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DADLE enhances viability and anti-inflammatory effect of human MSCs subjected to 'serum free' apoptotic condition in part via the DOR/PI3K/AKT pathway

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ABSTRACT

Aim: Nutritional deprivation and inflammation-rich zones are the major causative reasons for poor survivability of transplanted mesenchymal stem cells (MSCs). Therefore in the present study, we demonstrated the cytoprotective and anti-inflammatory effects of activated delta (δ)-opioid receptor (DOR) with synthetic peptide [D-Ala^2, D-Leu^5]-enkephalin (DADLE) treatment on human MSCs cultured in serum-starved condition.
Main methods: Cell viability was measured using MTT and Annexin V/PI assays. Expressions of pro-apoptotic (Bcl2) and anti-apoptotic genes (Bax/Bad), levels of activated p44/42 MAPK, Akt, PI3-kinase-p110γ and cleaved caspase-3 were determined by qPCR and western blot. Levels of secreted cytokines were measured by ELISA.
Key findings: In comparison to the control, DADLE significantly increased cell survivability under serum deprived condition as confirmed by MTT (71% vs 45%) and Annexin V/PI assays (25.9% vs 3.7%). Significant up-regulation of pro-apoptotic Bcl2 (~2.1 folds), down-regulations of anti-apoptotic Bax/Bad (~2.6/2.7 folds) as well as of cleaved caspase-3, increased expression of PI3kinase subunit p110γ and activation of Akt (Ser473) were observed following DADLE treatment in cells under 'serum deprivation' stress. In addition, DADLE treated hMSCs secreted increased levels of anti-inflammatory cytokines (IL10/IL4/TGF-β) under serum deprived condition. LPS stimulated macrophages showed abated release of pro-inflammatory cytokines (IL1/TNFα/IL6) when grown in hMSC conditioned 'serum deprived' media treated with DADLE. Both the cytoprotective and anti-inflammatory effects of DADLE were inhibited by the DOR specific antagonist naltrindole.
Significance: The DOR signaling pathway improved cell viability and enhanced anti-inflammatory effect of hMSCs subjected to 'serum deprivation' stress that could have potential therapeutic benefits in reparative medicine.

1. Introduction

Cell survivability is considered as a significant problem in post-transplantation therapies. The microenvironment of grafted cells at injured site is too harsh and most of these cells tend to die within few days after transplantation due to inflammation and inadequate nutritional supplements [1,2]. Muller-Ehmsen et al., demonstrated very low survival rate (only 1–2%) of bone marrow cells grafted in a myocardial infracted (MI) animal model and elucidated that the cell death was due to acute inflammatory response in the hostile infracted microenvironment [3]. Toma et al., observed only 0.44% of human MSCs to survive in the left ventricle of MI rodent models [4]. In a clinical trial, < 1% of myoblasts were found to survive in patients with ischemic damaged myocardium [5]. Hence, levels of inflammatory cytokines determine the viability of the grafted cells at ischemic sites. Anti-inflammatory cytokine such as IL-10 attenuates the generation of pro-inflammatory cytokines in macrophages [7,8]. Also, IL-10 has been shown to reduce the intake of opiod drugs in non-handled rodent models of early-life experience resulted in the reduction of neuroinflmmation in the nuclear accuembens [9]. Along with the regulation of immune response, IL-10 has been shown to thwart apoptosis by activating the PI3K/Akt signaling cascade and enhance the expression of anti-apoptotic signals: Bcl-2 and Bcl-xl, while attenuating that of caspase-3 [10,11]. Moreover IL-10 promotes neuronal cell survival by increased expression of anti-apoptotic signals Bcl-2, Bcl-xL and inhibition of cytochrome *c* release and caspase 3 cleavage in spinal cord injury model [12–14]. Thus, improvement of cell survival through the attenuation of pro-inflammatory cytokines is an important area of research for the long-term

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survival of grafts (cells/tissues) at injured sites.

G-protein coupled receptors (GPCRs) play a key role in the regulation of stem cell survival, proliferation, migration, and self-renewal [6]. Opioid receptors are a large super-family of GPCRs [7] that are generally of three major types; mu (μ), kappa (κ) and delta (δ), also known as Oprm1/MOR, Oprk1/KOR and Oprd1/DOR respectively. Among these opioid receptors, the δ-opioid receptor (DOR) has been consistently reported to provide cytoprotection especially in neuronal cells [8]. Recently, Higuchi et al., reported that activation of DOR by its agonist (SNC80) led to enhanced rat MSCs survivability on actinomycin-D exposure via activation of protein kinase C and its downstream signaling intermediates such as STAT3 and Mcl-1 [9]. Moreover, various reports have shown the protective effects of DOR on myocardial ischemia reperfusion injury in rodents [10,11]. Along with cytoprotective effect, delta opioids have immunomodulatory effects [12,13]. Recent data suggest that DOR activation with UFP-512 protects the brain from hypoxia-ischemia rat model by downregulating the expression of inflammatory cytokines [14]. However, the cytoprotective and anti-inflammatory effects of activated DOR if any, and its mechanism(s) in human MSCs remain unclear.

Serum starvation/deprivation is used as a tool to study various molecular mechanisms involved in cellular stress response, protein degradation, autophagy and apoptotic conditions and has been widely used as an *invitro* ischemic model [15]. However, prolonged exposure of MSCs to serum starvation can lead to cell demise [16]. Nutritional starvation is one of the major hindrances towards successful engraftment of transplanted MSCs, especially at injured/ischemic sites. Therefore, improving MSC's survival rate under such condition is crucial for the development of novel strategies in MSC-based tissue regeneration [17,18]. In this scenario, it is important to study the role of DOR in exerting cytoprotective effects which then could lead to increased cell survival. Thus in the current study, the cytoprotective and anti-inflammatory effect of the DOR agonist ([D-Ala^2, D-Leu^5]-enkephalin DADLE was elucidated in human umbilical cord derived MSCs (hMSCs) grown under serum starvation.

2. Materials and methods

2.1. Culture and characterization of hMSCs

Human umblical cord blood derived mesenchymal stem cells (hMSCs) were purchased from PromoCell (Germany) and cultured in α-minimum essential medium (α-MEM) (Gibco/Life Technologies, USA) supplemented with 10% FBS (Hi-Media, India) and 1% pen/strep (Himedia, India). The cultures were maintained in 5% CO_2 incubator at 37° C and with 95% humidified atmosphere. For all cell cultures, media was changed every third day. All experiments were carried out with cells of passage numbers between 2 and 5. Presence of DOR on hMSCs was elucidated in our recently published report [19].

2.1.1. Flow cytometric analysis of hMSCs

MSCs were characterized using cell surface markers based on the guidelines from the International Society for Cellular Therapy (ISCT) [20]. MSCs were stained with fluorescent-labeled monoclonal antibodies; positive markers: (FITC CD90, PerCP-Cy™5.5 CD105 and APC CD73) and negative markers: (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR) were analyzed using flow cytometry (BD Stemflow™, USA).

2.1.2. Differentiation potential of hMSCs

The mesoderm-lineage differentiation potency of hMSCs was confirmed by osteogenic and adipogenic differentiation by using specific differentiation media.

2.1.2.1. Adipogenic differentiation potential. To induce adipogenic differentiation, cells were treated with adipogenic differentiation medium (α-MEM (Gibco/Life technologies, USA) supplemented with 10% FBS (Himedia, India), 1% penstrip (Himedia, India) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA), 1 μM dexamethasone (Sigma, USA), 0.2 mM indomethacin (Sigma, Bangalore) and 10 μM h-insulin (SRL, India). Cultures maintained up to 21 days. Oil red O staining was performed to check the oil droplets from the differentiated culture [19].

2.1.2.2. Osteogenic differentiation potential. To induce osteogenic differentiation, cells were treated with osteogenic differentiation medium (α-MEM supplemented with 10% FBS, 1% Penstrep, 0.1 μM Dexamethasone (Sigma, USA) 10 mM β-glycerol phosphate (Sigma, USA), and 50 μM L-ascorbic acid 2- phosphate (sigma,) and cultures were induced up to 21 days. The calcium depositions were examined with 2% Alizarin red S (ARS) stain [19].

2.2. Cytoprotective effects of DOR under serum deprivation

2.2.1. Experimental groups

The groups were i. Control: Compete media (10% FBS), ii. Basal media (without FBS), iii. Basal media + DADLE, iv. Basal media + Naltrindole (Nal), v. Basal media + Nal + DADLE. To activate DOR, the agonist [D-Ala^2, D-Leu^5]-enkephalin acetate salt, DADLE (100 nM) (Sigma, USA) was used. To elucidate if DADLE is acting through the DOR, MSCs were pretreated (1 h) with a highly specific DOR antagonist called naltrindole (1 μM) (Sigma, USA) followed by DADLE treatment for a total of 48 h. All assays were performed 48 h post incubation with respective treatments.

2.2.2. MTT assay

MTT (2-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide) (Sigma, USA) assay was performed to determine the relative cell viability following DADLE treatment in serum deprived condition. HMSCs were seeded at a density of 1×10^4 cells/well into a 96-well plate and kept overnight at 37 °C with 5% CO_2. Cells were washed with PBS and medium was changed according to the experimental groups. MTT assay was performed after 48 h and the formed formazan crystals were solubilized by stop solution (10% SDS in 0.01 N HCl) according to the manufacturer's protocol (Sigma, USA). The absorbance of colored solution was measured at 570 nm.

2.2.3. Annexin-V/PI assay

DADLE mediated cyto-protection was further confirmed by Annexin-V apoptosis assay. For this experiment 5×10^4 cells were plated in 6 well plates and incubated for 24 h followed by the treatments mentioned in the experimental groups. Apoptosis was measured by using Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium Iodide (PI) (Thermofisher, USA) following the manufacturer's protocol. Briefly, the cells were trypsinized and centrifuged at 1500 rpm for 5 min. The pellet was washed with PBS for two times and re-centrifuged. The pellet was resuspended in 100 μL of 1 ×–Annexin binding buffer and then cells were conjugated with 5 μL of Annexin V Alexa Fluor® 488 and 1 μL of PI. Cells were mixed gently and incubated for 15 min at room temperature (RT) in the dark. After the incubation period, 400 μL of 1 × annexin-binding buffer was added, mixed gently, and kept on ice. Data was acquired by flow cytometry (BD FACSCelesta™, New Jersey, US).

2.3. Anti-inflammatory effect of DADLE/DOR under serum deprivation condition

2.3.1. Macrophage culture and maintenance

The murine macrophage cell line (RAW 264.7) was obtained from the NCCS, Pune (India). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% pen/strep and were maintained in 5% CO_2 incubator at 37 °C and with 95% humidified

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