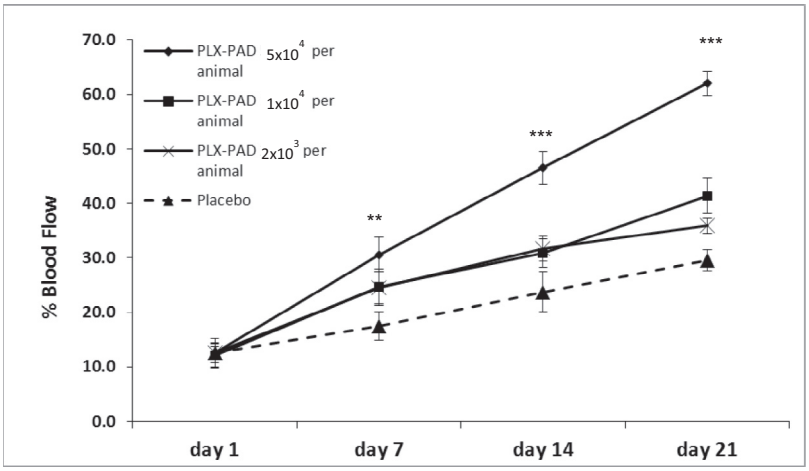

Figure 3. PLX-PAD cells improve blood flow in the HLI murine model in a dose-dependent fashion. Blood flow rates of the operated limb versus the contralateral limb were measured at various time points after administration of various concentrations of PLX-PAD cells or placebo, as indicated in the figure legend. n = 8 for each group. $F_{18,196} = 5.108$, $P < 0.0001$. **$P < 0.01$ and ***$P < 0.0001$ indicate significant difference in blood flow of treatment compared with the placebo at the same time point.

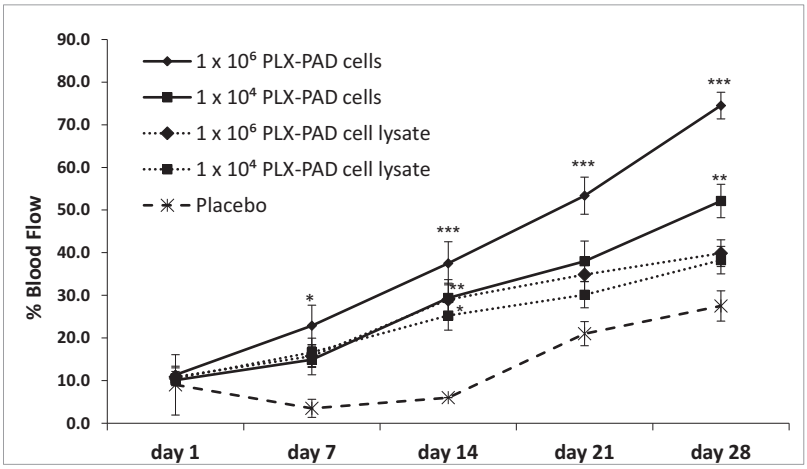

Figure 4. Blood flow is improved more by intact PLX-PAD cells than by cell lysate in the mouse HLI model. PLX-PAD cells (1×106 or 1×104, as indicated) or cell lysate (from the same number of cells, see Methods for details) were injected IM to the ischemic limb in two injections, and blood flow was monitored weekly up to day 28 post-operation. n = 8 for each group. $F_{32,235} = 2.767$, $P < 0.0001$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

can be achieved using a minimum of 0.05×10^6 cells/animal in the HLI model.

Intact PLX-PAD cells are more efficient than PLX-PAD cell lysate in rescuing blood flow in the HLI mouse model

Several previous reports have indicated that the mechanism of action of PLX-PAD cells involves the active secretion of paracrine factors [25,27] and that the conditioned medium can also be clinically effective [27]. Therefore, the efficiency of PLX-PAD cell lysate was compared with that of the intact cells in the HLI murine model. PLX-PAD cells or cell lysate were injected in two concentrations, 1×10^4 or 1×10^6 cells per animal, and blood flow was monitored up to 28 days after the induction of HLI.

The best rescue of blood flow was in response to administration of 1×10^6 intact cells per animal although the lower dose of cells also showed improvement (Figure 4). Blood flow was increased in a similar fashion by both PLX-PAD cells and cell lysate until day 14. From that time point on, the rate of blood flow resulting from administration of the PLX-PAD cell lysate seemed to plateau, whereas the effect of the PLX-PAD cells continued to increase. At day 28, blood flow was higher in the PLX-PAD cell treated group compared to the PLX-PAD cell lysate treated group (roughly a twofold increase in blood flow for the 1×10^6 cells versus cell lysate and 1.4-fold increase in blood flow when comparing the efficacy of 1×10^4 cells versus cell lysate). In a follow-up experiment, histological analyses of day 28 samples from mice treated with either 1×10^6 cells or placebo indicated a tendency to higher levels of CD34 in small (1–4 μm) capillaries (see Supplementary Figure S3).

PLX-PAD cells secrete pro-angiogenic cytokines by which they likely exert their therapeutic affect

As the proposed mechanism of action of PLX-PAD is one of paracrine and endocrine secretions, we wanted to ascertain *in vitro* which cytokines might be involved in the pro-angiogenic activities of PLX-PAD. We performed a high-throughput Luminex screening assay of 23 proteins to identify putative candidates involved in PLX-PAD mechanism of action (supplementary Tables SI–SIII) and verified preliminary candidates with follow-up ELISAs. In Figure 5, the

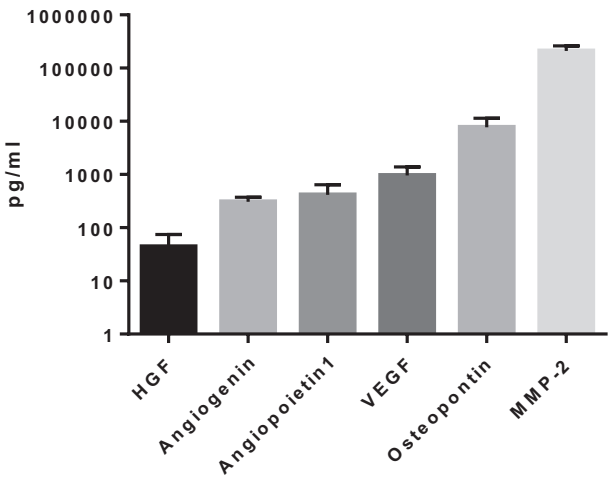

Figure 5. PLX-PAD secrete various pro-angiogenic proteins into conditioned medium. PLX-PAD cells were cultured for 24 h in a humidified incubator, with 5% CO_2 at 37°C, under hypoxic conditions (1% of O_2). Conditioned medium was collected and the protein content assayed by ELISA. Means of at least 11 runs from at least seven placentae are presented. Error bars indicate SD. VEGF, vascular endothelial growth factor.

secretion levels of five pro-angiogenic proteins by PLX-PAD *in vitro* can be seen. In addition, levels of MMP2, involved in extracellular matrix remodeling, an important aspect of angiogenesis, is quantitated. Taken together, it is likely that these proteins are at least partially responsible for the dose- and site-dependent rescue of blood flow by PLX-PAD.

Discussion

Despite advances in understanding of the underlying mechanisms of angiogenesis and pro-angiogenic medical therapy [29–31], treatment of PAD remains a challenging problem. For the growing number of patients who are not suitable candidates for conventional revascularization procedures, therapeutic angiogenesis using MSCs or MSC-like cells has been suggested as a promising treatment strategy [32,33]. The placenta-derived PLX-PAD cells used in this study have been shown to secrete factors known to exert anti-inflammatory, angiogenic and neuroprotective responses [10,26–28]. Here, we show that IM administration of PLX-PAD cells in the operated leg of mice with experimentally induced HLI damage significantly improved limb function and blood flow to the ischemic limb. This is similar to findings in recent publications also studying the effect of intramuscular administration of stromal cell populations (from the placenta and umbilical cord) to the HLI model [22,34]. In our study, we further show that blood flow to the operated limb was restored 60%–75% of the contralateral (unoperated) limb by day 21 post-surgery, using a maximal dose tested of 1×10^6 cells per animal. Studies of various MSC populations (e.g., from BM-MSC and placental MSCs) have indicated that these have similar levels of efficacy in the HLI model [12].

Blood flow restoration was shown to be dose-dependent, with as little as 50 000 cells per animal providing near-complete rescue of blood flow, and the minimal effective dose measured as 10 000 cells per animal. This illustrates an important aspect related to the safety of cell therapy: even a dose 20-fold higher than the effective dose (compare 1 mol/L with 50 000 cells) was safe in a preclinical setting. Although it is yet unclear what is the optimal methodology for extrapolating dose from mouse to human with respect to cell therapy given differences in biodistribution, metabolism and cell clearance, and microcapillary size between species [35], between species scaling based on body weight is frequently used. Given that 50 000 cells per animal, which translates to roughly 2 mol/L cells/kg, provided near-complete efficacy, the current dosing used in human clinical trials of 150–300 mol/L cells per human subject (usually 70–100 kg) is within range of the concentration expected to be necessary for efficacy (clinicaltrials.gov identifiers CLI: NCT03006770, total hip arthroplasty: NCT01525667, IC: NCT01679990). The rationale for performing clinical trials ranging from roughly 2–4 mol/L cells/kg, thereby assessing a dose slightly higher than the minimum efficacious dose in mice, is that the PLX-PAD administration site in the acute HLI model is closer in proximity to the area of blood occlusion than is expected in a human scenario, where the ischemic region is larger and more diffuse and may therefore need a higher cell dose for full efficacy.

To identify whether the effect seen on blood flow involved local cellular activity or was endocrine in nature, we compared blood flow restoration when cells were injected IM to the operated leg, injected IM to the contralateral leg, or given SC to the dorsal torso. A significant effect was still obtained when the cells were injected into the contralateral leg, but the maximal effect was seen when PLX-PAD was administered to the operated leg. Because PLX-PAD cells do not migrate [10], this indicates that the mode of action probably involves both a paracrine and endocrine effect, concordant with current opinion [17]. The endocrine effect is further supported by the fact that even SC injection of cells to the distal torso was able to mediate significant blood flow restoration. The beneficial effect of the cells is likely mediated not only by direct secretion of systemic proteins but also by indirect immunomodulatory activity. Such activity has previously been shown to be involved in the PLX-PAD mechanism of action, as well as in the mechanism of action of other MSC cells in mediating recovery from HLI [10,33]. It is likely that cells administered to the operated leg raised systemic levels of cytokines and performed immunomodulatory functions to an extent that rendered a second administration of cells unnecessary.

In the more distal administration routes, the benefit of a second dosing on final blood flow was demonstrated. Although the second administration had little to no effect on IM PLX-PAD administered to the operated leg, it also did no harm. Therefore, with respect to clinical translation, on the basis of these findings and that, in the clinical scenario, some of the ischemic regions are expected to be more distant relative to the injection site, a second administration is provided in current clinical trials. Past and ongoing clinical studies in patients with CLI and IC (a symptom of early-stage PAD) all involve IM delivery of cells to the infected leg for maximal efficacy; however, it may be convenient in certain clinical settings to deliver cells to the contralateral limb. Our studies here indicate that this may also provide clinical efficacy.

A comparison of the efficacy of injecting viable PLX-PAD cells with injection of cell lysate clearly indicated that the injection of live cells was more effective in restoring blood flow to the operated limb.

PAD-PLX cell lysate was, however, efficient to some extent, in contrast to another study that reported that nonviable placenta-derived cells had no effect [22]. Interestingly, cell lysate showed similar levels of efficacy in blood flow restoration in the first 14 days of the experiment, whereas later time points indicated a higher level of efficacy for live cells. Although some studies have suggested the use of conditioned medium from MSC cells as a therapeutic [36–41], our study indicates that maximal efficacy is reached upon injection of live cells and therefore argues against this notion, at least in the case of PLX-PAD cells. This finding is in agreement with previous studies showing that MSCs undergo additional changes *in vivo*, including secretome alterations, and also empower local resident cells to facilitate angiogenesis and tissue repair (reviewed in Wang *et al.*) [16]. It is therefore likely that, although the initial therapeutic effect of PLX-PAD cells may in part be mediated by factors immediately secreted by the cells, the full potency of the PLX-PAD product is mediated both by PLX-PAD cells that have been modified post-injection by local surroundings as well as by patient-derived cells activated by PLX-PAD to support healing processes. This is in agreement with our unpublished biodistribution studies as well as similar studies published on placenta-derived adherent cells used in the HLI model [22] indicating that clearance of the majority of injected cells occurs within several weeks of injection. These combined findings would suggest that PLX-PAD cells exert an effect more than that of conditioned medium alone by allowing themselves, and thereby their secretions and cell–cell interactions, to be mediated by the surrounding tissue. Subsequently, the modified PLX-PAD cells affect resident cells, resulting in pro-angiogenic activity that continues after the majority of PLX-PAD cells are no longer present in the tissue (Supplementary Figure S1).

This study shows that PLX-PAD rescue blood flow in a HLI mouse model in a dose- and site-dependent manner. Live PLX-PAD cells were shown to be more efficacious than cell lysate, indicating the importance of cells, and not just their content for maximal potency, likely because this enables secretion for longer time periods than a single cell lysate injection would allow. In support of the proposed mechanism of action whereby multifactorial PLX-PAD secretions activate secretory changes in endogenous cells, we show that numerous pro-angiogenic cytokines are released by PLX-PAD *in vitro*, likely playing a role in the *in vivo* rescue of blood flow. Interestingly, studies of another placenta-derived mesenchymal stromal–like cell population indicates similar pro-angiogenic cell secretions [22]. Taken together, the data presented here in conjunction with previous studies indicating PLX-PAD's ability to induce endothelial cell proliferation and to act in immune-modulatory mechanisms [25] support further clinical study of PLX-PAD to improve symptoms of PAD such as IC and CLI.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2017.09.010.