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Kaempferol slows intervertebral disc degeneration by modifying LPS-induced osteogenesis/adipogenesis imbalance and inflammation response in BMSCs

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ABSTRACT

Intervertebral disc (IVD) degeneration is a common disease that represents a significant cause of socio-economic problems. Bone marrow-derived mesenchymal stem cells (BMSCs) are a potential autologous stem cell source for the nucleus pulposus regeneration. Kaempferol has been reported to exert protective effects against both osteoporosis and obesity. This study explored the effect of kaempferol on BMSCs differentiation and inflammation. The results demonstrated that kaempferol did not show any cytotoxicity at concentrations of 20, 60 and 100 μM. Kaempferol enhanced cell viability by counteracting the lipopolysaccharide (LPS)-induced cell apoptosis and increasing cell proliferation. Western blot analysis of mitosis-associated nuclear antigen (Ki67) and proliferation cell nuclear antigen (PCNA) further confirmed the increased effect of kaempferol on LPS-induced decreased viability of BMSCs. Besides, kaempferol elevated LPS-induced reduced level of chondrogenic markers (SOX-9, Collagen II and Aggrecan), decreased the level of matrix-degrading enzymes, i.e., matrix metalloprotease (MMP)-3 and MMP-13, suggesting the osteogenesis of BMSC under kaempferol treatment. On the other hand, kaempferol enhanced LPS-induced decreased expression of lipid catabolism-related genes, i.e., carnitine palmitoyl transferase-1 (CPT-1). Kaempferol also suppressed the expression of lipid anabolism-related genes, i.e., peroxisome proliferators-activated receptor-γ (PPAR-γ). The Oil red O staining further convinced the inhibition effect of kaempferol on BMSCs adipogenesis. In addition, kaempferol alleviated inflammatory by reducing the level of pro-inflammatory cytokines (i.e., interleukin (IL)-6) and increasing anti-inflammatory cytokine (IL-10) via inhibiting the nucleus translocation of nuclear transcription factor (NF)-κB p65. Taken together, our research indicated that kaempferol may serve as a novel target for treatment of IVD degeneration.

1. Introduction

Intervertebral discs (IVDs) is composed of nucleus pulposus, annulus fibrosus and cartilage endplates [1]. The function of IVDs is to provide stability and flexibility to the spinal column [2]. However, under the condition of cell viability decrease, attenuation of proteoglycan and type II collagen synthesis, and dehydration of nucleus pulposus, IVD degeneration has been considered an irreversible process [3]. IVDs degeneration has also been considered as a significant contributor to the development of low back pain [4]. Adult stem cells have shown promise as a cell source for a variety of tissue engineering and cell therapy applications. Current preclinical studies show that mesenchymal stem cells (MSCs) have the capacity to repair degenerative discs by differentiation toward chondrocyte-like cells, which produce proteoglycans and type II collagen [5], convincing that MSCs transplantation help decrease IVDs degeneration.

The term MSCs refers to similar cell populations from various tissues that share the basic features of stem cells, but remain heterogeneous cells with variations among individual donors or clones [6]. MSCs are able to secrete a variety of soluble mediators and to be recruited chemotactically to injured tissues [7]. Adult MSCs possess the ability to self-renew, proliferate and differentiate into multiple mature cell types with immunomodulatory properties [8,9]. Numerous in vitro or in vivo studies have investigated the use of MSCs for the treatment of IVDs degeneration, for the reason that MSCs can differentiate into chondrocyte-like cells phenotypically similar to nucleus pulposus (NP) cells in chondrogenic conditions [10–12]. To be specific, MSCs may help improve IVD degeneration by secretion of growth factors and immuno-

Abbreviations: IVD, intervertebral disc; BMSCs, mesenchymal stem cells; LPS, lipopolysaccharide; PCNA, proliferation cell nuclear antigen; MMP, matrix metalloprotease; CPT-1, carnitine palmitoyl transferase-1; PPAR-γ, peroxisome proliferators-activated receptor-γ; IL, interleukin; NF-κB, nuclear transcription factor-κB; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; ACC, acetyl coenzyme A carboxylase; SREBP-1c, sterol regulatory element binding proteins-1c; FAS, fatty acid synthetase.

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modulate the inflammatory response. More importantly, MSCs can provide the lost or damaged cells, through directing differentiation into disc tissue-specific cells which contribute to the formation of extracellular matrix (ECM) [13]. Report indicated that MSCs can be readily harvested from multiple sites, and they are the only stem cell type that have been transplanted in human disc proving to be safe and being able to reduce LBP in patient [14].

Considering osteoblasts and adipocytes are derived from a common multipotential MSCs progenitor, the imbalance between osteogenesis and adipogenesis differentiation of MSCs has been suggested in osteoporosis [15,16]. The decrease in osteoblastogenesis associated with an increasing bone marrow adipogenesis has also been reported in IVD degeneration [17]. Nowadays, several agents, such as nutrients or metabolic signals, possess the ability to switch MSCs differentiation toward osteoblast formation [16]. Thus, novel regulator in the balance between osteogenesis and adipogenesis of MSCs may be a novel therapeutic target.

Flavonoids, small polyphenolic molecules commonly found in roots, leaves and fruits of dietary plants, have well-known antioxidant, anti-inflammatory, anticancer and antibacterial activities. Kaempferol ($C_{15}H_{10}O_6$), a kind of flavonol, which has been applied in traditional medicine in Asia for the treatment of hypertension, abdominal pain, headaches and rheumatism. Kaempferol has been reported to possess various osteogenic biological activities, such as inhibiting osteoclastogenesis [18] and inducing chondrogenesis [19]. However, the effect of kaempferol on regulating osteogenesis/adipogenesis balance of MSCs has been seldomly reported.

This study isolated bone MSCs (BMSCs) from New Zealand rabbits and treated with kaempferol at different concentrations. We found that kaempferol counteracted lipopolysaccharide (LPS)-induced decreased viability of BMSCs and inhibited LPS-induced matrix degradation, anabolism and lipid accumulation, and may through suppressing LPS-induced activation of the nuclear transcription factor (NF)-κB pathway in BMSCs.

2. Materials and methods

2.1. Animal ethics

New Zealand rabbits were euthanized via the abdominal injection a lethal dose of pentobarbital sodium. All of the animal work was conducted according to relevant national and international guidelines and was approved by the Animal Experimental Ethical Committee of the first people's Hospital of Huaihua.

2.2. BMSCs isolation and culture

BMSCs isolation and culture were conducted according to a previous report [20]. Briefly, Two New Zealand rabbits (female, 2 months) were euthanized and bone marrow solution was purified by density gradient centrifugation and adherence screening method. BMSCs were then incubated in phosphate buffered solution (PBS), supplemented with 2.5 g/l trypsin (3.0 ml), and placed in an incubator at 37 °C for two or 3 min. After digestion, BMSCs were incubated in serum-free L-DMEM, and placed in a plastic culture flask at 1.0×10^8/l. BMSCs was purified by density gradient centrifugation and adherence screening method. Cell morphology was observed under inverted microscope. The third passage of cells was used for the following experiments.

2.3. Cell viability assay

Kaempferol was purchased from Sigma (St. Louis, MO, USA), the purity of kaempferol was ≥95.0% by HPLC. The optimal dose was identified using multiple concentrations of kaempferol (20, 40, 60, 80, 100, 120, 140 μM) added in BMSCs culture medium and analyzed by CCK8. Higher concentrations (120 and 140 μM) reduced the viability of cells. Three concentrations of kaempferol (20, 60, 100 μM) were then selected for the following experiments. LPS at a dose of 10 μg/ml has been reported to induce inflammation and matrix degradation in IVD degeneration in many reports [21–23], thus, BMSCs in this study was induced by LPS at 10 μg/ml. A commercial kit (Cell Counting Kit-8, CCK-8; Dojindo) was used to evaluate the potential cytotoxic effect of kaempferol. BMSCs were plated in 96-well plates at a density of 6×10^3 per well, incubated with various concentrations of kaempferol (20, 60, 100 μM) for 2 h and stimulated with 10 μg/ml LPS for 24 h, and then administered 10 ml of CCK-8 solution and incubated for an additional 2 h. CCK8 kit was also used to measure the effect of kaempferol on cell growth in LPS-induced BMSCs. The surviving fractions were determined on day 1, 2, 3, 4 and 5. The optical density (OD) of each well was measured at 470 nm. The culture medium was used as a blank. Cell viability was calculated as follows: cell viability D [OD (with cordycepin) − OD (blank)]/[OD (without cordycepin) − OD (blank)].

2.4. Flow cytometric assay

Cell apoptosis was detected by the Annexin V Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, the medium containing the floating cells was collected, and the attached cells were washed and trypsinized. The floating and trypsinized cells were collected in the same centrifuge tubes. After centrifugation and washing, the cell pellets were fixed in cold 70% ethanol. The fixed cells were stained with propidium iodide (PI) and PE-Annexin V. Cell apoptosis percentage was reflected by Annexin V/PI ratio, detected by a flow cytometry machine (BD Bioscience, Shanghai, China).

2.5. Western blotting

Whole-tissue protein extracts were prepared by dissolving BMSCs in RIPA buffer containing protease inhibitors (cOmplete, Mini, EDTA-free; Roche, Welwyn Garden City, United Kingdom). Protein output was quantified using the BCA Protein Quantification Kit (Thermo scientific, USA). 25 μg of total protein in each sample was separated by 12% SDS-PAGE and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA) for immunoblotting. The following primary antibodies were used: *anti*-Ki67, *anti*-proliferating cell nuclear antigen (PCNA), *anti*-SOX-9, *anti*-Collagen II, *anti*-Aggrecan, *anti*-MMP-3, *anti*-MMP-13, anti- (a disintegrin and metalloproteinase with thrombospondin motifs) ADAMTS-4, *anti*-ADAMTS-5, *anti*-carnitine palmitoyltransferase-1 (CPT-1), *anti*-peroxisome proliferators-activated receptor (PPAR)-α, *anti*-acetyl coenzyme A carboxylase (ACC), *anti*-sterol regulatory element binding proteins-1c (SREBP-1c), *anti*-fatty acid synthetase (FAS), *anti*-PPAR-γ, *anti*-NF-κB p65, *anti*-*p*-NF-κB p65 and *anti*-GAPDH, which was used as the internal reference. After incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody, and were visualized with chemiluminescence (AlphaImager 2200).

2.6. Oil red O staining

The Oil Red O stock solution consisted of 0.5% Oil Red O in isopropyl alcohol. The working Oil Red O solution was prepared by adding 4 volumes of water to 6 volumes of Oil Red O stock solution. After treatment with sodium palmitate as described above, BMSCs were washed twice in phosphate-buffered saline (PBS), fixed for 30 min with 4% formaldehyde, and then stained with freshly diluted Oil Red O solution for 40 min at room temperature. The cells were then washed with 60% isopropyl alcohol once and with water twice. The nuclei of cells were counterstained with hematoxylin. The number of Oil Red O-positive cells was counted under a bright-field microscope in 10 replicates of 10 fields at 209 magnification and averaged.