Effects of Recombinant Overexpression of Bcl2 on the Proliferation, Apoptosis, and Osteogenic/Odontogenic Differentiation Potential of Dental Pulp Stem Cells

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Abstract

Introduction: The therapeutic usefulness of dental pulp stem cells (DPSCs) is severely limited by low survivability upon transplantation *in situ* because of the presence of various proapoptotic factors within damaged/diseased tissues (ie, hypoxia and inflammation). One strategy to enhance the survivability of grafted DPSCs could be recombinant overexpression of antiapoptotic genes, such as the B-cell lymphoma 2 gene (Bcl2). **Methods:** DPSCs were transfected with the Bcl2 and/or GFP gene. Cell density and mitotic activity of transfected DPSCs within *in vitro* culture were evaluated with the water soluble tetrazolium salt-8 (WST-8) and bromodeoxyuridine assay, respectively, whereas apoptosis was evaluated through the detection of cytoplasmic histone-associated DNA fragments. The osteogenic/odontogenic differentiation potential of these cells was evaluated with quantitative real-time polymerase chain reaction, alkaline phosphatase, and alizarin red staining. **Results:** Bcl2-transfected DPSCs exhibited consistently higher cell densities than the GFP-transfected control within *in vitro* culture, and this was not because of the higher mitotic rate but was instead attributed to enhanced cell survivability because of the inhibition of apoptosis by Bcl2. Recombinant overexpression of Bcl2 inhibited the osteogenic/odontogenic potential of DPSCs, as indicated by lower levels of alkaline phosphatase activity and mineralized calcium deposition, together with the down-regulated expression of several key osteogenic/odontogenic gene markers including collagen I, osteocalcin, dentin matrix protein-1, bone sialoprotein, and alkaline phosphatase. **Conclusions:** The results place a "caveat" or limitation on the use of recombinant Bcl2 overexpression as a therapeutic strategy for improving the survivability of grafted DPSCs in that the osteogenic/odontogenic potential of these cells may be compromised despite enhanced survival within the host. *(J Endod 2016;42:575–583)*

Key Words

Apoptosis, Bcl2, dental pulp stem cells, osteogenic differentiation, proliferation

Dental pulp tissue is responsible for maintaining the biological and physiological activity of teeth by functioning as a biosensor and continuously generating secondary and tertiary dentin in response to pathogenic stimuli and tooth abrasion (1). The endogenous adult stem cell niche within dental pulp tissue, commonly referred to as dental pulp stem cells (DPSCs), is of a neural crest origin and plays a crucial role in the homeostasis and self-renewal of dental pulp tissue (2). Besides the obvious therapeutic use of DPSCs in dental pulp regeneration (3), DPSCs have also shown much therapeutic potential for the repair and regeneration of a variety of nondental and nonoral tissues including bone, cartilage, skeletal muscle, myocardium, the spinal cord, and peripheral nerves (4).

Nevertheless, a major bottleneck limiting the therapeutic use of DPSCs is the low survivability of grafted cells upon transplantation *in situ* because of adverse proapoptotic factors being present within damaged/diseased tissues and organs such as hypoxia, inflammation, and accumulation of toxic metabolic waste products. This is best exemplified by the transplantation of DPSCs for dental pulp regeneration (3). Unlike other organs or tissues, the pulp chamber of teeth has a limited vascular connection through small openings (apical foramen) within the root canal (5). This lack of vascular exchange creates an unfavorable environment within the root canal space that is not conducive to graft survival. Indeed, some studies have shown low graft survivability upon implantation of DPSCs within the root canal space, with most of the cell death being attributed to apoptosis (6).

One strategy to enhance the survivability of grafted DPSCs could be recombinant overexpression of antiapoptotic genes. Of particular interest is the B-cell lymphoma 2 gene (Bcl2), a proto-oncogene that was first identified in human follicular lymphoma by Tsujimoto et al (7). Subsequent studies showed that the expression of this gene can prevent apoptosis and some forms of necrotic cell death (8, 9). Recombinant overexpression of the Bcl2 protein can be used to target diseases associated with increased apoptosis. For example, in an ischemic heart model, recombinant overexpression of Bcl2 has been shown to reduce cardiomyocyte death (10). Similar

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results were reported in an inherited condition of cardiomyopathy (11). Bcl2 has also been reported to exert a neuroprotective effect in focal cerebral ischemic injury (12). Hence, recombinant Bcl2 overexpression could be a promising strategy to enhance the survivability of grafted cells. Nevertheless, it is unknown whether recombinant Bcl2 overexpression could alter the proliferation, apoptosis, and differentiation potential of DPSCs.

Therefore, this study investigated the effects of recombinant overexpression of Bcl2 on the proliferation and apoptosis of DPSCs within *in vitro* culture. Additionally, the effects of recombinant Bcl2 overexpression on the osteogenic/odontogenic differentiation potential of DPSCs were also examined. The data obtained will be useful for evaluating whether recombinant Bcl2 overexpression could be a viable therapeutic strategy for tissue regeneration with transplanted DPSCs.

Materials and Methods

Culture Supplements, Media, Chemical Reagents, and Other Consumables

Unless explicitly stated otherwise, all culture supplements, serum, and media were purchased from Life Technologies (Carlsbad, CA). All labware consumables were purchased from Becton-Dickinson (Franklin Lakes, NJ), whereas all chemical reagents were purchased from Sigma-Aldrich (St Louis, MO).

Isolation and Expansion of Human DPSCs

All experiments using cells derived from human patients were reviewed and approved by the institutional review board of the University of Hong Kong, Pokfulam, Hong Kong. DPSCs were isolated from the extracted third molars of human dental patients between 18 and 25 years of age after obtaining informed consent. After cleaning the surfaces of the freshly extracted tooth, the pulp chamber was exposed by using a sterile fissure bur to cut at the cementum to the enamel junction. The pulp tissue was gently separated from the crown and root, and cell dissociation was achieved through enzymatic digestion in a 4 mg/mL dispase and 3 mg/mL collagenase type I solution for 1 hour at 37°C. Subsequently, a single-cell suspension was obtained by passing the digested pulp tissue through a 70-mm strainer. DPSCs from 1 donor were used. Some of the cells were subjected to flow cytometry analysis of the expression of typical adult stem cell markers (CD73, CD90, CD105, STRO-1, and CD45) as well as multilineage differentiation assays with osteo-/odontogenic, adipogenic, and neurogenic induction media as described previously (13). The "stemness" of the putative DPSCs was confirmed by positive results obtained from these various analyses, as reported previously (13). The remaining cells not subjected to these analyses were routinely passaged and expanded in 75-cm^2 culture flasks with alpha minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin antibiotic solution. Fresh culture media was replenished every 3 to 4 days, and confluent monolayers were dissociated with 0.5% (w/v) trypsin-EDTA for subculture. A humidified 5% CO_2 incubator was used for cell culture at 37°C.

Cell Transfection

The DPSCs were transfected with the Bcl2 and/or GFP (green fluorescent protein) gene through a lentiviral expression system (GenTarget Inc, San Diego, CA). DPSCs at 50% confluence within 24-well culture plates were incubated with 50 μL 1 $\times$ 10^8 infectious unit (IFU)/mL lentiviral particles per well, which carried human Bcl2, GFP, and antipuromycin genes. For simplicity, these cells would be referred to as Bcl2-transfected DPSCs. At the same time, another group of cells were transfected with lentiviral particles carrying only GFP and antipuromycin genes, which served as the control in future experiments. For simplicity, the control cells would be referred to as GFP-transfected DPSCs. Because the yield of the transfection process is not expected to be 100%, puromycin was added into the culture medium to cull non-transfected cells. The puromycin concentration required was determined from the antibiotic's killing curve, which was obtained by a pilot study. One month after lentiviral transfection, the puromycin-selected transgenic cells were subjected to quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analyses of Bcl2 expression.

WST-8 Assay of Cell Density of DPSCs within *In Vitro* Culture

DPSCs were seeded at a fixed seeding density of 2000 cells/cm^2 on 12-well plates (4.8 cm^2 per well) and cultured for a period of 11 days within a humidified 5% CO_2 incubator set at 37°C. The culture medium used was α-MEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin antibiotic solution. On days 1, 3, 5, 7, 9, and 11, the density of viable cells within each well was quantified with the water soluble tetrazolium salt-8 (WST-8) assay (CCK-8 Kit; Dojindo Molecular Laboratories Inc, Kumamoto, Japan). The kit uses WST-8 reagent, and the cells were incubated for 2 hours with 10% (v/v) WST-8 reagent supplemented in culture media (250 μL/well of a 12-well plate) before absorbance readings at 450 nm with a SpectraMAX 340 microplate reader (Molecular Devices, Sunnyvale, CA). Densities of adherent viable cells were calculated from standard curves. Cell density indices on days 3, 5, 7, 9, and 11 were calculated as a ratio of cell density on that particular day to that of the control (GFP-transfected DPSC) on day 1.

Bromodeoxyuridine Incorporation Assay for Assessment of Mitotic Activity

The Bcl2 and GFP-transfected DPSCs were subjected to the bromodeoxyuridine (BrdU) incorporation assay to compare the mitotic rate of these cells. The BrdU-based cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche GmbH, Heidelberg, Germany) was used according to the manufacturer's instructions. The 2 groups of cells were seeded into 96-well plates at a density of 2 $\times$ 10^4 cells/well. Twenty-four hours later, 10 μL BrdU solution was added into each well, which contained 100 μL culture medium. The cells were then incubated for 37°C for 4 hours to allow incorporation of the BrdU into the cellular DNA. Subsequently, the cells were fixed, and DNA was denatured with the fixative (FixDenat, Roche GmbH) that was provided by the kit. Antibody specific to BrdU was then added into each well, and a substrate solution was used to detect the immune complexes that developed. A colored solution was produced, and the absorbance was measured at a wavelength of 370 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Assessment of Cellular Apoptosis through Quantitation of Cytoplasmic Histone–associated DNA Fragments

Apoptosis of the transfected groups (Bcl2 and GFP and GFP only) was assessed with a Cell Death Detection ELISA PLUS Kit (Cat. No. 11774425001; Roche, Basel, Switzerland), which is based on quantitation of cytoplasmic histone–associated DNA fragments upon the induction of apoptosis. The 2 groups of cells were seeded into T75 flasks and cultured until approximately 50% confluent. The culture medium (α-MEM with supplements and FBS) was then replaced with a serum-free medium (α-MEM with supplements without FBS) to induce apoptosis through serum starvation. This is because under normal culture conditions in the presence of serum, the proportion of apoptotic