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Safety and Biodistribution Profile of Placental-derived Mesenchymal Stromal Cells (PLX-PAD) Following Intramuscular Delivery

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The administration of mesenchymal stromal cells (MSCs) provides an exciting emerging therapeutic modality for the treatment of peripheral arterial disease, a condition that is associated with critical limb ischemia as its end stage. Placental-derived MSCs, termed PLX-PAD cells, are stable adhesive stromal cells isolated from full-term human placentae, cultured on carriers, and expanded in a bioreactor called the PluriX. These cells can be expanded in vitro without phenotypic or karyotypic changes. We studied the safety and biodistribution properties of PLX-PAD cells following intramuscular administration in NOD/SCID mice. No significant clinical signs, hematological and biochemical parameters, or major pathological changes were found in PLX-PAD-treated animals in comparison to vehicle controls. Several animals in the control and PLX-PAD-treated groups developed thymic malignant lymphoma, first seen after one month, as expected in this mouse strain. In addition, both groups developed spontaneous mesenteric vessel inflammation. Real-time quantitative polymerase chain reaction (RT-qPCR) demonstrated that distribution of PLX-PAD cells was confined to the injection site. Placental-derived MSCs remained in this site with gradual decrease in concentration during a three-month period. In view of these data, we conclude that the administration of PLX-PAD cells is not associated with any adverse effects in NOD/SCID mice.

Keywords: limb ischemia; bone marrow transplant; safety profile; biodistribution; PLX-PAD.

Introduction

Peripheral arterial disease (PAD), a common disease associated with significant morbidity and mortality (Rosamond et al. 2007), affects approximately eight million people in the United States, with an estimated prevalence of 14%–29% among people older than 70 years (Hirsch et al. 2001; Rosamond et al. 2007; Selvin and Erlinger 2004). PAD frequently becomes the consequence of progressive narrowing of arteries in the lower extremities resulting from atherosclerosis (Selvin and Erlinger 2004). A less prevalent cause of limb ischemia is thromboangiitis obliterans, or Buerger's disease (Lazarides et al. 2006). The most severe clinical manifestation of PAD is critical limb ischemia (CLI), manifested as chronic ischemic rest pain, ulcers, or gangrene (Casserly 2008).

Conventional therapies for CLI include endovascular therapies such as balloon dilation and revascularization surgery; however, these procedures are suitable in fewer than half of patients and provide only partial results. In addition, large numbers of restenoses necessitate that many patients having to undergo additional procedures (Murphy et al. 2008). Against that background, alternative treatments have been extensively investigated. One therapeutic approach that has recently emerged as a promising treatment for this chronic, and potentially devastating, disease is mesenchymal stromal cell (MSC) therapy, which aims at the pathophysiological mechanisms underlying ischemic limb hypoperfusion. The administration of these cells into a hind limb ischemia-induced mouse model has been found to stimulate angiogenesis in the affected limb (Kinnaird, Stabile, Burnett, Shou et al. 2004).

To overcome some of the obstacles that limit the use of autologous cell treatments and allogeneic bone marrow MSCs, our group has been investigating placental-derived MSCs for the treatment of CLI. The cells, termed PLX-PAD cells, were found to be stable adhesive stromal cells that expressed typical MSCs markers (e.g., CD105, CD75, CD90) and did not express hematopoietic markers as shown by flow cytometry.

Studies using cell therapy for the treatment of PAD have generally shown a reassuring safety profile (Hirsch 2006). Nevertheless, one case series reported major adverse events, including one death, worsening of leg ulceration and rest pain, and arteriovenous shunt formation (Miyamoto et al. 2006). This case series, however, was criticized as suffering from several major trial design weaknesses (e.g., very small sample size) (Hirsch 2006). An additional study using mice as an experimental model has raised concerns regarding the potential of bone marrow and endothelial progenitor cells to increase atherosclerotic lesion size and to influence plaque stability

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Abbreviations: 2DCS, 2D-Cell Stock; CLI, critical limb ischemia; IM, intramuscular; MSC, mesenchymal stromal cell; NOD/SCID, nonobese diabetic/severe combined immunodeficient; PAD, peripheral arterial disease; RT-qPCR, real-time quantitative polymerase chain reaction.

(George et al. 2005). In addition, any use of stem cells has the potential for increasing the risk of uncontrolled growth and tumorigenicity (Halme and Kessler 2006).

The purpose of this study was to assess the potentially toxic effects of PLX-PAD cells in target and nontarget tissues at various time points during a three-month period, following single or multiple intramuscular (IM) injections to male and female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (this phase of the study was termed the toxic phase). The IM route of administration is in consideration. Additionally, we performed a biodistribution assay for the detection of PLX-PAD cells in selected tissues (the biodistribution phase).

Materials and Methods

Manufacturing Process of PLX-PAD Cells

The production of PLX-PAD cells was performed in a state-of-the-art clean room facility according to GMP regulations. The facility and utility systems provided a 125-m^2 clean room production area, a QC lab, storage room, and cold storage areas.

The production process was composed of several major steps: after receipt of a donor human placenta, it was cut into pieces, washed with Hank's buffer solution, and incubated for three hours at 37°C with 0.1% collagenase. The digested tissue was roughly filtered, washed, and seeded in full-2D-medium in 80 cm^2 flasks and incubated at 37°C in a tissue culture incubator under humidified conditions supplemented with 5% CO_2. After two to three days, in which the cells were allowed to adhere to the flask surface, they were washed with PBS and full-2D-medium was added. The culture medium was composed of the following ingredients: Dulbecco's Modified Eagle's Medium (DMEM), 1 g/L D-glucose (Sigma), 8% fetal bovine serum (HyClone), 45 μg/mL gentamicin–IKA (Teva Medical), 0.25 μg/mL fungizone (Invitrogen), and 2 mM L-glutamine (Sigma).

The cells were harvested and stored in liquid nitrogen as 2D-cell stock (2DCS), which was considered to be an in-process intermediate product and was tested for sterility, *Mycoplasma sp*. contamination, immunophenotype, and viability. A *Mycoplasma sp*. contamination test was done prior to the first passage using a polymerase chain reaction (PCR) method (EZ-PCR Mycoplasma kit, Biological Industries, Israel). For immunophenotype characterization, the cells were stained with monoclonal antibodies for MSC-positive markers CD73, CD29, and CD105 and for negative markers CD34, CD45, CD19, CD14, and HLA-DR, and analyzed using the FC 500 flow cytometry system (Beckman Coulter) with CXP analysis software. The immune phenotype test specifications were set as ≥90% for all positive markers and ≤3% for all negative markers. The following monoclonal antibodies were used: FITC-conjugated anti-human CD29 (eBioscience); PE-conjugated anti-human CD73 (Bactlab Diagnostic); PE-conjugated anti-human CD105 (eBioscience); Cy7-conjugated anti-human CD45 (IQProduct); PE-conjugated anti-human CD19 (IQProducts); PE-conjugated anti-human CD14 (IQProduct); PE-conjugated anti-human CD34 (IQProduct); and FITC-conjugated anti-human HLA-DR (IQProduct). Cell viability was evaluated by counting the cells with Trypan-Blue (CEDEX).

Upon meeting the 2DCS-release specifications, the appropriate number of cells was thawed, washed, and seeded onto carriers within a bioreactor (automatic CelliGen Plus, New Brunswick Scientific, Edison, NJ) to create a three-dimensional environment. After one to two weeks of growth in the bioreactors, cells were harvested, tested again for phenotypic and karyotypic changes, and cryopreserved in liquid nitrogen as PLX-PAD cells.

Animals, Treatments, and Experimental Procedures

Male and female NOD/SCID mice (strain NOD.SCID/NCrHsd-Prkdc^{scid}) seven to eight weeks of age were obtained from Harlan Laboratories (Rehovot, Israel) and maintained on standard chow (Harlan Teklad diet 2018S, Madison, WI, USA). They were allowed free access to drinking water, supplied to each cage via polyethylene bottles with stainless steel sipper tubes. The water was filtered (0.1-μ filter), chlorinated, and acidified. During the acclimation period and throughout the entire study duration, animals were housed within a limited access rodent facility and kept in groups of maximum five animals cage in polypropylene cages (36.5 × 20.7 × 14.0 cm) that were fitted with solid bottoms and filled with wood shavings as bedding material (7093 Harlan Teklad Shredded Aspen). The mice were allowed a six-day acclimation period to facility conditions (20°C–24°C, 30%–70% relative humidity, and a twelve-hour light/dark cycle) prior to inclusion in the study. Animal care and administration of PLX-PAD cells were conducted at a GLP-certified site (Harlan Biotech Israel Ltd., Rehovot, Israel), and approved by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals of the Hebrew University, Jerusalem, Israel.

Placental-derived MSCs were thawed and prepared immediately before each injection. The cells were thawed by placing the frozen PLX-PAD cell vials in a 37°C water bath and were then transferred into tubes containing the vehicle. From the suspension, 30 μL of the PLX-PAD cell diluent was sampled and well mixed with 30 μL of trypan blue, and 20 μL of the mixture was loaded onto two loading chambers of a hemocytometer for estimation of cell density. While cells were being counted, the tubes were centrifuged at 4°C at 1200 rpm (300 *g*) for ten minutes. The number of viable cells and the number of dead cells were counted in at least two fields (sixteen squares each) of each side of the hemocytometer. The supernatant from the centrifuged cells was discarded, and the pellet of PLX-PAD cells was resuspended in the vehicle to give a final concentration of 1×10^6/50 μL PLA (20×10^6/mL). The cell suspension was then divided and placed in Eppendorf tubes (one tube per mouse). The stock cell suspension was mixed to avoid an uneven cell concentration within the suspension, and each Eppendorf tube contained about 80 μL of cell suspension. The

TABLE 1.—Experimental study design – toxicity and biodistribution phases.

Group no.	No. of animals per group	Treatment			
		Test material	Dose (cells/50 μL)	Frequency and duration	Study period
Toxicity phase					
1	10 (5 males and 5 females)	Vehicle control	0	3 IM injections	8 days
2	10 (5 males and 5 females)	Vehicle control	0	3 IM injections	1 month
3	10 (5 males and 5 females)	Vehicle control	0	3 IM injections	3 months
4	10 (5 males and 5 females)	PLX-PAD	1×10^6	3 IM injections	8 days
5	10 (5 males and 5 females)	PLX-PAD	1×10^6	3 IM injections	1 month
6	10 (5 males and 5 females)	PLX-PAD	1×10^6	3 IM injections	3 months
7	10 (5 males and 5 females)	PLX-PAD	1×10^6	1 IM injection	8 days
8	10 (5 males and 5 females)	PLX-PAD	1×10^6	1 IM injection	1 month
9	10 (5 males and 5 females)	PLX-PAD	1×10^6	1 IM injection	3 months
Biodistribution phase					
10	10 (5 males and 5 females)	Vehicle control	0	1 IM injection	1 day
11	10 (5 males and 5 females)	Vehicle control	0	1 IM injection	8 days
12	6 (3 males and 3 females)	Vehicle control	0	1 IM injection	1 month
13	6 (3 males and 3 females)	Vehicle control	0	1 IM injection	3 months
14	10 (5 males and 5 females)	PLX-PAD	1×10^6	1 IM injection	1 day
15	10 (5 males and 5 females)	PLX-PAD	1×10^6	1 IM injection	8 days
16	6 (3 males and 3 females)	PLX-PAD	1×10^6	1 IM injection	1 month
17	6 (3 males and 3 females)	PLX-PAD	1×10^6	1 IM injection	3 months

Abbreviations: IM, intramuscular; PLX-PAD, placental-derived mesenchymal stromal cells.

cells in the Eppendorf tubes were kept on ice and used within two hours.

Because the IM route of administration corresponds to the anticipated route in projected forthcoming clinical trials, it was selected as the method of dosing, and the PLX-PAD cells and vehicle were administered by IM injection into the right thigh musculature. For technical reasons, male mice were injected on days 0, 3, and 6 and females on days 1, 4, and 7, but the duration of exposure was identical for both sexes.

The Toxic Phase

The toxic phase of the study included five male and five female mice per group. Animals in each group were subjected to sequential study termination at seven days, one month, and three months after the first IM dosing session (Table 1). In the toxicity phase, only a control group for the three-injection procedure was used. There were no controls for the single-injection procedure. In all instances, PLX-PAD cells were administered at a single dose of 1×10^6 cells at a constant dose volume of 50 μL/animal. The total volume was divided into two injection sites in the right thigh musculature. Measurements of food consumption per cage confined to animals of all toxicity-designated groups were initially carried out during the acclimation period (prior to the first dosing session), followed by weekly measurements throughout the entire observation period. The last food consumption measurement was carried out prior to the respective scheduled termination. Surviving animals were euthanized by CO_2 asphyxiation prior to the scheduled necropsy. Animals euthanized for humane reasons were sacrificed by the same method.

The Biodistribution Phase

For the biodistribution phase, five male and five female mice groups were subjected to study termination at one day and seven days postinjection, and three male and three female mice groups were subjected to study termination at one month and three months postinjection (Table 1).

The control groups for both phases included five male and five female mice per group, except for the biodistribution phase, where control groups subjected to study termination at one month and three months postinjection included three male and three female mice at each time point. They were administered the vehicle, composed of PlasmaLyte (Baxter Healthcare Corp, Deerfield, IL), 10% DMSO (Waco Chemicals, Dessau-Thornau, Germany), and 5% albumin (Biotest Pharma, Dreieich, Germany) without PLX-PAD cells, under identical experimental conditions.

Animals were randomly assigned to the various study groups according to a computer-generated randomization output. Body weights were measured at randomization, prior to the first injection, and weekly thereafter. The last body weight determination was carried out prior to scheduled termination. All mice were observed for abnormal clinical signs once daily and for morbidity and mortality twice daily (six days/week). Observations included changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions (e.g., diarrhea), and autonomic activity (e.g., lacrimation, salivation, piloerection, unusual respiratory pattern). Changes in gait, posture, and response to handling, as well as the presence of bizarre behavior, tremors, convulsions, sleep, and coma, were also observed and recorded. Any local reaction at the injection site was recorded as well.

Hematology and Biochemistry

Blood for hematology and biochemistry parameters was collected just prior to euthanasia. Blood samples (at least 100 μL whole blood, collected into EDTA-coated tubes for hematology, and at least 150 μL serum, collected into noncoated tubes for biochemistry) were obtained by retro-orbital sinus bleeding under light CO_2 anesthesia and following food deprivation of at least three hours. The tubes were kept at 2°C–8°C until transported to the analytical laboratory. The samples were assayed for hematology using the Sysmex KX-21 Hematology Analyzer (Kobe, Japan) and for biochemistry using the Roche/Hitachi Modular P800 analyzer (Roche Diagnostics, Almere, The Netherlands).

Necropsy and Tissue Handling

Complete necropsy and macroscopic examinations were performed on all treated and control animals. For the toxicity phase, samples from the following tissues and organs were collected and fixed in 10% neutral buffered formalin: brain; pituitary; optic nerve; spinal cord; heart; aorta; trachea; lungs; kidney; tongue; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; testes; epididymides; seminal vesicles; urinary bladder; prostate; femur; knee joint; injection site (right thigh); skeletal muscle (left thigh); lacrimal gland; salivary gland; pancreas; liver; gall bladder; mandibular, mesenteric, and right inguinal lymph nodes; skin; mammary gland; spleen; sternum with bone marrow; thymus; and adrenals, thyroid, and parathyroids. Eyes and Harderian glands were fixed in Davidson's solution. In addition, any other organ/tissue with gross macroscopic change(s) was collected, recorded, and preserved in 10% neutral buffered formalin.

Tissues were trimmed, dehydrated in graded ethanols, cleared in toluene (xylene), embedded in paraffin wax, and sectioned to approximately 5 μm thickness, and stained with hematoxylin and eosin (H&E). The injection site (right-thigh musculature) was trimmed in the middle and on both sides (right and left) about 2 mm from the middle section. All of the prepared tissue sections were examined microscopically.

For the biodistribution phase, the following tissues/organs were collected using a sterile scalpel for each animal for subsequent PCR assay to determine the presence of any persisting PLX-PAD cells: injection site (muscle), brain, heart, lungs, liver, kidney, spleen, testis/ovary, and femur for bone marrow. Samples were frozen in liquid nitrogen and kept at −70°C to −80°C until transported to the analytical laboratory. Whole blood in EDTA tubes was also collected.

Lesion Grading

Histopathological changes, such as PLX-PAD cell hyperplasia, inflammatory cell infiltration, fibrosis, blood vessel inflammation, and thrombosis, were scored using a semiquantitative grading of five grades (0–4): 0 = *no lesion,* 1 = *minimal change,* 2 = *mild change,* 3 = *moderate change,* 4 = *marked change.*

Development and Quantitation of a qPCR Assay to Detect Human Alu Repeat Sequences

A quantitative polymerase chain reaction (qPCR) assay was used to measure the distribution of PLX-PAD cells in the tissues and blood of the NOD/SCID mice. This qPCR assay was developed and performed by Althea Technologies Inc. (San Diego, CA, USA). In real-time PCR, the intensity of the sequence-specific fluorescence probe signal is proportional to the number of copies of the target sequence in the reaction. A TaqMan-based assay has been developed to detect and quantify human Alu Y DNA sequence in mouse tissue. The assay measures the mass of human genomic DNA (gDNA) by amplifying a 231 base pair sequence of the human Alu Y repeat sequence using the ABI Prism 7900 Sequence Detection System. The assay's amplicon is specific for placental gDNA and does not amplify mouse genomic DNA. The mass of gDNA detected in 1 μg of mouse gDNA extracted from each tissue was quantified using serial dilutions of gDNA as standards. The standard curve was created using placental gDNA in a background of mouse liver gDNA. The limits of detection and quantification were determined as lower limit of detection: 7 pg (1 cell equivalent) of PLX-PAD cells/μg DNA. The lower limit of quantification was 10 pg (1.43 cell equivalents) of PLX-PAD cells/μg DNA.

The mass of human DNA detected can be converted into cell equivalents to display the detection of the number of human placental cell equivalents per number of mouse cell equivalents. This value was obtained by dividing the mass of human DNA detected by 6.67 pg, the approximate mass of DNA in diploid cells.

Ki-67 Immunohistochemistry

The injection site (right thigh musculature) was evaluated for the presence of human proliferating cells by performing Ki-67 immunostaining in an equal number of selected male and female day 8–sacrificed animals. Immunostaining was performed using an automated slide stainer (Ventana NexES). Slides were incubated with monoclonal antibodies against Ki-67 (Neomarkers, Fremont, CA, MB 67, 1:300 dilution, 32 min).

Statistical Analysis

For the toxicity phase, data analysis of all measurable parameters was performed using one-way analysis of variance (ANOVA)-Dunnett multiple comparison test. No statistical evaluation was performed for the biodistribution phase.

RESULTS

Clinical Observation

Toxicity Phase: No mortality in reaction to treatment occurred in any of the animals prior to the scheduled study termination. Four animals developed exophtalmus, piloerection, and dyspnea. In addition, one animal exhibited moderately decreased

TABLE 2.—Weights and selected organ weights of male and female NOD/SCID mice in the toxicity phase following a single injection or 3 injections of PLX-PAD cells or the vehicle control.

	Treatment								
	8-day study			1-month study			3-month study		
Parameter	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection
Males									
Terminal body weight (g)	23.6 (1.67)	24.0 (2.43)	23.3 (1.17)	24.9 (1.88)	24.9 (2.33)	25.3 (0.75)	28.5 (3.11)	29.2 (1.66)	29 (1.81)
Organ weight (g)									
Brain	0.402 (0.011)	0.423 (0.035)	0.405 (0.018)	0.412 (0.022)	0.417 (0.024)	0.404 (0.02)	0.469 (0.032)	0.439 (0.038)	0.440 (0.011)
Liver	1.3 (0.096)	1.22 (0.158)	1.22 (0.14)	1.39 (0.2)	1.27 (0.147)	1.34 (0.046)	1.51 (0.27)	1.57 (0.136)	1.66 (0.164)
Spleen	0.036 (0.016)	0.033 (0.005)	0.034 (0.005)	0.038 (0.021)	0.036 (0.007)	0.034 (0.004)	0.042 (0.013)	0.101 (0.009)	0.095 (0.003)
Organ-to-body-weight ratio (mg organ weight/g body wt)									
Brain	17.34 (1.32)	18.15 (2.63)	17.7 (0.49)	16.88 (0.98)	17.01 (1.69)	16.3 (0.7)	17.27 (0.31)	15.27 (0.64)**	15.56 (0.78)*
Liver	56.02 (2.43)	51.53 (1.98)	53.46 (5.69)	56.51 (4.29)	51.36 (1.32)*	54.12 (1.68)	55.35 (5.1)	54.6 (2.34)	58.34 (2.84)
Spleen	1.55 (0.72)	1.39 (0.18)	1.47 (0.21)	1.53 (0.71)	1.45 (0.26)	1.35 (0.17)	1.54 (0.43)	1.81 (0.42)	2.06 (0.3)
Females									
Terminal body weight (g)	18.4 (1.23)	18.7 (0.76)	18.8 (0.37)	21.1 (0.85)	20.2 (1.58)	20.1 (1.6)	21.2 (1.61)	23.3 (1.11)	23.8 (1.95)
Organ weight (g)									
Brain	0.393 (0.033)	0.411 (0.026)	0.406 (0.015)	0.427 (0.012)	0.425 (0.015)	0.418 (0.025)	0.426 (0.012)	0.452 (0.011)	0.449 (0.015)
Liver	0.96 (0.088)	0.99 (0.076)	0.96 (0.037)	1.13 (0.061)	1.06 (0.143)	0.99 (0.164)	1.03 (0.076)	1.23 (0.182)	1.29 (0.115)
Spleen	0.028 (0.006)	0.038 (0.009)	0.032 (0.005)	0.039 (0.002)	0.042 (0.008)	0.037 (0.008)	0.045 (0.006)	0.064 (0.026)	0.066 (0.011)
Organ-to-body-weight ratio (mg organ weight/g body wt)									
Brain	22.01 (2.77)	22.56 (1.41)	22.15 (0.88)	20.89 (0.75)	20.6 (1.43)	21.53 (1.43)	20.56 (2.38)	19.94 (0.7)	19.31 (1.06)
Liver	53.19 (2.2)	54.12 (2.23)	52.48 (1.99)	55.21 (2.86)	50.87 (2.5)	50.46 (4.66)	49.39 (2.18)	54.31 (8.44)	55.29 (0.49)
Spleen	1.53 (0.22)	2.08 (0.4)*	1.73 (0.26)	1.91 (0.1)	2 (0.28)	1.92 (0.39)	2.15 (0.3)	2.83 (1.26)	2.83 (0.29)

Abbreviation: PLX-PAD, placental-derived mesenchymal stromal cells.

Note: Values are mean (*SD*).

* Indicates statistical significance at $p \leq .05$. ** Indicates statistical significance at $p \leq 0.01$.

TABLE 3.—Weights of male and female NOD/SCID mice in the biodistribution phase following single or 3 injections of PLX-PAD cells or the vehicle control.

	Treatment							
	1-day study		8-day study		1-month study		3-month study	
Parameter	Vehicle single injection	PLX-PAD single injection	Vehicle single injection	PLX-PAD single injection	Vehicle single injection	PLX-PAD single injection	Vehicle single injection	PLX-PAD single injection
Males								
Terminal body weight (g)	22.3 (2.54)	22.0 (1.86)	22.3 (2.41)	22.7 (1.30)	24.1 (2.38)	25.5 (3.51)	26.2 (3.38)	27.9 (3.05)
Females								
Terminal body weight (g)	17.7 (0.55)	18.2 (1.25)	18.4 (0.85)	18.2 (1.33)	19.9 (0.9)	18.7 (0.81)	23.3 (0.85)	22.9

Note: Values are mean (*SD*).

motor activity and slight emaciation. These animals included two female mice from the vehicle-treated group (Table 1, Group 3) that were removed from the study on days 32 and 76 (twelve and eighteen weeks old, respectively). From the PLX-PAD-treated mice, one female treated with three IM injections (Table 1, Group 6), and one male treated with a single IM injection (Table 1, Group 9) were removed on days 71 and 62 (eighteen and sixteen weeks old), respectively. The animals were later diagnosed as suffering from malignant lymphoma, which is a common pathological finding of the NOD/SCID mouse strain, and therefore was considered as an incidental finding.

Biodistribution Phase: No mortality occurred in any of the animals prior to the scheduled study terminations.

Body Weights

There was no effect of PLX-PAD cells on the body weight or body weight gain of the mice (Tables 2 and 3). Statistically significant differences were noted in a small number of time points and were considered incidental findings with no toxicological significance. The statistically significant differences versus controls were as follows: an increase ($p < .05$) in mean body weight gain was noted in the PLX-PAD-treated females subjected to a single injection at the end of the three-month study period. An increase ($p < .05$ or $p < .01$) in mean calculated percentage change in body weight versus the first dosing session of both PLX-PAD cell-treated males was seen during weeks 4, 6, 7, and 8. At week 11, the statistically significant increase ($p < .05$) was confined to the single-injection, PLX-PAD cell-treated group. An increase ($p < .05$ or $p < .01$) in mean calculated percentage change in body weight versus the first dosing session of PLX-PAD cell-treated females subjected to a single injection was noted during weeks 1, 4, 7, 8, 11, and 12.

Organ Weights

There was no effect of PLX-PAD cells on body weight or body weight gain at seven days, one month, or three months following the first dosing session. Statistically significant differences noted in a small number of organs were considered incidental findings (Table 2).

Food Consumption

Food consumption measurements of PLX-PAD-treated groups were similar to those of the vehicle control group throughout the eight-day, one-month, or three-month study duration.

Hematology and Biochemistry

There were no treatment-related effects on any of the hematological or biochemical parameters measured in this study (Tables 4 and 5).

Macroscopic and Histopathological Findings

Injection Site: A hemorrhage-like lesion at the injection site was noted in two males in the eight-day study period subjected to three IM injections of PLX-PAD cells or vehicle, respectively. Histopathologically, treatment-related changes seen in the injected skeletal muscle consisted of increased severity of PLX-PAD cell hyperplasia, seen only in the eight-day study period, following single and three IM injections of PLX-PAD cells (Table 6). The hyperplasia was assessed semiquantitatively, taking into consideration the relative increase in PLX-PAD cells. The cells had round to oval irregular nuclei, prominent nucleoli, and abundant acidophilic cytoplasm. The severity of PLX-PAD cell hyperplasia was generally of grade 2 (mild) degree in the three-injection group, and of grade 1 (minimal) degree in the group injected only once (Figure 1a and 1b and Table 6). Evaluation of the injection site of the animals sacrificed at eight days by Ki-67 immunostaining for the presence of human proliferating cells confirmed the presence of human cells only following three IM injections of PLX-PAD cells. This finding correlates with grade 2 (mild) degree of PLX-PAD cell proliferation seen at histology (data not shown).

Mesenteric Blood Vessels: In several mice, inflammation associated with or not associated with thrombosis was noted in

TABLE 4.—Selected hematology and biochemistry analyses of male NOD/SCID mice in the toxicity phase following single injection or 3 injections of PLX-PAD cells or the vehicle control.

	Treatment								
	8-day study			1-month study			3-month study		
Parameter	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection
No. of animals analyzed	5	5	5	5	4	5	3	4	4
White blood cells ($\times 10^3/\mu L$)	4.2 (2.09)	3.3 (1.29)	2.8 (0.45)	5.3 (2.71)	3.6 (0.45)	4.1 (0.47)	4.8 (1.67)	5.7 (2.93)	6.9 (2.38)
Monocytes (%)	1 (1.6)	0 (0.5)	0 (0)	1 (0.7)	1 (1)	0 (0.9)	0 (0)	1 (0.5)**	0 (0)
Eosinophils (%)	2 (1.8)	1 (1.8)	2 (1.6)	1 (0.8)	1 (1)	0 (0.5)	1 (1.7)	2 (2.2)	1 (1)
LDH (IU/L)	2491 (397)	1679 (392)*	1952 (550)	1945 (282)	1893 (338)	1697 (336)	2363 (318.5)	2341 (863)	3704 (2350)
TRIG (mg/dL)	94 (17.9)	78 (9.9)	80 (20.8)	129 (19.4)	63 (6.1)**	99 (21)**	98 (8.7)	113 (17.4)	114 (23.4)
Cholesterol (mg/dL)	145 (8)	137 (6.8)	137 (7.2)	131 (11.4)	139 (11.2)	122 (6.6)	112 (1.7)	132 (5.5)*	124 (11.9)

Note: Values are mean (*SD*).

Abbreviations: LDH, lactate dehydrogenase; PLX-PAD, placental-derived mesenchymal stromal cells; TRIG, triglycerides.

* Indicates statistical significance at $p \leq .05$.

** Indicates statistical significance at $p \leq .01$

TABLE 5.—Selected hematology and biochemistry analyses of female NOD/SCID mice in the toxicity phase following single or 3 injections of PLX-PAD cells or the vehicle control.

	Treatment								
	8-day study			1-month study			3-month study		
Parameter	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection
No. of animals analyzed	5	5	5	5	5	4	3	4	4
White blood cells ($x10^3/\mu L$)	3.2 (1.54)	2.7 (0.84)	3.2 (0.8)	3.5 (0.84)	3.4 (0.31)	3.5 (1.1)	4.6 (1.29)	4.5 (0.71)	4.4 (1.21)
Monocytes (%)	0 (0)	0 (0.5)	1 (0.9)*	1 (1.3)	1 (0.8)	2 (1.8)	0 (0)	0 (0.5)	1 (1.4)
Eosinophils (%)	0 (0.5)	0 (0.9)	1 (2.2)	2 (0.8)	2 (1.1)	1 (1.9)	2 (0)	0 (0.5)**	0 (0)**
Urea (mg/dl)	49.8 (5.64)	39.5 (4.37)**	45.4 (4.08)	46.8 (3.74)	40.9 (4.32)*	45.2 (1.95)	50.8 (3.22)	48.2 (3.52)	51.3 (3.23)
Na (mEq/L)	151 (1.7)	153 (1.1)	153 (1.5)	154 (1.1)	154 (0.8)	153 (0.8)	154 (1.2)	157 (1.3)*	155 (0.8)
Total protein (g/dl)	6.31 (0.229)	5.47 (0.42)**	6.1 (0.299)	5.73 (0.185)	5.67 (0.149)	5.74 (0.084)	5.9 (0.085)	5.59 (0.227)	5.64 (0.357)
Albumin (g/dl)	4.58 (0.259)	4.2 (0.166)*	4.39 (0.194)	4.08 (0.098)	3.99 (0.072)	4.07 (0.054)	4.13 (0.076)	3.74 (0.162)*	3.83 (0.252)
AST (IU/L)	525 (292.5)	223 (93.8)*	247 (108.7)	121 (22.5)	185 (43.8)	179 (61.6)	308 (52)	456 (275.6)	215 (74.5)
ALP (IU/L)	214 (12.6)	205 (11.5)	200 (15.2)	180 (13.7)	160 (5)*	176 (16.2)	156 (18.6)	133 (35.9)	132 (28.4)
TRIG (mg/dl)	107 (12.1)	74 (13.8)**	84 (15.7)*	131 (16.3)	84 (16.2)*	110 (35.3)	115 (33.5)	76 (9.7)	152 (94.5)
Phosphorus (mg/dl)	9.4 (0.86)	10.8 (0.95)	11.1 (1.79)	8.8 (0.99)	9.7 (1.09)	10 (0.78)	7.7 (0.31)	9.9 (0.7)*	9.7 (1.25)*
LDH (IU/L)	2641 (965.2)	1554 (309)*	2190 (241.4)	1396 (325.5)	1588 (183.2)	1450 (230.2)	2313 (147.4)	2707 (587.9)	2269 (121.2)

Note: Values are mean (*SD*).

Abbreviations: ALP, alkaline phosphatase AST, aspartate transaminase; LDH, lactate dehydrogenase; PLX-PAD, placental-derived mesenchymal stromal cells; TRIG, triglycerides.

* Indicates statistical significance at $p \leq .05$.

** Indicates statistical significance at $p \leq .01$

TABLE 6.—Histopathological findings in the male and female NOD/SCID mice in the toxicity phase.

	Histopathological FindingsMean severity (Number Affected / Total Number of Animals)								
	8-day study			1-month study			3-month study		
Organ/tissue	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection	Vehicle 3 injections	PLX-PAD single injection	PLX-PAD 3 injection
Males									
Injection site—right thigh musculature									
Inflammation subchronic, multifocal	1.0 (5/5)	1.0 (5/5)	1.0 (5/5)	(0/5)	(0/5)	(0/5)	0.4 (1/5)	(0/5)	0.2 (1/5)
Stromal cell hyperplasia, multifocal	(0/5)	1.0 (5/5)	2.0 (5/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)
Fibrosis—interstitial, multifocal	(0/5)	(0/5)	(0/5)	0.2 (1/5)	0.4 (2/5)	1.0 (5/5)	0.2 (1/5)	(0/5)	0.4 (2/5)
Mesenteric lymph nodes									
Mesenteric blood vessels—periarterial inflammation subchronic, multifocal	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	0.8 (3/5)	1.8 (4/5)	1.4 (4/5)
Mesenteric blood vessels—thrombus, multifocal	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	0.2 (1/5)
Stromal cell hyperplasia, multifocal	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	0.2 (1/5)	(0/5)	0.2 (1/5)
Multiple or single organ									
Lymphoma, malignant	(0/5)	(0/5)	(0/5)	(0/5)	1/5	1/5	2/5	1/5	2/5
Females									
Injection site—right thigh musculature									
Inflammation subchronic, multifocal	1.0 (5/5)	1.0 (5/5)	1.0 (5/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)
Stromal cell hyperplasia, multifocal	(0/5)	1.0 (5/5)	2.0 (5/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)
Fibrosis—interstitial, multifocal	(0/5)	(0/5)	(0/5)	0.2 (1/5)	0.2 (1/5)	0.4 (2/5)	(0/5)	0.2 (1/5)	0.4 (2/5)
Mesenteric lymph nodes									
Mesenteric blood vessels—inflammation subchronic, multifocal	(0/5)	0.4 (1/5)	(0/5)	(0/5)	0.2 (1/5)	0.6 (3/5)	1.2 (3/5)	1.0 (3/5)	1.0 (4/5)
Mesenteric blood vessels—thrombus, multifocal	(0/5)	0.4 (1/5)	(0/5)	(0/5)	0.2 (1/5)	0.6 (3/5)	0.6 (2/5)	0.2 (1/5)	0.2 (1/5)
Stromal cell hyperplasia, multifocal	(0/5)	(0/5)	(0/5)	(0/5)	0.2 (1/5)	0.6 (3/5)	0.6 (2/5)	0.4 (1/5)	0.2 (1/5)
Multiple or single organ									
Lymphoma, malignant	(0/5)	(0/5)	(0/5)	1/5	0/5	1/5	2/5	1/5	2/5

blood vessels located in the mesenterium–close to the mesenteric lymph nodes (Figure 1c and 1f and Table 6). In the male group, these lesions were seen only in the three-month period in the control and treated groups (11/15). In the female group, one case was already noted in the eight-day period group in the PLX-PAD cell-treated group; another four cases in the one-month period group and ten cases in the three-month period group were observed in both the treatment and the control groups. The inflammation mainly involved the medium- and large-diameter arteries and was characterized by infiltration of the media and adventitia by predominantly mixed mononuclear cells, with rarely seen polymorphonuclear cells. Occasional focal smooth muscle cell proliferation occurred within the media. No fibrinoid necrosis was detected in the affected vessels. The morphology of the lesions was comparable in both the control and PLX-PAD cell-treated groups. In both groups, the inflammation seen in the three-month study period was more florid. No vascular inflammation was seen in any of the other organs examined.

Malignant Lymphoma: Several animals from all groups manifested macroscopic lesions of enlarged thymus, spleen, mesenteric, mandibular and inguinal lymph nodes, and irregular kidneys, which were all confirmed to be consistent with malignant lymphoma (Table 6).

PLX-PAD Cells Biodistribution

Distribution of PLX-PAD cells, determined by RT-qPCR evaluation, was confined to the muscle at the injection site. No human DNA was detected in any of the tissues evaluated, including the blood and femur bone marrow. The concentration of DNA was highest after one day of PLX-PAD cell injection and was followed by a gradual decrease in concentration in male and female groups (Figure 2).

DISCUSSION

Although several hypotheses have been suggested, the exact mechanism by which MSCs exert their beneficial properties on ischemic tissues remains vague. Until recently, the prevalent belief was that MSCs become incorporated into capillaries, differentiating into endothelial cells and thus helping in the regeneration of the blood supply to the injured tissue (Wang et al. 2001). Many studies have shown that improved perfusion to

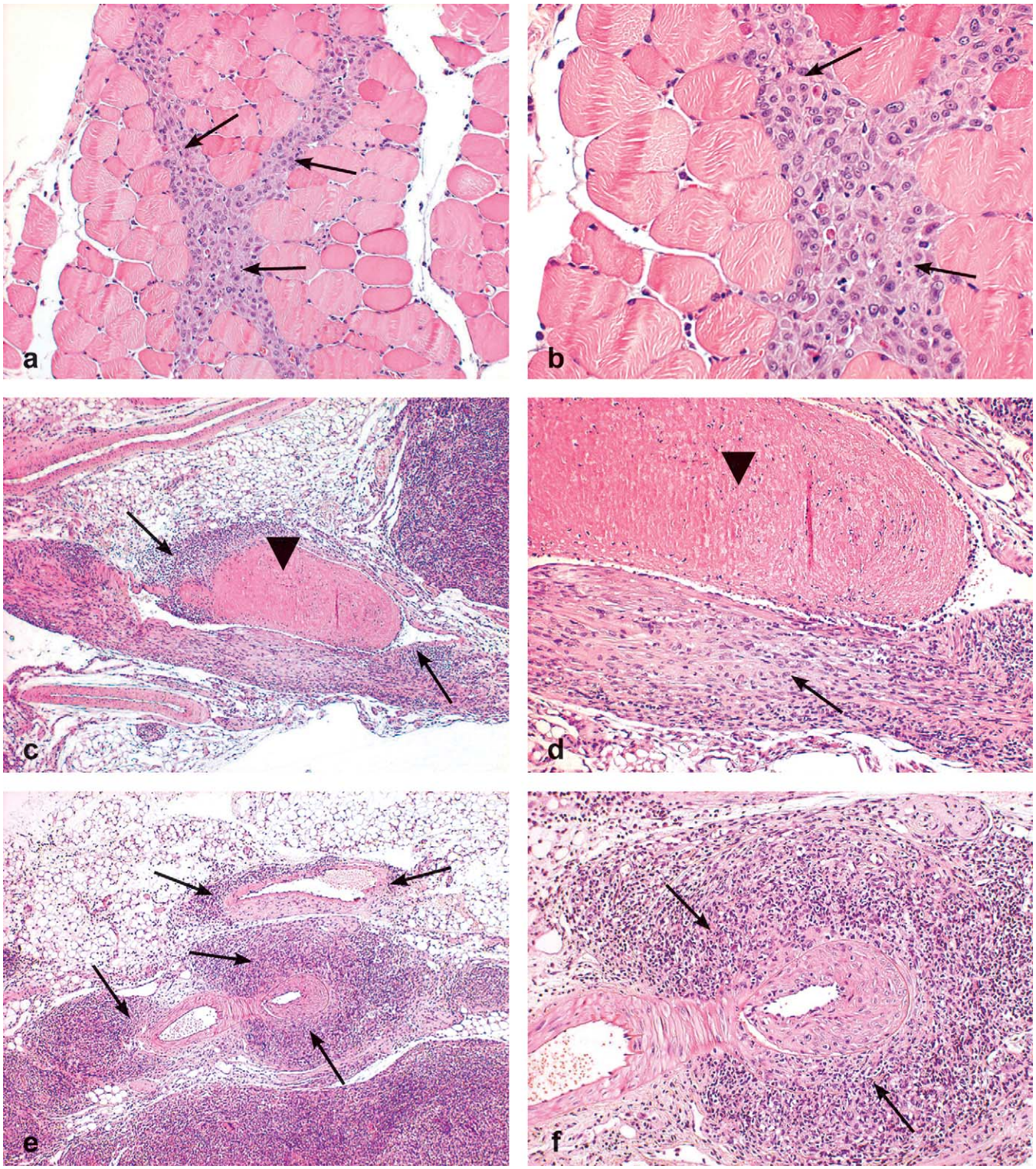

FIGURE 1.—(a, b) Grade 2 (mild) PLX-PAD cell hyperplasia (arrows). The PLX-PAD cells have round to oval irregular nuclei, prominent nucleoli, and abundant acidophilic cytoplasm; injection site of male mouse sacrificed eight days following IM injection. H&E, ×100 (a); ×200 (b). (c, d) Mesenteric blood vessel thrombosis (arrowheads) associated with stromal cell hyperplasia and inflammation (arrows) in female mouse sacrificed one month following IM injection. H&E, ×40 (c); ×100 (d). (e, f) Mesenteric blood vessel periarteritis (arrows) associated with stromal (smooth muscle) cell hyperplasia in male mouse sacrificed three months following IM injection. H&E, ×40 (e); ×100 (f).

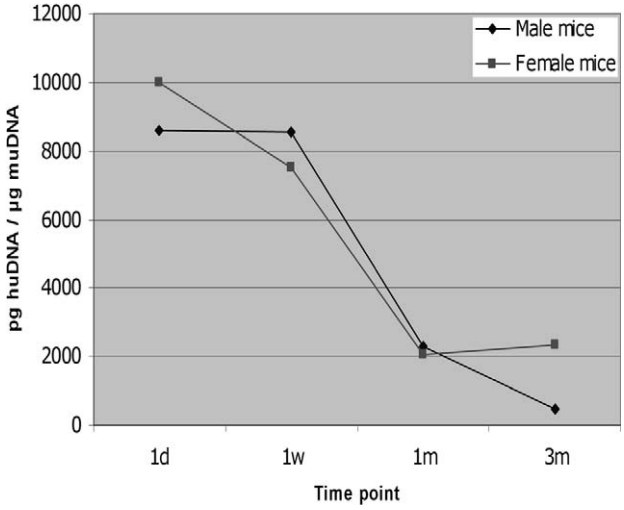

FIGURE 2.—Ratio between human DNA transcripts and mouse DNA transcripts in injection site muscle, determined by RT-qPCR. Each time point is the mean ratio of five male or female mice (one day and one week) or three male and female mice (one and three months). Ratios higher than 10,000 were regarded as 10,000 for mean calculation.

ischemic tissue was achieved with little incorporation of MSCs into the blood vessels (Iba et al. 2002; Tomita et al. 1999; Wang et al. 2001); however, that belief has been repudiated. One study showed that MSC injection in the mouse hind limb ischemia model caused significant improvement, without incorporation of MSCs into the regenerating blood vessels (Kinnaird, Stabile, Burnett, Shou et al. 2004). Another study using MSCs to treat chronic wounds in the mouse excisional wound splinting model demonstrated enhanced wound healing with the cells found in close proximity to the vascular structures, but not in the structures themselves (Wu et al. 2007). Other studies have suggested that MSCs act by fusion with tissue-specific cells (Reinecke et al. 2004; Ying et al. 2002); however, no data support the fact that this fusion phenomenon contributes functionally to collateral vascular development (Kinnaird, Stabile, Burnett, and Epstein 2004). Researchers have suggested, therefore, that MSCs promote collateral vascular remodelling by other mechanisms.

One such hypothesis emphasizes the supportive roles of MSCs in improving the response of blood vessels to ischemia. These cells may contribute to the local microenvironment by secreting angiogenic growth factors such as VEGF, MCP-1, Ang-1, and others (Fuchs et al. 2001; Kamihata et al. 2001). These cells also secrete a wide array of supportive cytokines. The medium collected from MSC cultures promoted in vitro proliferation and migration of endothelial cells in a dose-dependent manner, and its injection led to clinical improvement in a hind limb ischemia model (Kinnaird, Stabile, Burnett, Shou et al. 2004). In addition, MSC-conditioned medium was found to promote endothelial cell formation (Wu et al. 2007). Taken together, PLX-PAD cells appear to exert their effects through a diverse array of biological mechanisms. Placental-derived MSCs may exert many, if not all, of their effects via paracrine mechanisms, by supplying the necessary environment for a host tissue to repair itself without excessive scar tissue formation. Although very promising, the ability of PLX-PAD culture medium alone to exert beneficial effects has not been extensively studied. Considering the fact that the effects of PLX-PAD cells may be seen only when injected into the ischemic milieu and in view of their potentially complex and different mechanisms of action, it is not known to what extent the cell culture alone is able to replicate the beneficial effects seen with the injection of the PLX-PAD cells themselves.

The biodistribution phase of the current study shows that all PLX-PAD cells were confined to the injection site muscle. No PLX-PAD cells were found in the blood or in the femur bone marrow. The concentration of cells in the muscle decreased gradually with time. These findings are in agreement with the histopathological data, demonstrating PLX-PAD cell hyperplasia in the injection site only in animals of the eight-day period. No PLX-PAD cell hyperplasia was found in later time points. Immunostaining of the injection site of the eight-day study period animals for Ki-67 was positive only after three IM injections of PLX-PAD cells. The lack of positivity in the single IM injections of PLX-PAD cells may be explained by the low grade (minimal) of proliferation of the cells seen at histology at this time point. Our data support the rising theory that MSCs exercise their important roles not by incorporation into or fusion to regenerating vessels, but probably by secretion of beneficial factors acting locally and later degrading.

The reduced incidence and severity of PLX-PAD cell hyperplasia at the injection site, compared to the eight-day post-injection sacrifice, suggest that the injected cells do not have the ability for progressive, long-term autonomous proliferation. This suggestion is supported by the gradual decrease of human DNA found in the injection site, thus emphasizing that these cells are not capable of uncontrolled growth in the mouse strain.

Spontaneous thymic lymphoma that progresses to systemic distribution is a common pathological finding of the NOD/SCID mouse strain, although the mechanism of lymphomagenesis remains unclear (Chiu et al. 2002). The incidence of thymic lymphoma varies from 67% to 76% in mice forty weeks of age (Chiu et al. 2002; Prochazka et al. 1992; Serreze et al. 1995), with clear dominance in females. In our study, the first lymphoma case appeared at the age of twelve weeks, and the incidence rose to 33% at twenty weeks. Interestingly, no clear increased female susceptibility to lymphoma in our study suggested that the reported female predominance of this pathology may appear only at later stages as the mice age.

In summary, in view of our reported findings and under the conditions of this study, we conclude that the administration of PLX-PAD cells following single or three IM injections at intervals of three days to male and female NOD/SCID mice, and at one dose of 1×10^6 cells/50 µL, was not associated with any

adverse effects. Only relatively acute toxicity was evaluated in the present study because of the three-month limit of this study. Further studies are needed to evaluate reproductive toxicity and toxicity in older animals, since the latter would more accurately model human patients receiving PLX-PAD cells. Although the mechanism of the action of PLX-PAD cells is still unknown, biodistribution data from this study show that the cells were confined to the injection site, with no proliferation or spread to, or incorporation into, other tissues, suggesting a supportive, paracrine mechanism of action.

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