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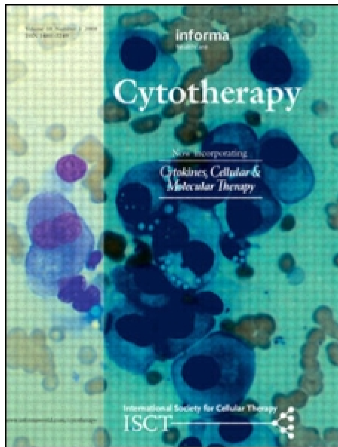

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The role of placental-derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia

William R. Prather[1], Amir Toren[1], Moran Meiron[1], Racheli Ofir[1], Carsten Tschope[2] and Edwin M. Horwitz[3]

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Background aims

Mesenchymal stromal cells (MSC) are spindle-shaped plastic-adherent cells isolated from bone marrow (BM), adipose tissue and other organs, including the placenta. Autologous BM-derived MSC have been studied in animals with experimentally induced critical limb ischemia (CLI) as a model of end-stage peripheral vascular disease. While demonstrating therapeutic benefit, the use of these cells is limited by the need to surgically extract BM and the fear of thrombosis secondary to the use of granulocyte–colony-stimulating factor (G-CSF) to mobilize the cells.

Methods

We studied the use of placental-derived adherent stromal cells (ASC) in a standard limb ischemia model of male Balb/c mice. These placental-derived cells, termed PLX-PAD, share the adherence and marker expression of BM-derived MSC but lack their differentiation potential. PLX-PAD are isolated from human placenta following a Caesarean section and cultured in a bioreactor, termed the PluriX™ System. The PluriX™ System provides a three-dimensional (3-D) microenvironment that enables the large-scale growth of these cells. PLX-PAD are stable adhesive cells that can be expanded in vitro *without the loss of phenotype and without showing signs of karyotypic changes.*

Results

The intramuscular (i.m.) administration of PLX-PAD in our model significantly improved blood flow (BF) ($P = 0.0008$*), increased capillary density (* $P = 0.021$*), reduced oxidative stress (* $P = 0.034$*) and reduced endothelial damage (* $P = 0.004$*), while increasing limb function versus the administration of a phosphate-buffered saline (PBS) control vehicle in the affected limb.*

Conclusions

Allogeneic placental-derived ASC may provide an off-the-shelf supply of therapeutic cells that would need no histocompatible tissue matching and be potentially less expensive and considerably more convenient than BM or adipose-derived MSC.

Keywords

Allogeneic transplantation, cell therapy, critical limb ischemia (CLI), mesenchymal stromal cell (MSC), peripheral artery disease (PAD), PLX-PAD.

Introduction

Peripheral artery occlusive disease (PAOD), also known as peripheral vascular disease (PVD) or, more commonly, peripheral artery disease (PAD), is a collator for diseases caused by the obstruction of large peripheral arteries resulting from atherosclerosis or other inflammatory processes that can lead to acute or chronic ischemia. Critical limb ischemia (CLI) is the severe subset and natural end-point of PAD.

Analysis of data from the 1999–2000 National Health and Nutrition Examination Survey indicated that 4.3% of the USA population older than 40 years of age suffers from PAD [1], which currently translates into approximately 7 million people with the disorder. The incidence

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of PAD increases significantly with age, rising to approximately 20% of the population older than 70 [1], and indicates a growing market for therapies intended to treat this disorder. It has been estimated that CLI affects approximately 1.1 million USA patients and is anticipated to grow to approximately 1.4 million patients by 2015 [2]. This translates into approximately 160–200,000 PAD amputations performed annually in the USA [2].

Although endovascular therapies such as balloon dilation and revascularization surgery can be quite helpful for selected patients, it has been estimated that approximately 25% of CLI patients are not suitable for such procedures [3]. Additionally, many patients with CLI have a stable or slowly progressive presentation. A review of the data revealed that patients with chronic CLI have a 3-year limb loss rate of approximately 40% [4]. This suggests that a substantial proportion of patients with the disease are not at risk of imminent limb loss with most patients tending to have an inexorable downhill course. This is important because it allows for stand-alone or adjunctive therapies that have no role in the emergent treatment of CLI but addresses the underlying pathophysiology of hypoperfusion via mechanisms other than attacking the diseased vessel. One such therapy that has been found to be useful is mesenchymal stromal cell (MSC) therapy, which stimulates angiogenesis in the CLI-involved extremity.

In an effort to supply patients with an allogeneic, off-the-shelf supply of MSC that would need no histocompatible tissue matching and would be potentially less expensive and considerably more convenient than bone marrow (BM) or adipose-derived MSC. Pluristem Therapeutics Inc. (Haifa, Israel) is studying placental-derived mesenchymal-like adherent stromal cells (ASC) for the treatment of CLI. While most of the work performed on MSC has been derived from autologous BM, Pluristem Therapeutics Inc. has focused on using ASC derived from placenta and used allogeneically.

Pluristem's ASC exhibit many markers common to MSC isolated from other tissues, including CD105, CD73, CD90 and CD29, and lack the expression of hematopoietic, endothelial and trophoblastic-specific cell markers. These placental-derived mesenchymal-like stromal cells are expanded in Pluristem's proprietary PluriX™ System, which imitates the natural microstructure of the bone marrow and does not require supplemental growth factors or other exogenous materials. Recent evidence suggests that the resultant PLX (PLacental eXpanded) cell's efficacy may be related to the secretion of cytokines or other potent immune modulators. Furthermore, PLX-PAD cells are stable adhesive cells that can be expanded *in vitro* without the loss of phenotype and without showing signs of karyotypic changes. They are immune privileged and have immunomodulatory properties that may result in anti-inflammatory qualities.

Methods

Cell manufacturing

ASC were isolated from full-term human placentas [5,6]. All placentas obtained by Pluristem are received from a maternity ward following scheduled Caesarean sections, under approval of the Israeli Ministry of Health Helsinki Committee. All placenta donors sign an informed consent before donor screening and testing are performed. The placentas are transported from the medical center to Pluristem under controlled conditions: they are placed in a sterile plastic bag and then into a Styrofoam box with ice packs, and delivered to Pluristem. At Pluristem the placentas are placed in a quarantine area until released for use by quality control (QC) and quality assurance (QA). Placenta-processing initiation occurs ≤ 4 h following the Caesarean section. Thus the placenta provides an ethically uncontroversial and easily accessible source of cells for future experimental and clinical applications.

The production of PLX-PAD is performed in a state-of-the-art clean room facility according to good manufacturing practice (GMP) regulations. The facility and utility systems provide a 125-m^2 clean room production area, a QC laboratory, a storage room and cold storage areas. The production process is composed of several major steps, as illustrated in Figure 1, that includes receipt of the placenta, recovery and processing of ASC, growth of the cells in tissue culture flasks [two-dimensional (2-D) cultures] and harvest and storage of the cells in liquid nitrogen as 2-D cell stock (2DCS). The 2DCS is considered to be an in-process intermediate product and is tested for sterility, mycoplasma, immunophenotype and viability. Upon meeting 2DCS release specifications, the appropriate amount of 2DCS is thawed, washed and seeded onto carriers in bioreactors for further expansion in three-dimensional (3-D) culture. After 1–2 weeks of growth in the bioreactors, the cells are harvested and cryopreserved in liquid nitrogen as PLX-PAD.

During the manufacturing process an in-process control (IPC) is established based on FDA guidelines. PLX-PAD

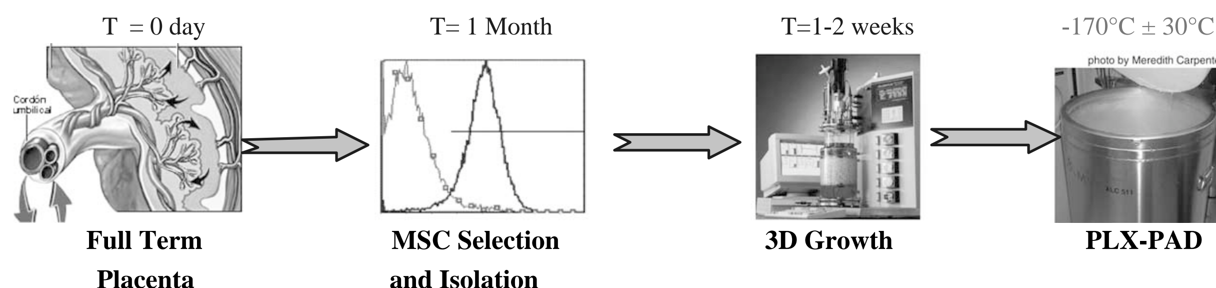

Figure 1. *PLX-PAD production scheme*

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are manufactured and released under a QC program including IPC testing and a battery of product-release tests and specifications, such as visual appearance, viability, immunophenotype, mycoplasma, endotoxin, sterility and an *in vitro* potency assay.

Flow cytometry analysis of cell membrane marker phenotype

In order to characterize the surface antigens/markers expressed by PLX-PAD, the cells were stained with monoclonal antibodies (Table I) for the characteristic MSC-positive markers CD73, CD90 CD29, CD105 and negative markers CD34, CD45, CD19, CD14 and HLA-DR. The immunophenotype test specifications were set at $\geq 90\%$ for all positive markers and $\leq 3\%$ for all negative markers. Positive and negative markers and their release specifications were determined based on cumulative experimental data and according to BM MSC literature characterization. All the membrane marker tests were performed using the FC 500 Flow cytometry system (Beckman Coulter, Fullerton, CA, USA) with CXP analysis software. This flow cytometer conducts five-color analysis from a single laser excitation.

Table I. Immunophenotype of PLX-PAD

Membrane marker	% positive cells
CD105	99.5
CD73	98.9
CD90	99.2
CD29	98.5
CD34	0.21
CD45	1.84
CD19	2.3
HLA-DR	1.74
CD14	0.3
CD31	1.52
KDR	1.24
D7-Fib	99.5

PLX-PAD mixed lymphocyte reaction assay

PLX-PAD mixed lymphocyte reaction (MLR) tests were performed with 2×10^5 cord blood-derived mononuclear cells (MNC) stimulated with equal amounts of irradiated (3000 Rads) peripheral blood (PB)-derived MNC, in the presence of different amounts of irradiated PLX-PAD. Three replicates of each group were seeded in 96-well plates. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 20% fetal bovine serum (FBS). Plates were pulsed with 1 μC [^{3}H]thymidine during the last 18 h of 5-day culturing. Cells were harvested over fiberglass filters and thymidine uptake was quantified using a scintillation counter.

PLX-PAD biodistribution

PLX-PAD were stably infected with a lentiviral construct expressing the luciferase gene under the cytomegalovirus (CMV) promoter. Efficiency rates of infection were close to 100%. Evaluation of luminescence in living cells and living mice was performed using the IVIS Lumina Imaging System (Xenogen Corporation, Hopkinton, MA, USA), which includes a highly sensitive CCD camera that captures the luciferase luminescence signal. Cells continued to divide following infection, and expression levels of luciferase in the growing cells remained strong and stable. Two weeks post-infection 1×10^6 PLX-PAD cells were injected intramuscularly (i.m.) into NOD/SCID and Balb/c mice. Injected cells were monitored using the IVIS system.

In vivo *hind limb ischemia model*

This experiment was performed in order to asses the therapeutic potential of PLX-PAD in a widely accepted animal model for the treatment of CLI, the Balb/c mouse

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hind limb ischemia model. In all efficacy experiments, hind limb ischemia was induced in Balb/c mice. Balb/c mice were used under the assumption that immune-competent animals serve as the most suitable model for humans who do not suffer from immune deficiency. Additionally, as our data suggested the mode of action of PLX-PAD is through a paracrine secretion mechanism, it may require interaction with the immune system and an inflammatory environment which relates to limb ischemia, and thus the use of immune-competent animals.

The induction of hind limb ischemia was performed as described elsewhere [7]. This proximal artery ligation model is considered to be an appropriate model to induce severe ischemic damage, as in critical limb ischemia. In this model, ligation of the femoral artery initiates inflammatory processes caused by hypoxic conditions. Under anesthesia, the mouse is placed ventral side up and a 1–1.5-cm incision is made in the skin in the inguinal area. The femoral artery is ligated twice with 6-0 silk and transected distal to the ligature. The wound is closed with 3-0 silk and the mouse allowed to recover.

In all instances, the PLX-PAD test product and phosphate-buffered saline (PBS) vehicle control were administered by i.m. injection into the right thigh musculature 5 h post-induction of limb ischemia in a volume of 50 μL/animal divided into two 25-μL injections. The administration of the cell suspension vehicle (PBS) served as a baseline control for the evaluation of improvement of various parameters. The rationale for administering PLX-PAD cells 5 h following surgery was to enable animals to recover from the trauma of surgery and anesthesia prior to test product or vehicle control administration. The contra-lateral (left) hind limb served as control. The study included 20 mice (10 in each group) and was performed via the administration of 1×10^6 PLX-PAD cells/mouse/50 μL. The study lasted 21 days, at which time the animals were sacrificed and histologic examination of the limbs conducted.

Evaluation of efficacy

Animals were randomly assigned to groups to evaluate limb functionality and blood flow (BF). All measures and evaluations were performed in a blinded manner.

Limb functionality

Semi-quantitative assessments of functionality of the ischemic limb were performed serially (3, dragging of foot; 2, no dragging but no plantar flexion of the foot; 1, plantar flexion of the foot; 0, flexing the toes in response to gentle traction of the tail). Limb function was graded as not applicable in the case of partial or full limb necrosis. In this study BF measurements from the foot area were not included in the statistical analysis.

Blood flow

Blood flow of legs from both the ischemic-induced and normal limb were measured three consecutive times with a non-contact laser Doppler just after the operation (day 1) and at 1-week intervals post-operation until the end of experiment. Blood flow measurements were expressed as a ratio of the flow in the ischemic limb to that in the normal limb.

Statistical analysis

Data analysis of all measurable parameters was carried out using a *t*-test comparison test. A probability of 5% ($P \leq 0.05$) was regarded as significant.

Results

PLX-PAD cell immunophenotype

In order to characterize the surface antigens expressed by PLX-PAD, the cells were assessed by flow cytometry for the recommended markers for BM-derived MSC. Similar to fibroblasts, PLX-PAD are spindle-shaped cells and express typical fibroblast markers on the surface of the cell membrane. PLX-PAD cultures do not include any impurities of endothelial cells (EC), as shown by negative staining for the endothelial markers CD31 and KDR. Table I presents a summary of typical membrane markers analyzed performed on the PLX-PAD batch that was used in the *in vivo* hind limb ischemia study.

Immunogenicity of PLX-PAD cells

To investigate the immunogenicity as well as the immunomodulation properties of PLX-PAD, MLR reactions, utilizing PB samples from two donors (PB-A and PB-B) and PLX-PAD, were performed. The addition of PLX-PAD to PB-A evoked no response and suggested PLX-PAD cells were immunoprivileged, while the addition of PB-A to PB-B evoked a considerable stimulation of lymphocytes. Thereafter, there was a dose-dependent reduction in lymphocyte stimulation with the addition of PLX-PAD cells, suggesting PLX-PAD cells were immunosuppressive. The experiments demonstrated that these

cells could both escape allorecognition and reduce the T-cell response, as measured by thymidine incorporation. Figure 2A shows the reduction of lymphocyte proliferation (c.p.m. measurement) in the presence of an increasing amount of PLX-PAD in a dose-dependent manner. PLX-PAD also reduced lymphocyte proliferation to mitogenic stimuli, such as phytohemagglutinin (PHA) and concanavalin A (Con A), and non-specific stimulation by anti-CD3 and anti-CD28 (data not shown).

PLX-PAD trans-well assays

In order to better understand the mechanism of action and determine whether the immunomodulation effect of PLX-PAD on lymphocyte proliferation occurred via cell–cell interaction or soluble mediators, PB-derived MNC were stimulated by PHA using the trans-well method, which prevents cell–cell contact but enables the diffusion of cytokines through the membrane between the two compartments. The inhibition of proliferation remained

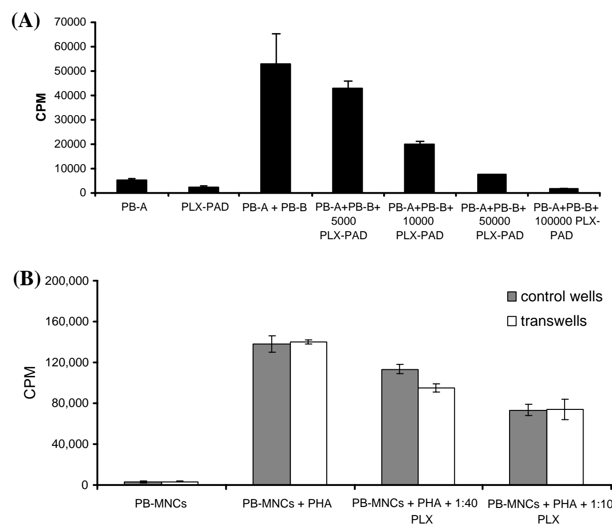

Figure 2. *(A) PLX-PAD MLR tests performed with 2×10^5 cord blood-derived MNC stimulated with equal amounts of irradiated (3000 Rads) PB-derived MNC, in the presence of different amounts of irradiated PLX-PAD. Three replicates of each group were seeded in 96-well plates. (B) PB-derived MNC were stimulated with PHA (2 mg/mL) using a trans-well system. Increasing amounts of PLX-PAD cells were added to the culture. Three replicates of each group were seeded in 96-well plates. In both experiments, cells were cultured in RPMI-1640 medium containing 20% FBS. Plates were pulsed with 1μC [^{3}H]thymidine during the last 18 h of 5-day culturing. Cells were harvested over a fiberglass filter and thymidine uptake was quantified with a scintillation counter.*

the same when cell–cell contact was prevented by separating the cell cultures (Figure 2B).

PLX-PAD biodistribution

PLX-PAD were stably infected with a lentiviral construct expressing the luciferase gene under the CMV promoter. Efficiency rates of infection were *c.* 100%. The evaluation of luminescence in living cells and living mice was performed using the IVIS Lumina Imaging system, which includes a highly sensitive CCD camera that captures the luciferase luminescence signal. Two weeks following infection, 1×10^6 PLX-PAD cells were administered i.m. into five NOD/SCID and five Balb/c mice.

The biodistribution of PLX-PAD is depicted in Figure 3. Once PLX-PAD cells were administered into both immunocompetent and immunocompromised (i.e. Balb/c and NOD/SCID) mice, the biodistribution pattern showed that the cells persisted only at the injection site and did not distribute to additional organs. PLX-PAD cells retained consistently high levels of luciferase expression, *in vitro*, for up to 3 weeks.

PLX-PAD therapy of experimental limb ischemia

In this study, the i.m. administration of PLX-PAD was performed in order to determine whether the implantation of these cells can reduce ischemic damage in the mouse hind limb ischemia model and mice would demonstrate clinical and motor function improvement in the ischemic limb. The results demonstrated that the administration of these cells significantly improved BF ($P = 0.0008$), capillary density ($P = 0.021$), oxidative stress ($P = 0.034$) and endothelial damage ($P = 0.004$) while increasing limb function versus the administration of a PBS control vehicle in the affected limb.

In terms of BF, this effect could be demonstrated 9 days following the injection of PLX-PAD and observed throughout the entire study. In the hip region, BP in the PLX-PAD-treated group ranged from $24 \pm 2.3\%$ to $80 \pm 4.7\%$, while in the control vehicle-treated group BF ranged from $35 \pm 2\%$ to $54 \pm 4.5\%$ (day 0 versus day 21, respectively) ($P = 0.0008$). To a lesser extent, and similar to the observed effect in the hip region, an increase in BF could be demonstrated in the foot region. In the vehicle-treated group, BF ranged from $12 \pm 0.6\%$ to $46 \pm 4.9\%$, while in the PLX-PAD group BF increased from $10 \pm 0.7\%$ to $52 \pm 5.5\%$ (day 0 versus day 21, respectively) ($P = 0.054$), as shown in Figure 4.

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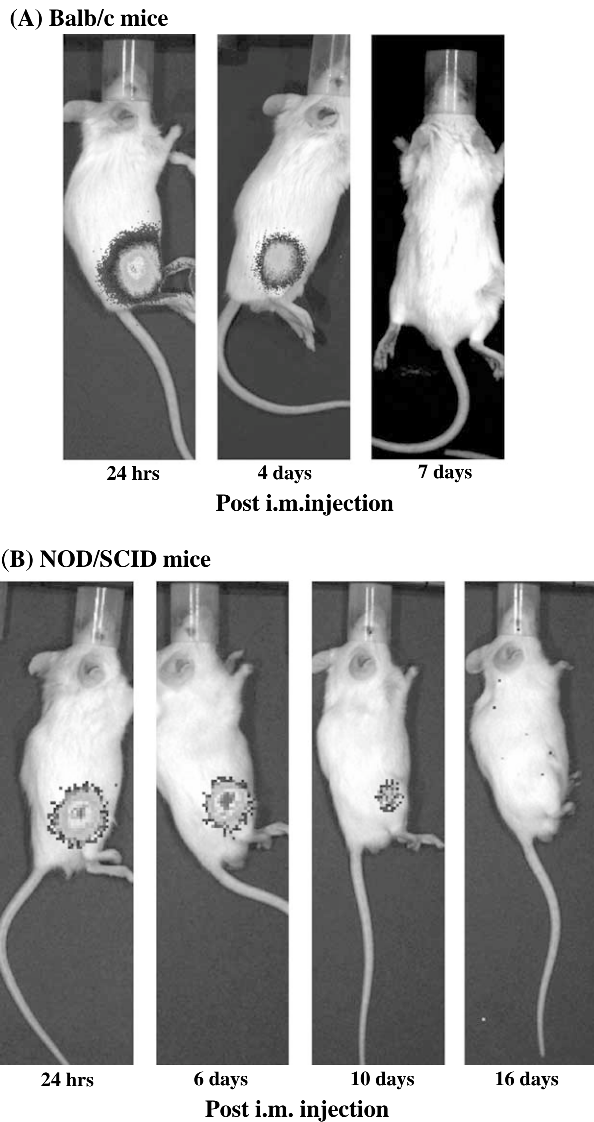

Figure 3*. Biodistribution of PLX-PAD in (A) Balb/c and (B) NOD/SCID mice.*

A slight difference in limb function (2.5 ± 0.2 vs. $2.1 \pm 0.2\%$ control vs. PLX-PAD group respectively, $P = 0.433$) (Figure 5) could be demonstrated between the control vehicle and PLX-PAD-treated groups. Macroscopic assessment of ischemic severity revealed that, in the control vehicle-treated group, necrosis limited to the toes could be observed in two animals on day 6. In the PLX-PAD-treated group, necrosis limited to the toes could be demonstrated in only one animal after 14 days.

Histologic evaluation of limbs

Post-mortem immunohistochemical analysis of the limbs treated with PLX-PAD indicated a significant increase in the number of new capillaries (vessels) supplying the limb compared with controls ($P = 0.021$), suggesting PLX-PAD has the ability to promote angiogenesis (Figure 6).

Additionally, a decreased oxidative stress ($P = 0.034$) and a reduction in endothelial inflammation ($P = 0.004$) (which is a surrogate parameter for improved endothelial function) in the treated animals were observed in the PLX-PAD-treated mice compared with controls (Figure 7).

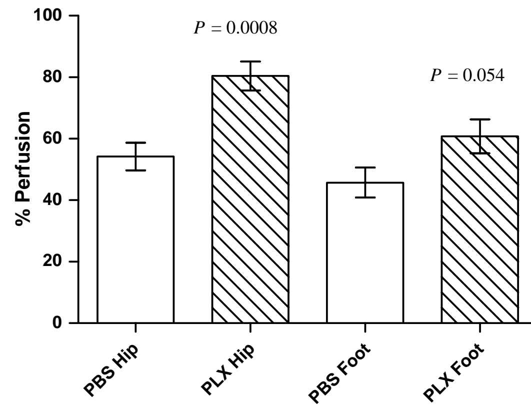

Figure 4*. Improved BF in mice treated with PLX-PAD, but not in control mice treated with PBS, following induced limb ischemia, as demonstrated by laser Doppler technology.*

Toxicity

Compared with control PBS-injected mice, none of the PLX-PAD-injected mice exhibited any adverse clinical signs or symptoms in response to i.m. cell administration.

Discussion

MSC have been shown to differentiate into EC [8]. In addition, emerging experimental results with MSC have

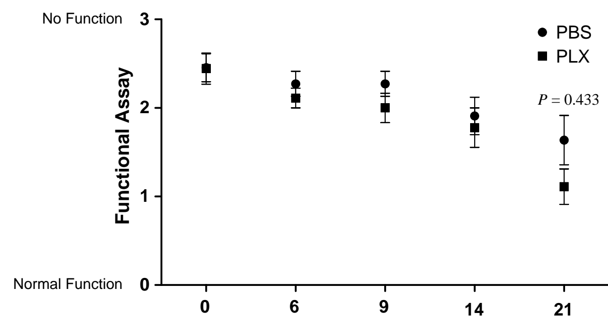

Figure 5*. Improved leg functionality* (P = 0.433) *in mice treated with PLX-PAD cells, but not in control mice- treated with PBS, at days 0, 6, 9, 14 and 21 following induced limb ischemia an evaluated by macroscopic examination.*

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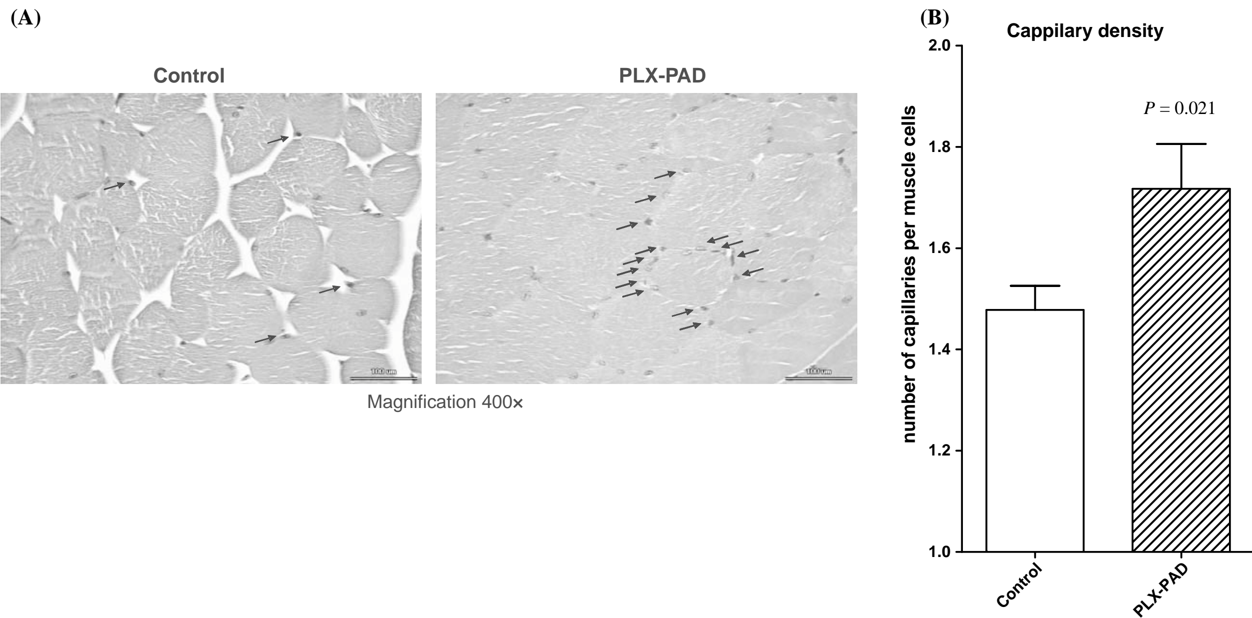

Figure 6. Increased capillary density in mice treated with PLX-PAD cells, but not in control mice treated with PBS (P = 0.021), *following induced limb ischemia, as demonstrated by specific capillary staining. (A) H&E staining; arrows mark new capillaries. (B) Quantitation of the number of capillary vessels per myofiber, as counted for all the muscles in the study.*

offered novel mechanistic insights into vascular regenerative therapy in general. For example, adipose tissue-derived stromal cells co-cultured with EC induce a significant increase in EC viability and migration, mainly through the secretion of angiogenesis cytokines such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [9].

There are numerous examples in the literature citing the efficacy of MSC in patients with vascular insufficiency, although most of these studies used an autologous supply of mononuclear cells. In 2002, Esato *et al.* [10] published the results of an eight-patient pilot study in patients with PAD in whom surgical bypass procedures or long-term medication had failed. Four out of the eight patients had arteriosclerosis obliterans; the other four cases suffered from severe Buerger's disease affecting five of eight limbs. Patients were treated with autologous BM-derived mononuclear cells and evaluated after 4 weeks and 1 year. Seven of the eight cases reported improvement in ischemic rest pain after 4 weeks. Increased collateralization was observed by angiography in two of eight cases and six of nine limbs showed an improvement in Fontaine stage after 4 weeks. These improvements were sustained for at least 1 year post-treatment [10]. In 2003, Taguchi *et al.* [11] published a case report on a patient with non-healing ulcers of both hands. Treatment with autologous BM cells resulted in the dilation of existing arteries and new collateral vessel formation. Systolic pressures in the fingers were increased; skin color changed from ischemic red to pink and the healing ulcers were covered with skin [11]. In 2006, Kim

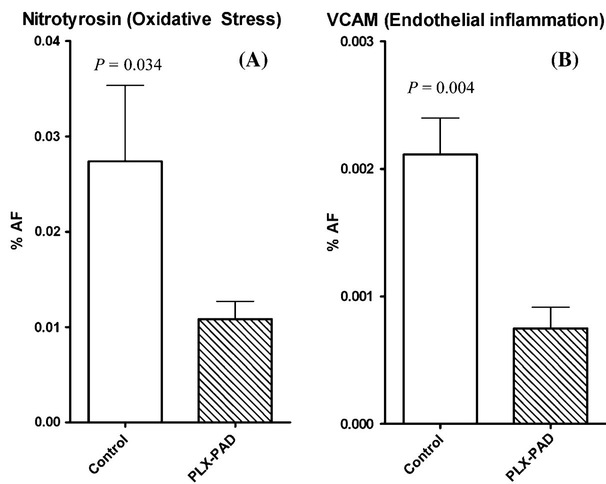

Figure 7. (A) Reduced oxidative stress, as measured using nitrotyrosin staining (P = 0.034), *and (B) endothelial inflammation, measured by VCAM evaluation* (P = 0.004), *due to an increased oxygen supply in mice treated with PLX-PAD cells but not in control mice treated with PBS, following induced limb ischemia.*

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et al. [12] administered umbilical cord blood-derived MSC to four men with Buerger's disease who had already received medical and surgical therapy, resulting in significant relief in ischemic rest pain. Other studies have indicated that there have been no adverse events associated with local implantation of BM-derived mononuclear cells [13].

This experiment offers the first study using mesenchymal-like stem cells derived from the human placenta (ASC) and expanded in a 3-D manner without the use of growth factors or other adulterants for the indication of CLI. Under the conditions of the present study, it was demonstrated that PLX-PAD induces significant increases in BF, probably as a result of angiogenesis, as supported by histologic evaluation of the damaged limbs. In addition, the delay in development of necrosis and the difference in number of affected animals are suggestive of a clinical response in this model, thereby supporting the BF and angiogenesis findings.

The use of these so-called PLX-PAD cells may be a cellular therapeutic modality that addresses the concerns of using BM-derived MSC in the treatment of PAD. These concerns include primarily the surgical procedure needed to extract BM and the use of granulocyte–colony-stimulating factor (G-CSF) to mobilize BM-derived MSC, which it is feared potentially predisposes patients afflicted with CLI to thrombosis [14]. There are several other advantages in using PLX-PAD, placenta-derived mesenchymal-like ASC versus BM cells. In contrast to BM, placentas are considered to be 'biologic waste' and can be obtained without the need of surgical intervention or any morbidity whatsoever to the donor. Additionally, placental cells are harvested from younger tissues compared with BM-derived MSC obtained from adult donors, and it has been demonstrated that the *in vitro* proliferation capacity of MSC declines as the donor's age rises [15]. Because of the immune-modulating properties noted with these cells, PLX-PAD may offer CLI patients the first allogeneic off-the-shelf product that needs no histocompatible tissue matching for treatment of this disorder.

Declaration of interest: William Prather, Amir Toren, Moran Meiron and Racheli Ofir are shareholders and employees of Pluristem Therapeutics Inc. Edwin Horowitz is a shareholder and member of the Scientific Advisory Board of Pluristem Therapeutics Inc.

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