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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:

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