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Human PLacental eXpanded (PLX) mesenchymal-like adherent stromal cells confer neuroprotection to nerve growth factor (NGF)-differentiated PC12 cells exposed to ischemia by secretion of IL-6 and VEGF

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ABSTRACT

Mesenchymal stem cells are potent candidates in stroke therapy due to their ability to secrete protective anti-inflammatory cytokines and growth factors. We investigated the neuroprotective effects of human placental mesenchymal-like adherent stromal cells (PLX) using an established ischemic model of nerve growth factor (NGF)-differentiated pheochromocytoma PC12 cells exposed to oxygen and glucose deprivation (OGD) followed by reperfusion. Under optimal conditions, 2×10^5 PLX cells, added in a trans-well system, conferred 30–60% neuroprotection to PC12 cells subjected to ischemic insult. PC12 cell death, measured by LDH release, was reduced by PLX cells or by conditioned medium derived from PLX cells exposed to ischemia, suggesting the active release of factorial components. Since neuroprotection is a prominent function of the cytokine IL-6 and the angiogenic factor $VEGF_{165}$, we measured their secretion using selective ELISA of the cells under ischemic or normoxic conditions. IL-6 and $VEGF_{165}$ secretion by co-culture of PC12 and PLX cells was significantly higher under ischemic compared to normoxic conditions. Exogenous supplementation of 10 ng/ml each of IL-6 and $VEGF_{165}$ to insulted PC12 cells conferred neuroprotection, reminiscent of the neuroprotective effect of PLX cells or their conditioned medium. Growth factors as well as co-culture conditioned medium effects were reduced by 70% and 20% upon pretreatment with 240 ng/ml Semaxanib (anti $VEGF_{165}$) and/or 400 ng/ml neutralizing anti IL-6 antibody, respectively. Therefore, PLX-induced neuroprotection in ischemic PC12 cells may be partially explained by IL-6 and $VEGF_{165}$ secretion. These findings may also account for the therapeutic effects seen in clinical trials after treatment with these cells.

1. Introduction

Amid various cell therapy strategies for treating neurodegenerative diseases including brain trauma and ischemic disorders, mesenchymal stem cells (MSC) transplantation is considered a promising therapeutic modality [1]. The human placental expanded (PLX) mesenchymal-like adherent stromal cells, characterized by highly selective expression of typical markers such as CD 73 (ecto-5′-nucleotidase), CD 90 (Thy-1 cell surface antigen) and CD 29 (Integrin β-1) [2], have been tested for their therapeutic effect in experimental models to treat stroke outcome in rats [2] and limb ischemia in mice [3]. PLX cells at low and high doses are currently evaluated for therapy in several ischemic disorders and are used in human clinical trials for treating peripheral artery disease, accelerate regeneration of injured gluteal musculature after total hip arthroplasty and improved intermittent claudication (http://www.clinicaltrials.gov) due to their pro-angiogenic, immnomodulatory and reparative beneficial therapeutic effects [2,4]. Compelling evidence exist that MSC confer beneficial therapeutic effects after transplantation alone or together with hematopoietic stem cells [4] through the secretion of immune modulatory and neurotropic factors released in a paracrine fashion. The paracrine theory is promoting novel pharmacological outlooks by which neuroprotection of the injured neuronal tissue may be achieved by the secretome of the transplanted MSC, calling for the identification of particular cytokines, chemokines and growth factors secreted by MSC and their involvement in neurotherapeutic effects [5].

Abbreviations: 2D, two dimensional; 3D, three dimensional; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FU, fluorescence unit; H_2O_2, hydrogen peroxide; HS, horse serum; IL-6, interleukin 6; LDH, lactate dehydrogenase; Mab, monoclonal antibody; MSC, mesenchymal stem cells; NGF, nerve growth factor; NP-Index, neuroprotective index; OGD, oxygen and glucose deprivation; PC12, pheochromocytoma cells; PE, phycoerythrin type R; PLX, Human PLacental eXpanded mesenchymal-like adherent stromal cells; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; VEGF, vascular endothelial growth factor

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In the present study we have adapted PLX cells to grow in monolayer culture in a trans-well co-culture system with a rat adrenergic neural tumor pheochromocytoma cell line PC12 that has been extensively used as a model for dopaminergic neurons and neuronal differentiation in general. NGF-differentiated PC12 cells have been subjected to a brief (4 h) oxygen and glucose deprivation phase (OGD) followed by a reperfusion phase (18 h) to mimic clinical situations of ischemia in order to study the possible beneficial effects of PLX cells on this insult. The feasibility of the PC12 cell model system was validated recently by our laboratory in diverse insults [6] and found suitable to investigate the neuroprotective effects of different drugs such as the calcium channel blockers nifedipine and nimodipine [7], the brain endogenous neuroprotective histidine dipeptides carnosine and homocarnosine [8], the superoxide dismutase mimetic and antioxidant neuroprotective compound, Tempol [9] and the monoamine oxidase B inhibitor with neuroprotective effects, used in Parkinson therapy, Rasagiline [10].

Using the present pharmacological model, we now demonstrate a robust neuroprotective effect of PLX cells on ischemic PC12 cells and identify stimulus-secreted specific cytokines by the former under ischemic conditions. These findings should pave the way for alternative therapeutic options for ischemic disorders using human IL-6 and $VEGF_{165}$ recombinant proteins.

2. Materials and methods

2.1. Chemicals, growth factors and antibodies

4-Hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (Tempol), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), 1, 1, 3, 3-tetraethoxypropane (TEP) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO). NGF was purchased from Alomone Labs (Jerusalem, Israel). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), horse serum (HS), penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). Collagen type I from rat tail was purchased from BD Biosciences (Bedford, MA, USA). Alamar blue was purchased from Invitrogen Corporation (Grand Island, NY, USA). Recombinant human IL-6, recombinant human $VEGF_{165}$ and rabbit anti-human IL-6 were purchased from PeproTech (Rehovot, Israel) while Semaxanib was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Quantikine human IL-6 ELISA kit and quantikine human/mouse VEGF ELISA kit were purchased from R&D Systems (Minneapolis, MN, USA). Specific antibodies towards CD antigens labeled with either FITC or PE: CD29, CD54, CD90 and CD166, CD44, CD51, CD106 and CD73 were purchased from Immunotech (Vaudreuil-Dorion, Canada) and Southern Biotech (Alabama USA), respectively.

2.2. Cell cultures

2.2.1. PC12 pheochromocytoma cultures

PC12 cells were propagated in 25 cm^2 flasks in growth medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal calf serum (FCS), 7% horse serum (HS), 10,000 U/ml penicillin and 100 μg/ml streptomycin, as previously described [11]. The medium was replaced every second day and cells were grown at 37 °C, in a humidified atmosphere of 6% CO_2.

2.2.2. PLX cell cultures

PLX cells were manufactured by Pluristem Therapeutics, Ltd. (Haifa, Israel) [3,4]. Briefly, full term human placentas were collected from healthy donor mothers following elective cesarian section and after informed consent in the frame of the Helsinki program. Placental stromal cells isolated from placenta tissue were propagated as two dimensional (2D) cultures followed by 3D culture on fibrous carriers in a bioreactor [4]. The 3D cultured cells were harvested and cryopreserved in liquid nitrogen. The characterization of PLX cells was performed by positive labeling with the following conjugated anti-human antibodies: CD29-FITC, and CD73 (SH3)-FITC and CD105. One day before the experiment, PLX cells from three different donor-derived batches were thawed and 2×10^5 cells were plated on membrane of Falcon culture inserts (polyethylene terephthalate track-etched membranes, 1 μm pore size, diameter 12 mm) from Becton Dickinson Co. (Franklin Lakes, NJ, USA) and grown in DMEM supplemented with 7% FBS, 7% HS, 10,000 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C, in a humidified atmosphere of 6% CO_2. For experiments, the cells were harvested and adjusted to constant densities using hemocytometry before plating.

2.3. Ischemic insult protocol using NGF-differentiated PC12 cells

Actively proliferating PC12 cells (2×10^5 cells/well) were seeded onto 12-well plates pre-coated with 200 μg/ml collagen type-I and differentiated with 50 ng/ml Nerve Growth Factor (NGF) for 7 days. Fresh medium-containing NGF was added every second day. In all experiments, only NGF-differentiated PC12 cells were used. On the day of the experiment, cell medium was replaced to glucose-free DMEM (hypoglycemic insult) and cultures introduced into an ischemic chamber with oxygen level below 1% (anoxic insult) for 4 h at 37 °C under oxygen and glucose deprivation (OGD) as previously described [6]. In order, to mimic in vivo reperfusion conditions due to renewal of blood supply, at the end of the OGD insult, 4.5 mg/ml glucose was added and cultures were incubated for 18 h under normoxic conditions (reperfusion/reoxygenation) to complete the ischemic insult. Operationally, ischemic insult represents therefore a combination of both OGD and reperfusion phases. Control cultures were maintained under regular atmospheric conditions (normoxia) in the presence of 6% CO_2. The antioxidant Tempol was added to appropriate wells, before the OGD insult, unless otherwise stated [8]. Addition of PLX cells (2×10^5 cells/well) to the double chamber co-culture system [6] was routinely performed prior to OGD or whenever stated after OGD, before reperfusion. At the end of the reperfusion cell death was measured as detailed below. All experiments ($n = 15$) were carried out under good laboratory practice conditions using a clean room, regulated according to ISO7 requirements (10,000 particles/m^3).

2.4. Conditioned medium preparation

Cultures of PC12 cells (2×10^5/well) or PLX cells (2×10^5/well) and co-cultures of both cell lines were exposed to OGD for 4 h followed by 18 h reperfusion. At the end of the ischemic insult the medium was collected, filtered (0.2 μm Whatman GmbH, Dassel, Germany) and frozen at −20 °C. The filtered medium denoted as "conditioned medium" was frozen and aliquots were used for designated experiments.

2.5. Determination of cell death by Lactate dehydrogenase (LDH) release

Cell death was evaluated by measuring the leakage of LDH into the medium as previously described [8]. LDH activity was determined at 340 nm using a spectrofluorimeter (TECAN, SPECTRA Fluor PLUS, Salzburg, Austria). Basal LDH release was measured in both PC12 and PLX cultures maintained under normoxic conditions. Under OGD insult, LDH release representing cell death was expressed as percent of total LDH released into the medium upon subtracting the basal values of LDH release. Total LDH (extracellular + intracellular) was obtained by freezing and thawing the cultures. The neuroprotective effect, defined as the percent decrease in LDH release in the presence of PLX cells or Tempol was normalized to untreated ischemic cultures and is depicted below:

$$\text{Cell death } (\%) = (LDH_{(\text{ischemia - basal})}/(LDH_{\text{total}}) \times 100.$$

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Additionally, a neuroprotection index defined as the fractional ratio of cell death in treated versus control PC12 cells was calculated as noted below.

NP-Index = 1 −(% cell death in treated well / % cell death in control well).

The rescue capacity on a scale of 0–1 is maximal at 1.

2.6. Lipid peroxidation by measurement of TBARS

Malondialdehyde-like metabolites released into the culture medium were collected and reacted with thiobarbituric acid (TBA) reagent as previously described [12]. Briefly, the TBA reagent was prepared by dissolving 0.67% TBA (wt./vol.) and 0.01% (wt./vol.) butylated hydroxytoluene (BHT) in 50% acetic acid. Aliquots of 0.5 ml culture medium were collected after ischemic insult or normoxic conditions and TBA-reagent (0.5 ml) was added. The solution was heated in boiling water for 15 min. The samples were cooled with tap water and the developed color read at 535 nm excitation and 553 nm emission using spectrofluorimetry. For quantification of TBARS, a standard curve was prepared by using a 100 μM 1,1,3,3-tetraethoxypropane (TEP) stock solution diluted in PBS [12].

2.7. PLX cells metabolic status using the Alamar blue assay

Viability of PLX cell growth in PC12 medium was assessed using the Alamar Blue assay [13]. Fluorescence in arbitrary units was measured using spectrofluorimetry at an excitation wavelength of 540 nm and emission wavelength of 595 nm.

2.8. Measurements of IL-6 and VEGF levels using human and mouse selective ELISA

Aliquots of media from controls (normoxia) or from ischemic PC12 cells or PLX cells as well as co-cultures of both were collected and submitted to ELISAs selective for human recombinant IL-6 and $VEGF_{165}$ and for mouse $VEGF_{164}$, as per the manufacturer's instructions. IL-6 levels were estimated using the quantikine human IL-6 ELISA kit according to the manufacturer's instructions. The amount of IL-6 in pg/ml was calculated from a linear standard curve, with a sensitivity of 3.12 to 300 pg/ml. The anti-IL-6 polyclonal antibody used in the assay cross-reacted with rat and human IL-6, but shows <0.5% cross-reactivity with other cytokines and <50% cross-species reactivity was observed. The samples were also analyzed for human and mouse VEGF using quantikine human/mouse VEGF ELISA kit according to the manufacturer's instructions. Using a human VEGF ELISA kit, the amount of VEGF-A_{165} in pg/ml was calculated from a human $VEGF_{165}$ standard curve, with a sensitivity of 15.6 to 1000 pg/ml. The antibody exhibited a sensitivity of detection up to >4000 pg/ml and 0.5% cross reactivity with murine $VEGF_{165}$. Using a mouse $VEGF_{164}$ ELISA kit, the amount of $VEGF_{164}$ in pg/ml was calculated from a mouse $VEGF_{164}$ standard curve, with a sensitivity of 7.8 to 500 pg/ml. The antibody exhibited a sensitivity of detection up to >2500 pg/ml and 0.2% and 25% cross reactivity with recombinant human $VEGF_{165}$ and rat $VEGF_{164}$, respectively. Preparation of plates and solutions and ELISA protocols was performed according to the manufacturer's instructions.

2.9. Statistical analysis

Each experiment was performed 3–5 times in sixplicate (n = 6) wells. The results are presented as mean ± S.E. Statistical comparisons between experimental groups were determined by using analysis of variance program (ANOVA) followed by Dunnett's multiple comparison test. P value of 0.05 or less was considered significant for all comparisons.

3. Results

3.1. The impact of PLX cells on PC12 cell death following ischemic insult

In order to optimize the response of the NGF-differentiated PC12 cells to the ischemic insult, we first assessed to some detail the effect of cell density and duration of the OGD exposure on cell death. As shown in Fig. 1, cell death as assessed by LDH release showed a strong cell-density and time-of OGD insult dependency which attained a maximum at approximately 5 h at an average density of approximately 4×10^5 cells/well. An EC_{50} value for cell death in the range of 1.6–3.3×10^5 cells/well could be extrapolated and an optimal density of 2×10^5 PC12 cells and was used throughout this study.

We then examined the effect of PLX cells addition on PC12 cell death. As shown in Fig. 2 (panel A), the presence of PLX cells in the co-culture system (see drawing) during the OGD phase or during the reperfusion phase (added after OGD), caused a substantial decrease in cell death by 46% and 38%, respectively. Addition of the potent antioxidant Tempol at millimolar concentrations, prior to OGD and throughout reperfusion, significantly reduced cell death by 31%. Tempol was however less effective when added after the OGD insult, during the reperfusion phase only. Based on an average cell death value of 39 ± 2% we could extrapolate a relative neuroprotective-index (NP-Index) for comparison between the two agents. Thus as shown in Fig. 2 (panel B), the NP-Index values of PLX cells and Tempol were 0.45 and 0.31 respectively, when the agents were present during the entire ischemic insult. When PLX cells were added during the reperfusion phase only, the NP-Index remained practically the same suggesting an equally effective rescue capacity while Tempol failed to provide rescue under these conditions. These data most likely indicate a different mechanism of protective action of these two seemingly different experimental modalities.

The use of PLX cells in this particular co-culture system deemed an adaptation of cell growth in monolayers as they are usually propagated in 3D bioreactors on fibrous matrices (3). Therefore we set to examine their rates of proliferation in the presence of PC12 cell medium components. As assessed by the Alamar blue vital stain, at 3 days in vitro, PLX cell doubling time was highly dependent on the initial amount seeded and decreased relatively with increasing plating density most likely due to plating efficiency (Table 1). At 6 days, cells still continued to proliferate. These results demonstrate that PLX cells survived and even proliferated in these monolayer conditions. Furthermore, the PLX cells appeared to be resistant towards the OGD insult applied in this study (data not shown), similar to the resistance previously reported for

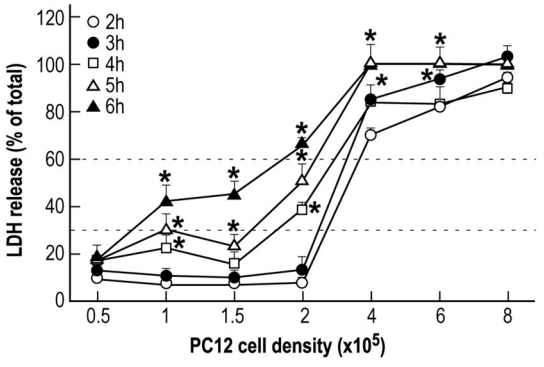

Fig. 1. Cell-density and duration of OGD dependency of LDH release by PC12 cells. PC12 cells were seeded at designated densities in 12-well plates and differentiated for 7 days in the presence of NGF. Cells were exposed to OGD insult for various time periods up to 6 h as detailed under Materials and methods. Aliquots from the culture media were taken for LDH release measurements. Values expressed as percent LDH released in the medium out of total culture LDH is mean ± SE (n = 18).*p < 0.05 vs 2 h OGD at each time point at the respective density. An operational defined range of cell death and duration of OGD is underlined.

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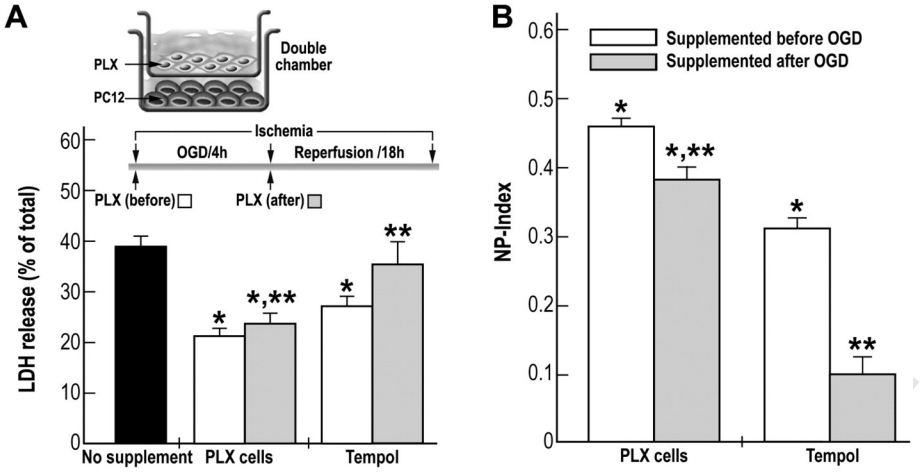

Fig. 2. Effect of PLX cells and Tempol on ischemia-induced PC12 cell death. PC12 cells (2×10^5 cells/well) were seeded in 12-well plates and differentiated for 7 days in the presence of NGF. Designated wells were supplemented with a platform of PLX cells (2×10^5 cell/insert, see scheme panel A) or 3 mM Tempol before (white bars) or after (gray bars) the 4-h OGD insult. Cultures were subjected to18 h reperfusion and medium aliquots taken for LDH quantification. Cell death expressed as % LDH release out of total LDH (panel A) are mean ± SE (n = 30); *p < 0.01 vs no supplement; **p < 0.01 vs PLX before OGD. Panel B expressed as NP-Index from zero tone was extrapolated from panel A as detailed in Materials and methods.

different mesenchymal stem cells exposed to hypoxia and serum deprivation [14].

The neuroprotective effect of PLX cells on NGF-differentiated PC12 cultures was further evaluated by the appearance of lipid peroxidation products measured as TBARS, a marker known to increase in PC12 cells after oxidative stress [12,15,16]. As expected, under normoxic conditions, treatment of PC12 cells for 2 h with 0.5 mM H_2O_2, which was used as positive control for lipid peroxidation, increased by 2.4 fold TBARS formation compared to that of untreated controls (Fig. 3). When subjected to ischemic insult, generation of TBARS in the cell medium increased 1.6 folds. In the presence of PLX cells TBARS were reduced (25%) to 0.06 μM/mg protein from a level of 0.081 μM/mg protein. Tempol on the other hand, did not reduce TBARS levels furthering the likelihood of a different mechanism of action. PLX cells before or after ischemia released practically the same amounts of TBARS in the medium (0.055 μM/mg protein — data not shown). Notably, in the presence of PLX cells, TBARS levels were significantly reduced (25%) in the medium. In contrast, Tempol addition did not reduce the amount of TBARS generated (2%),

Stem cell intervention for treating the consequences of ischemia is usually confined to the stage that follows the post traumatic event. Therefore, it was interesting to evaluate the time window for neuroprotection upon addition of PLX cells during the reperfusion period (Fig. 4 panels A, B) as well as establish the duration of the neuroprotective effect during the reperfusion period (Fig. 4 panel C). Our data demonstrate that addition of PLX cells immediately after OGD insult at the beginning of the reperfusion period (Fig. 4 panel A), was the most appropriate time to acquire neuroprotection. This is in contrast to Tempol which basically was ineffective when added after the OGD insult (Fig. 4 panel B). However, when Tempol was added at the beginning of the OGD insult, it provided neuroprotection even after 3 days following reperfusion, to the same degree as PLX cells did when added at the beginning of the reperfusion phase (Fig. 4 panel C). These results unambiguously indicate that the significant neuroprotective effect of PLX cells is sustained during the reperfusion phase for at least 3 days. Fig. 4 (panel D) illustrates a typical density-dependent neuroprotection effect of PLX cells with an EC_{50} corresponding to a cell dose of 6×10^4 PLX cells.

3.2. The neuroprotective effect of PLX cells is mediated by cell-released factors including IL-6 and VEGF

The effect observed in the presence of PLX cells raised the possibility that compounds actively or passively released into the medium after ischemia are responsible for PC12 cells neuroprotection. Therefore, in pilot studies variations of conditioned media were collected from cultures exposed to ischemia, and aliquots tested in fresh PC12 cells subjected to ischemia. As shown in Fig. 5 (panel A) a significant decrease in cell death of 25 ± 6%, 25 ± 4% and 52 ± 3% was found upon treatment with either conditioned medium derived from PC12 cells or from PLX cells alone or from co-cultures of both, respectively. For comparison, Tempol reduced PC12 cell death by 31 ± 3%. TBARS levels were also reduced by 32% using the ischemic conditioned medium generated from PC12/PLX co-cultures (Fig. 5 panel B). These results indicate that

Table 1
Density-dependent PLX cells proliferation in PC12 growth medium.

Seeding density ($\times 10^4$)	Day 3 (FU × 10^3)	Day 6 (FU × 10^3)	FU × 10^3/day
1	3.5 ± 0.08	7.7 ± 0.1	1.1
5	4.0 ± 0.2	8.1 ± 0.2	1.3
10	4.4 ± 0.1	10.5 ± 0.04	1.4
20	6.2 ± 0.08	15.5 ± 0.2	2.0

PLX cells were grown at different densities in PC12 cells medium for 6 days and metabolic activity was evaluated by Alamar blue assay at day 3 and day 6 in the same wells. Fluorescence in arbitrary units (FU × 10^3) is presented as mean ± SEM (n = 9). *p < 0.05 vs day 3.

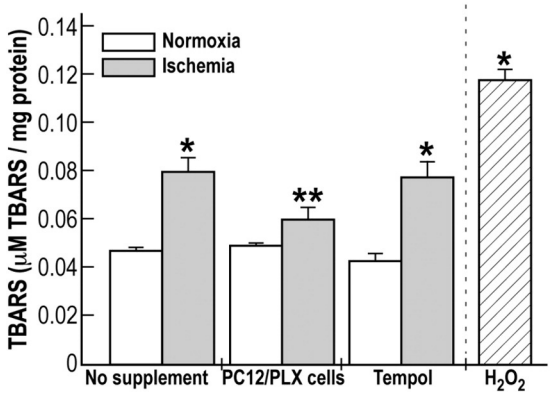

Fig. 3. Effects of PLX cells and Tempol on ischemia-induced lipid peroxidation. Experimental details were identical to those in Fig. 2. Designated wells were supplemented with 3 mM Tempol or 0.5 mM H_2O_2 (striped bar) before OGD or with PLX cells after 4 h OGD. After 18 h reperfusion medium aliquots were collected and analyzed for TBARS. Values, expressed as μM TBARS normalized to mg protein, are mean ± SE (n = 12); *p < 0.05 vs no supplement in normoxia; **p < 0.01 vs no supplement.

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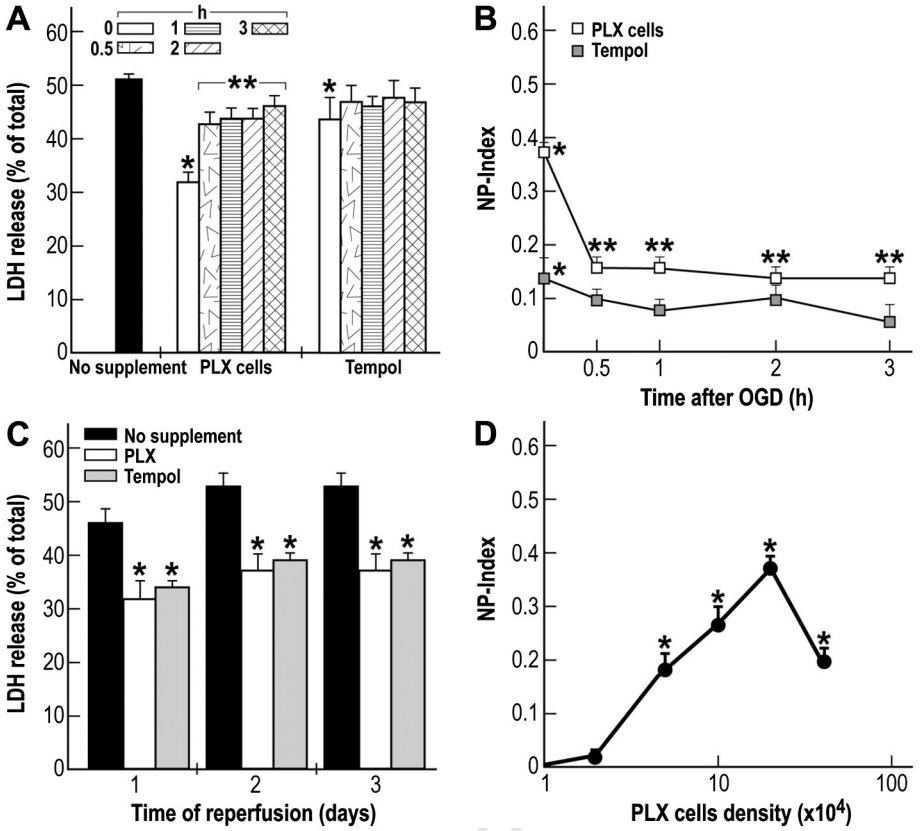

Fig. 4. Cell density and time of treatment dependency of PLX cells and Tempol neuroprotective actions. PC12 cells were exposed for 4 h to OGD and subsequently to different periods of reperfusion in the presence of PLX cells or 3 mM Tempol as detailed in Fig. 2. Panel A shows % LDH release as mean ± SE (n = 16);*p < 0.01 vs no supplement; **p < 0.01 vs time zero following supplements at designated time after OGD. Black bars indicate cell death without supplements. Panel B depicts the time course of the NP-Index of PLX cells and Tempol after OGD and was extrapolated from panel A. Panel C illustrates the long term effects of the supplements on % LDH release (±SE, n = 12) after prolonged reperfusion; *p < 0.05 vs control. Panel D shows a density-dependence of the NP-Index on the seeding density (log scale) of the PLX cells in the co-cultures. NP-Index value on a scale from zero (no protection) to one (complete protection) are means ± SE (n = 48);*p < 0.01 compared to ischemia-treated PC12 cells.

pre-conditioned medium of monolayer cultures of PLX cells, but also of PC12 cells alone and/or in combination, possess the capacity to provide neuroprotection as noticed by reduced LDH release (cell death) and TBARS levels (lipid peroxidation).

Prompted by the likelihood that mesenchymal stem cells as recently indicated, possess the capacity to secrete several growth factors [17–19], we set up an ELISA protocol to detect some of these factors in the current ischemic model. For these studies we applied an anti-mouse $VEGF_{164}$ with a partial (15%) cross reactivity to rat VEGF but no cross reactivity to human $VEGF_{165}$ and a second, highly selective human $VEGF_{165}$ antibody as well as an anti human IL-6 with no cross reactivity to rat. Fig. 6 depicts the levels of VEGF (panel A) and IL-6 (panel B) in the conditioned medium arising from either PC12 cells or PLX cells alone or their co-culture under control or ischemic

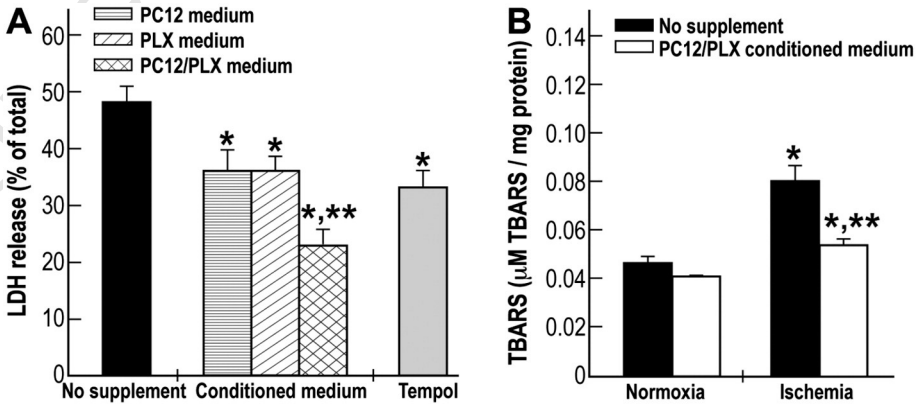

Fig. 5. Effect of pre-conditioned medium on LDH release and TBARS production in PC12 cells following ischemic insult. PC12 cells were exposed to 4 h OGD and 18 h reperfusion under similar conditions as detailed in Fig. 2 in the presence or absence of 50 μl conditioned media (added before OGD) which was previously collected from wells of PC12 cells or PLX cells alone or from PC12/PLX co-culture subjected to OGD. Cell death expressed as % LDH release (panel A) are mean ± SE (n = 16);*p < 0.01 vs no supplement; **p < 0.01 vs PLX conditioned medium. Aliquots of the culture medium were taken for TBARS determination (panel B) as described in Fig. 3 and expressed as μM TBARS per mg protein (±SEM, n = 12);*p < 0.05 vs no supplement (normoxia); **p < 0.01 vs no supplement in ischemia.

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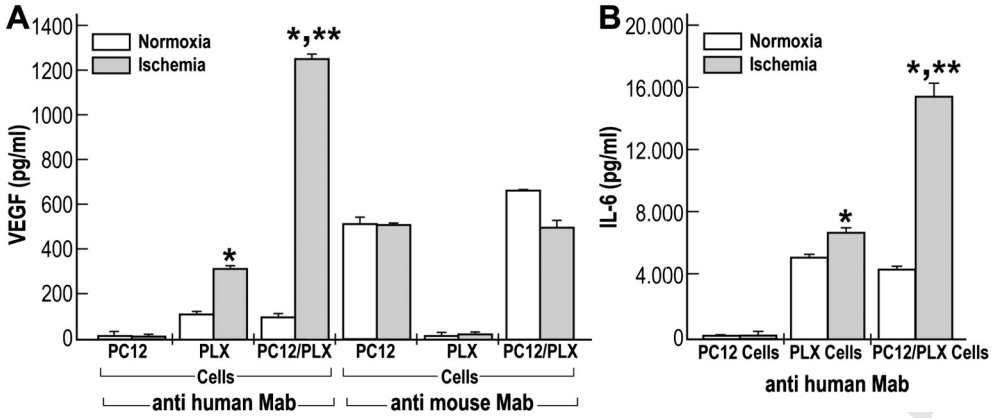

Fig. 6. VEGF and IL-6 identification in pre-conditioned medium following ischemia. PC12 cells were subjected to ischemic or normoxic conditions in the presence or absence of PLX cells as detailed in Fig. 2. Cultures of PLX cells alone, in the absence of PC12 cells, were also subjected to ischemic or normoxic conditions. Conditioned medium was collected and aliquots used for VEGF measurements, using an anti-mouse or a human Mab (panel A) and for IL-6 measurements using an anti-human Mab (panel B). Values expressed as pg/ml medium are mean ± SE (n = 12); *p < 0.01 vs PLX cell normoxia;**p < 0.01 vs PLX cell ischemia.

conditions. Notable, under normoxic, control conditions, PLX cells secrete 110 ± 5 pg/ml $VEGF_{165}$. This basal value is elevated by nearly 3 fold (315 ± 10 pg/ml) after the ischemic insult. Conditioned medium derived from PC12/PLX co-cultures after ischemic insult, shows a further robust increase up to 1245 ± 21 pg/ml in $VEGF_{165}$ indicative for a strong synergistic effect. Notably, PC12 cells release a basal level, independent of ischemia, of rat VEGF. That may suggest that the constitutive rodent levels of VEGF are not sufficient to protect the PC12 cells after the ischemic insult. Fig. 6 (panel B) also shows a remarkable basal level of IL-6 secreted from PLX cells as attested by the human Mab (5.25 ± 2 ng/ml) but only a small increase (about 23%) following ischemia. In great contrast, an ischemic insult applied to co-cultures of both PC12 and PLX cells triggered release of IL-6 to a value of approximately 16 ng/ml which constitutes a 4 fold increase over the basal levels. Taken together, these data strongly indicate that the ischemic insult episode caused enhanced synthesis and secretion of both growth factors by the PLX cells and that the presence of the PC12 cells was necessary to trigger the amplitude of this activation.

Further confirmation for the neuroprotective effect of $VEGF_{165}$ and IL-6, was obtained from direct supplements of either 10 ng/ml of recombinant human $VEGF_{165}$ and/or IL-6 to PC12 cells subjected to ischemia (Fig. 7 panel A). A robust neuroprotection (NP-Index = 0.35, Fig. 7 panel A, insert) was attained upon treatment of the cultures with either IL-6 or $VEGF_{165}$ before or immediately after OGD presumably because the immediate availability of the growth factors. Interestingly, no synergistic effect was observed upon treating PC12 cells with a combination of both growth factors. Treatment of the PC12 cultures with human recombinant IL-6, $VEGF_{165}$ and IL-6/$VEGF_{165}$ in the presence of selective antagonists, i.e., IL-6 neutralizing monoclonal antibody, $VEGF_{165}$ receptor 2 antagonist Semaxanib or a combination of both, canceled the growth factor neuroprotective effect, causing again an increase in cell death by 33%, 33% and 27%, respectively (Fig. 7 panel B). Treatment of PC12 cells with antagonists in the absence of agonists had no protective effects (data not shown).

Further examination on the role of each of these PLX cells-secreted growth factors prompted us to measure the neuroprotective effect of

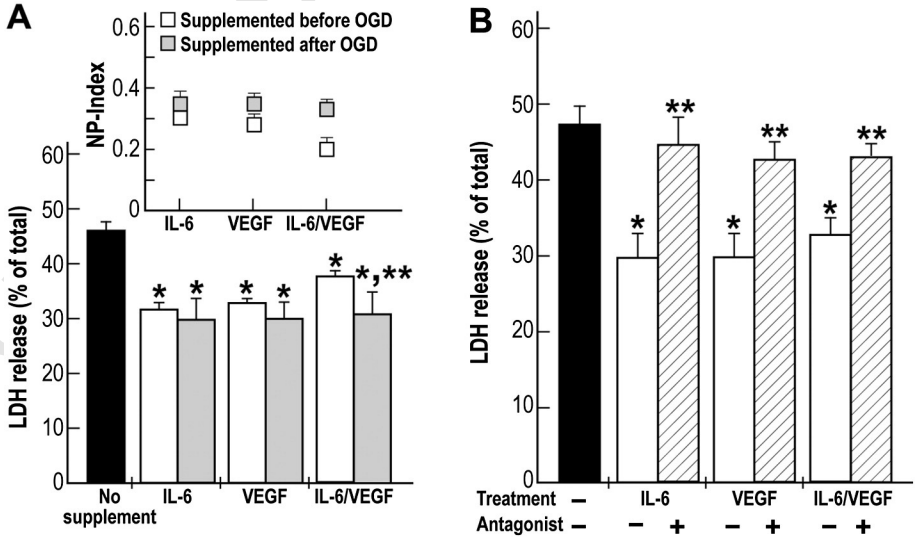

Fig. 7. Effect of $VEGF_{165}$ and IL-6 supplement and their antagonists on ischemia-induced PC12 cell death. PC12 cells were exposed to ischemia as detailed in Fig. 2 in the presence of recombinant human IL-6 (10 ng/ml) or recombinant human $VEGF_{165}$ (10 ng/ml) or a mixture of both (10 ng/ml each). The growth factors were added 1 min before (white bars) or immediately after OGD (gray bars). Cell death expressed as % LDH release (panel A) and NP-index (scheme) are mean ± SE (n = 20); *p < 0.01 vs no supplement; **p < 0.01 vs IL-6/VEGF before OGD. Panel B depicts the partial neutralization of the protective effect of IL-6 and/or VEGF (striped bars) by either IL-6 neutralizing monoclonal antibody (400 ng/ml) added concomitantly with the IL-6 or Semaxanib (240 ng/ml), a potent VEGFR2 antagonist added 3.5 h before $VEGF_{165}$, or a mixture of both. Values expressed as % LDH release are mean ± SE (n = 20). *p < 0.01 vs no treatment (−); **p < 0.01 vs growth factor alone without antagonist.

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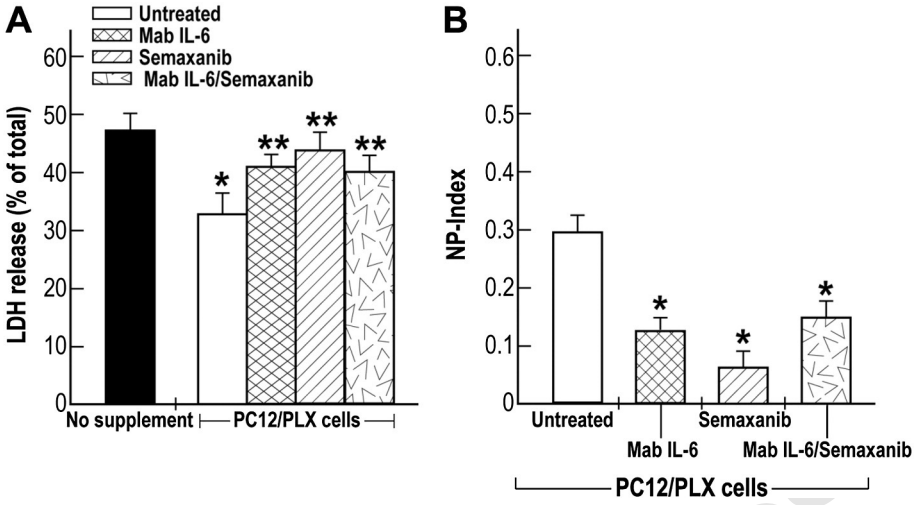

Fig. 8. IL-6 monoclonal antibody and Semaxanib reverse the PLX neuroprotective effect on PC12 cells subjected to ischemia. PC12 cells were exposed to ischemia in the presence of PLX cells, added after OGD, as detailed in Fig. 2. The antagonists including anti-human IL-6 (400 ng/ml), Semaxnib (240 ng/ml) or a mixture of both at the same concentration were added before OGD. Cell death expressed as % LDH release (panel A) and NP-index (panel B) are mean ± SE (n = 12); *p < 0.01 vs no supplement; **p < 0.05 vs untreated PLX cells.

PLX cells on PC12 cells in the presence of the selective antagonists. As shown in Fig. 8 (panels A, B) addition of the IL-6 antibody or administration of Semaxanib, inhibited the protective effect of PLX cells by 57% and 78%, respectively. A combination of both antagonists, although not synergistic, was also highly effective (50%). These findings demonstrate the involvement of IL-6 and $VEGF_{165}$ in the PLX-induced neuroprotective effect.

4. Discussion

This work establishes that human placental expanded (PLX) mesenchymal-like adherent stromal cells adapted to grow in monolayer cultures are able to confer protection to a NGF-differentiated neural cell line PC12 of pheochromocytoma origin, which has been subjected to an acute ischemic insult. The ischemic insult triggered by sustained glucose and oxygen deprivation (OGD) followed by reperfusion where both glucose and oxygen level have been replenished, caused robust cell damage as attested by the release of LDH from the cells into the medium as well as by production of substantial levels of TBARS, both indicative of oxidative cell damage (Figs. 2 and 3). Notably, a remarkable PLX concentration-dependent neuroprotective effect was evident (Fig. 4).

During and after the OGD insult, the PLX mesenchymal-like adherent stromal cells, secrete into the culture medium at least two growth factors, IL-6 and VEGF, that have been identified and characterized. Several lines of evidence are indicative for the involvement of these factors in the rescue process of PC12 cells. Firstly, the PLX cells and not the PC12 cells actively secrete both factors into the growth medium upon ischemia as assessed by a highly sensitive and specific immunoassay (Fig. 6). Interestingly, the relatively high level of the basal rodent VEGF compared to human VEGF after ischemia is fairly striking (Fig. 6A); yet the rodent VEGF is neither enhanced nor able to provide protection to the PC12 cells. That may be reasoned by a difference in the affinities of the rodent and human VEGF or that additional VEGF, above the basal levels, is needed to enable neuroprotection. Secondly, direct addition of human recombinant VEGF or IL-6 at nanogram concentrations was able to reproduce the neuroprotective effect obtained in the PLX/PC12 co-culture system (Fig. 7). Thirdly, inhibition of IL-6 and VEGF neuroprotective activity by specific antagonists was attained to either exogenously added peptide.

Mesenchymal stem cells have been shown recently to secrete a variety of protective anti-inflammatory cytokines and growth factors and also exert a protective capacity in vivo in stroke therapy [17–19] and in traumatic brain injury [20–22]. In line with these studies, implantation of PLX cells in a mouse model of critical limb ischemia, reduced ischemic damage as expressed by improved blood flow to the damaged limb, increased capillary density, decreased oxidative stress and endothelial damage and increased functionality of the insulted limb [3].

We have expanded these observations to a neuronal cell line model and now demonstrate that PLX cells can secrete such growth factors under monolayer culture conditions. This is the first time, to our knowledge, that such capability of the PLX cells in monolayer cultures to secrete ex vivo specific growth factors has been reported. The use of co-cultures offers the unique opportunity to investigate cell–cell interaction events mediated via the medium under amenable conditions. In that respect, we firstly show that there is a reciprocal interaction between the ischemia-targeted PC12 cells and the potency of the PLX mesenchymal-like adherent stromal cells to enhance secretion (Fig. 5). This is clearly evident from the fact that OGD-insulted PLX cells alone show little but significant enhanced secretory activity. Furthermore, the dependency of PLX cell secretion by PC12 cells highly suggests a possible release of active substances and/or low molecular weight signals from the ischemically-insulted PC12 cells. The nature of these substances is presently unknown. In this context it is possible that the protective activity of the PC12 conditioned medium alone (Fig. 5) is due to certain factors released by the PC12 cells which are beneficial. Secondly, the time window of the stimulation of release appears to be fairly large, provided cells are sufficiently close to the time of the OGD insult (i.e. 30 min, see Fig. 4 panel A). The inability of PLX cells to rescue PC12 cells after a certain time point (Fig. 4 panels A, B), could be attributed to the fact that triggering molecules arising from the PC12 cells i.e. release of ATP [10] or anti-oxidants [6] are rendered cryptic, such that within a short period (1 h), PLX cells are unable to secrete growth factors. This raises the importance of identifying the therapeutic window for the use of these cells for in vivo transplantation.

Thirdly, we provide solid evidence that a neurally-differentiated cell line under the influence of NGF appears robustly responsive to cues and signals which are derived from a stem cell line, but also appear to influence the latter. Indeed stem cells secrete a broad spectrum of signaling molecules but these are likely to be highly selective and quantitatively controlled depending on the micro environmental cues that the target cells confer in their domains. Thus stem cells challenged with cues from kidney or heart may provide a different spectrum of growth factors than those challenged by brain or other peripheral tissues. Such an advantage should be underscored

when compared to the general application of synthetic growth factors, the latter of which are of transient nature.

The molecular mechanism underlying the neuroprotective effects still remains elusive and several second messenger pathways have been implicated but not completely elaborated. Some laboratories suggested that the neuroprotective effects are mediated by attenuation of the excessive calcium influx, a primary trigger for excitotoxic cascade during ischemic insult and/or inhibition of stress kinases such as Jnk and p-38 [23,24]. Other studies indicate a mechanism of action which includes modulation of free ROS and up regulation of cellular signaling cascades [25]. The latter possibility is also supported by recent studies from our laboratory indicating that cord blood derived neuronal progenitors confer neuroprotection following a robust reduction of ROS level and secretion of different anti-oxidant molecules into the culture medium [6]. Moreover mesenchymal cells derived from various origins, provide rescue to PC12 cells from diverse cytotoxic insults such as hydrogen peroxide, radiation, chronic ethanol damage and hypoxia via downregulation of apoptotic proteins and upregulation of PI3K/Akt kinase pathway [26–30].

In this report we provide substantial evidence that IL-6 and $VEGF_{165}$ constitute a major mechanism involved in PC12 cell neuroprotection from ischemic insult. IL-6 in particular, appears beneficial in preventing death in PC12 cells for it has been shown to rise in a spectrum of stress events such as serum deprivation or addition of toxic agents such as calcium ionophore [31,32], 6-hydroxydopamine, 4-hydroxynonenal [33,34] and MPP + or tetrahydro-isoquinoline [35]. In all these studies reduction of the oxidative damage has been attributed to activation of free radical detoxifying mechanisms such as catalase [34] and glutathione levels [33] possibly accompanied by upregulation of phosphorylation pathways such as phosphatidylinositol 3-kinase/Akt and STAT3 [31].

$VEGF_{165}$ on the other hand, apart from being a major angiogenic factor, is also known for its neurotropic activity in PC12 cells [36] after beta-amyloid [37], glutamate [28] and hypoxia-induced [27] toxicity. VEGF is also beneficial in OGD insult applied to primary cultures of rat neurons [38,39] and in rat model of cerebral ischemia [40]. These trophic effects are believed to be mediated through the PI3K/Akt/FoxO1 survival pathway [27,28,38].

In this study we took advantage of the well-established antioxidant Tempol [41,42] as a comparative, positive marker for its antioxidant characteristics. Clearly, protection by Tempol appears to be attained through a different mechanism as attested by its shorter window of opportunities to provide rescue to cells (Figs. 2 and 4) and by its continuous presence at relatively high millimolar concentrations. Nevertheless it was instrumental in determining the magnitude of protection in comparison to the PLX cells.

Considering that at this time PLX cells constitute an important tool in clinical trials for therapy of different ischemic disorders, it would be tempting to propose that the principal mode of action of these cells involves the active secretion of IL-6 and $VEGF_{165}$. This possibility is plausible since mesenchymal stem cell transplantation has been shown to be beneficial in ischemic disorders via trophic mechanisms [43,44]. Trophic support is generally of transient nature as opposed to a cell substitution mechanism and is relatively fast and diminished proportionally to the survival rate of the transplant [45]. A large variety of trophic factors, which may be beneficial to injured and/or regenerating neurons, have been detected and isolated from secreted proteomes derived of human MSC fetal and adult tissues including placenta, umbilical cord blood, umbilical stroma, adult bone marrow, and peripheral blood [46,47].

In conclusion, PLX-induced neuroprotection towards NGF-differentiated PC12 cells exposed to ischemia is strongly correlated to secretion of IL-6 and $VEGF_{165}$ which is in line with the neuroprotective effect of these factors in hypoxic/ischemic injuries both in vitro and in vivo models [48,49]. Therefore, this correlation may explain part of the neuroprotective mechanism of PLX cells upon transplantation in ischemic models [2,3]. Furthermore, our present studies add to the notion that pre-triggering of PLX cells before transplantation may enhance the effectiveness of the stem cell therapy [44,50]. Future investigations on the secretome of PLX cells, should reveal additional anti-inflammatory cytokines and/or growth factors with neuroprotective effects beneficial for treating ischemic disorders.

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References

[1] A. Trounson, R.G. Thakar, G. Lomax, D. Gibbons, Clinical trials for stem cell therapies, BMC Med. 9 (2011) 52.
[2] A. Kranz, D.C. Wagner, M. Kamprad, M. Scholz, U.R. Schmidt, F. Nitzsche, Z. Aberman, F. Emmrich, U.M. Riegelsberger, J. Boltze, Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats, Brain Res. 1315 (2010) 128–136.
[3] W.R. Prather, A. Toren, M. Meiron, R. Ofir, C. Tschope, E.M. Horwitz, The role of placental-derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia, Cytotherapy 11 (2009) 427–434.
[4] W.R. Prather, A. Toren, M. Meiron, Placental-derived and expanded mesenchymal stromal cells (PLX-I) to enhance the engraftment of hematopoietic stem cells derived from umbilical cord blood, Expert. Opin. Biol. Ther. 8 (2008) 1241–1250.
[5] D. Drago, C. Cossetti, N. Iraci, E. Gaude, G. Musco, A. Bachi, S. Pluchino, The stem cell secretome and its role in brain repair, Biochimie 95 (2013) 2271–2285.
[6] H. Arien-Zakay, S. Lecht, M.M. Bercu, R. Tabakman, R. Kohen, H. Galski, A. Nagler, P. Lazarovici, Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors, Exp. Neurol. 216 (2009) 83–94.
[7] S. Lecht, E. Rotfeld, H. Arien-Zakay, R. Tabakman, H. Matzner, R. Yaka, P.I. Lelkes, P. Lazarovici, Neuroprotective effects of nimodipine and nifedipine in the NGF-differentiated PC12 cells exposed to oxygen-glucose deprivation or trophic withdrawal, Int. J. Dev. Neurosci. 30 (2012) 465–469.
[8] R. Tabakman, P. Lazarovici, R. Kohen, Neuroprotective effects of carnosine and homocarnosine on pheochromocytoma PC12 cells exposed to ischemia, J. Neurosci. Res. 68 (2002) 463–469.
[9] T. Lipman, R. Tabakman, P. Lazarovici, Neuroprotective effects of the stable nitroxide compound Tempol on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in the nerve growth factor-differentiated model of pheochromocytoma PC12 cells, Eur. J. Pharmacol. 549 (2006) 50–57.
[10] S. Abu-Raya, R. Tabakman, E. Blaugrund, V. Trembovler, P. Lazarovici, Neuroprotective and neurotoxic effects of monoamine oxidase-B inhibitors and derived metabolites under ischemia in PC12 cells, Eur. J. Pharmacol. 434 (2002) 109–116.
[11] R. Tabakman, H. Jiang, R.A. Levine, R. Kohen, P. Lazarovici, Apoptotic characteristics of cell death and the neuroprotective effect of homocarnosine on pheochromocytoma PC12 cells exposed to ischemia, J. Neurosci. Res. 75 (2004) 499–507.
[12] E. Schonfeld, I. Yasharel, E. Yavin, A. Brand, Docosahexaenoic acid enhances iron uptake by modulating iron transporters and accelerates apoptotic death in PC12 cells, Neurochem. Res. 32 (2007) 1673–1684.
[13] M.M. Bercu, H. Arien-Zakay, D. Stoler, S. Lecht, P.I. Lelkes, S. Samuel, R. Or, A. Nagler, P. Lazarovici, U. Elchalal, Enhanced survival and neurite network formation of human umbilical cord blood neuronal progenitors in three-dimensional collagen constructs, J. Mol. Neurosci. 51 (2013) 249–261.
[14] Y.C. Huang, Z.M. Yang, X.H. Chen, M.Y. Tan, J. Wang, X.Q. Li, H.Q. Xie, L. Deng, Isolation of mesenchymal stem cells from human placental decidua basalis and resistance to hypoxia and serum deprivation, Stem Cell Rev. 5 (2009) 247–255.
[15] H. Hong, G.Q. Liu, Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin, Life Sci. 74 (2004) 2959–2973.
[16] I. Kruman, A.J. Bruce-Keller, D. Bredesen, G. Waeg, M.P. Mattson, Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis, J. Neurosci. 17 (1997) 5089–5100.
[17] N. Joyce, G. Annett, L. Wirthlin, S. Olson, G. Bauer, J.A. Nolta, Mesenchymal stem cells for the treatment of neurodegenerative disease, Regen. Med. 5 (2010) 933–946.
[18] A. Uccelli, F. Benvenuto, A. Laroni, D. Giunti, Neuroprotective features of mesenchymal stem cells, Best Pract. Res. Clin. Haematol. 24 (2011) 59–64.
[19] J.Y. Hsieh, H.W. Wang, S.J. Chang, K.H. Liao, I.H. Lee, W.S. Lin, C.H. Wu, W.Y. Lin, S.M. Cheng, Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis, PLoS One 8 (2013) e72604.
[20] S.A. Acosta, N. Tajiri, K. Shinozuka, H. Ishikawa, P.R. Sanberg, J. Sanchez-Ramos, S. Song, Y. Kaneko, C.V. Borlongan, Combination therapy of human umbilical cord blood cells and granulocyte colony stimulating factor reduces histopathological

and motor impairments in an experimental model of chronic traumatic brain injury, PLoS One 9 (2014) e90953.
[21] I. Dela Pena, P.R. Sanberg, S. Acosta, N. Tajiri, S.Z. Lin, C.V. Borlongan, Stem cells and G-CSF for treating neuroinflammation in traumatic brain injury: aging as a comorbidity factor, J. Neurosurg. Sci. 58 (2014) 145–149.
[22] N. Tajiri, S.A. Acosta, M. Shahaduzzaman, H. Ishikawa, K. Shinozuka, M. Pabon, D. Hernandez-Ontiveros, D.W. Kim, C. Metcalf, M. Staples, T. Dailey, J. Vasconcellos, G. Franyuti, L. Gould, N. Patel, D. Cooper, Y. Kaneko, C.V. Borlongan, P.C. Bickford, Intravenous transplants of human adipose-derived stem cell protect the brain from traumatic brain injury-induced neurodegeneration and motor and cognitive impairments: cell graft biodistribution and soluble factors in young and aged rats, J. Neurosci. 34 (2014) 313–326.
[23] S. Ryu, G.H. Seol, H. Park, I.Y. Choi, Trans-anethole protects cortical neuronal cells against oxygen–glucose deprivation/reoxygenation, Neurol. Sci. (2014).
[24] F.C. Barone, E.A. Irving, A.M. Ray, J.C. Lee, S. Kassis, S. Kumar, A.M. Badger, J.J. Legos, J.A. Erhardt, E.H. Ohlstein, A.J. Hunter, D.C. Harrison, K. Philpott, B.R. Smith, J.L. Adams, A.A. Parsons, Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia, Med. Res. Rev. 21 (2001) 129–145.
[25] D. Jia, B. Han, S. Yang, J. Zhao, Anemonin alleviates nerve injury after cerebral ischemia and reperfusion (i/r) in rats by improving antioxidant activities and inhibiting apoptosis pathway, J. Mol. Neurosci. 53 (2014) 271–279.
[26] C.C. Tate, C. Fonck, M. McGrogan, C.C. Case, Human mesenchymal stromal cells and their derivative, SB623 cells, rescue neural cells via trophic support following in vitro ischemia, Cell Transplant. 19 (2010) 973–984.
[27] Q. Zhong, Y. Zhou, W. Ye, T. Cai, X. Zhang, D.Y. Deng, Hypoxia-inducible factor 1-alpha-AA-modified bone marrow stem cells protect PC12 cells from hypoxia-induced apoptosis, partially through VEGF/PI3K/Akt/FoxO1 pathway, Stem Cells Dev. 21 (2012) 2703–2717.
[28] S. Lu, C. Lu, Q. Han, J. Li, Z. Du, L. Liao, R.C. Zhao, Adipose-derived mesenchymal stem cells protect PC12 cells from glutamate excitotoxicity-induced apoptosis by upregulation of XIAP through PI3-K/Akt activation, Toxicology 279 (2011) 189–195.
[29] L. Liu, J.X. Cao, B. Sun, H.L. Li, Y. Xia, Z. Wu, C.L. Tang, J. Hu, Mesenchymal stem cells inhibition of chronic ethanol-induced oxidative damage via upregulation of phosphatidylinositol-3-kinase/Akt and modulation of extracellular signal-regulated kinase 1/2 activation in PC12 cells and neurons, Neuroscience 167 (2010) 1115–1124.
[30] S. Francois, M. Mouiseddine, B. Allenet-Lepage, J. Voswinkel, L. Douay, M. Benderitter, A. Chapel, Human mesenchymal stem cells provide protection against radiation-induced liver injury by antioxidative process, vasculature protection, hepatocyte differentiation, and trophic effects, Biomed. Res. Int. 2013 (2013) 151679.
[31] H. Kunioku, K. Inoue, M. Tomida, Interleukin-6 protects rat PC12 cells from serum deprivation or chemotherapeutic agents through the phosphatidylinositol 3-kinase and STAT3 pathways, Neurosci. Lett. 309 (2001) 13–16.
[32] H. Umegaki, K. Yamada, M. Naito, T. Kameyama, A. Iguchi, T. Nabeshima, Protective effect of interleukin-6 against the death of PC12 cells caused by serum deprivation or by the addition of a calcium ionophore, Biochem. Pharmacol. 52 (1996) 911–916.
[33] A. Nakajima, K. Yamada, L.B. Zou, Y. Yan, M. Mizuno, T. Nabeshima, Interleukin-6 protects PC12 cells from 4-hydroxynonenal-induced cytotoxicity by increasing intracellular glutathione levels, Free Radic. Biol. Med. 32 (2002) 1324–1332.
[34] K. Yamada, H. Umegaki, I. Maezawa, A. Iguchi, T. Kameyama, T. Nabeshima, Possible involvement of catalase in the protective effect of interleukin-6 against 6-hydroxydopamine toxicity in PC12 cells, Brain Res. Bull. 43 (1997) 573–577.
[35] N. Shimma, N. Akiyama, M. Umezawa, Y. Okuma, Y. Nomura, T. Saito, S. Horie, T. Murayama, Possible role of interleukin-6 in PC12 cell death induced by MPP+ and tetrahydroisoquinoline, J. Pharmacol. Sci. 93 (2003) 471–477.
[36] P. Lazarovici, C. Marcinkiewicz, P.I. Lelkes, Cross talk between the cardiovascular and nervous systems: neurotrophic effects of vascular endothelial growth factor (VEGF) and angiogenic effects of nerve growth factor (NGF)—implications in drug development, Curr. Pharm. Des. 12 (2006) 2609–2622.
[37] S.P. Yang, B.O. Kwon, Y.S. Gho, C.B. Chae, Specific interaction of VEGF165 with beta-amyloid, and its protective effect on beta-amyloid-induced neurotoxicity, J. Neurochem. 93 (2005) 118–127.
[38] K.L. Jin, X.O. Mao, D.A. Greenberg, Vascular endothelial growth factor: direct neuroprotective effect in *in vitro* ischemia, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 10242–10247.
[39] C.V. Borlongan, Y. Kaneko, M. Maki, S.J. Yu, M. Ali, J.G. Allickson, C.D. Sanberg, N. Kuzmin-Nichols, P.R. Sanberg, Menstrual blood cells display stem cell-like phenotypic markers and exert neuroprotection following transplantation in experimental stroke, Stem Cells Dev. 19 (2010) 439–452.
[40] D.F. Emerich, E. Silva, O. Ali, D. Mooney, W. Bell, S.J. Yu, Y. Kaneko, C. Borlongan, Injectable VEGF hydrogels produce near complete neurological and anatomical protection following cerebral ischemia in rats, Cell Transplant. 19 (2010) 1063–1071.
[41] P.K. Chatterjee, S. Cuzzocrea, P.A. Brown, K. Zacharowski, K.N. Stewart, H. Mota-Filipe, C. Thiemermann, Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat, Kidney Int. 58 (2000) 658–673.
[42] C.S. Wilcox, Effects of tempol and redox-cycling nitroxides in models of oxidative stress, Pharmacol. Ther. 126 (2010) 119–145.
[43] F. Scheibe, O. Klein, J. Klose, J. Priller, Mesenchymal stromal cells rescue cortical neurons from apoptotic cell death in an in vitro model of cerebral ischemia, Cell. Mol. Neurobiol. 32 (2012) 567–576.
[44] L. Hao, Z. Zou, H. Tian, Y. Zhang, H. Zhou, L. Liu, Stem cell-based therapies for ischemic stroke, Biomed. Res. Int. 2014 (2014) 468748.
[45] A.M. Parr, C.H. Tator, A. Keating, Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury, Bone Marrow Transplant. 40 (2007) 609–619.
[46] H. Kupcova Skalnikova, Proteomic techniques for characterisation of mesenchymal stem cell secretome, Biochimie 95 (2013) 2196–2211.
[47] S.K. Sze, D.P. de Kleijn, R.C. Lai, E. Khia Way Tan, H. Zhao, K.S. Yeo, T.Y. Low, Q. Lian, C.N. Lee, W. Mitchell, R.M. El Oakley, S.K. Lim, Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells, Mol. Cell. Proteomics 6 (2007) 1680–1689.
[48] B. Svensson, M. Peters, H.G. Konig, M. Poppe, B. Levkau, M. Rothermundt, V. Arolt, D. Kogel, J.H. Prehn, Vascular endothelial growth factor protects cultured rat hippocampal neurons against hypoxic injury via an antiexcitotoxic, caspase-independent mechanism, J. Cereb. Blood Flow Metab. 22 (2002) 1170–1175.
[49] T. Yamashita, K. Sawamoto, S. Suzuki, N. Suzuki, K. Adachi, T. Kawase, M. Mihara, Y. Ohsugi, K. Abe, H. Okano, Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of Stat3 activation in the protection of neurons, J. Neurochem. 94 (2005) 459–468.
[50] H. Sakata, P. Narasimhan, K. Niizuma, C.M. Maier, T. Wakai, P.H. Chan, Interleukin 6-preconditioned neural stem cells reduce ischaemic injury in stroke mice, Brain 135 (2012) 3298–3310.

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