Food and Chemical Toxicology 62 (2013) 238–245

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Kaempferol induces chondrogenesis in ATDC5 cells through activation of ERK/BMP-2 signaling pathway

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ARTICLE INFO

Article history:
Received 25 February 2013
Accepted 14 August 2013
Available online 26 August 2013

Keywords:
Kaempferol
Chondrogenesis
MAP kinases
BMP-2
ATDC5 cells

ABSTRACT

Endochondral bone formation occurs when mesenchymal cells condense to differentiate into chondrocytes, the primary cell types of cartilage. The aim of the present study was to identify novel factors regulating chondrogenesis. We investigated whether kaempferol induces chondrogenic differentiation in clonal mouse chondrogenic ATDC5 cells. Kaempferol treatment stimulated the accumulation of cartilage nodules in a dose-dependent manner. Kaempferol-treated ATDC5 cells stained more intensely with alcian blue staining than control cells, suggesting greater synthesis of matrix proteoglycans in the kaempferol-treated cells. Similarly, kaempferol induced greater activation of alkaline phosphatase activity than control cells, and it enhanced the expression of chondrogenic marker genes, such as collagen type I, collagen type X, OCN, Runx2, and Sox9. Kaempferol induced an acute activation of extracellular signal-regulated kinase (ERK) but not c-jun N-terminal kinase or p38 MAP kinase. PD98059, an inhibitor of MAPK/ERK, decreased in stained cells treated with kaempferol. Furthermore, kaempferol greatly expressed the protein and mRNA levels of BMP-2, suggesting chondrogenesis was stimulated via a BMP-2 pathway. Taken together, our results suggest that kaempferol has chondromodulating effects via an ERK/BMP-2 signaling pathway and could potentially be used as a therapeutic agent for bone growth disorders.

1. Introduction

Chondrogenesis is a multi-step cellular process that leads to differentiation of mesenchymal cells into chondrocytes during the growth and development of vertebrates. This process includes cell migration, aggregation, condensation, and chondrocyte differentiation (Cancedda et al., 1995; Hall et al., 2000). Through a sequential process of cell proliferation, extracellular matrix synthesis, cellular hypertrophy, matrix mineralization, vascular invasion, and eventually, apoptosis, cartilage is continually replaced by bone at the end of chondrogenesis (Shum and Nuckolls, 2002). The regulation of longitudinal growth at the growth plate generally occurs through tight interactions between circulating systemic hormones and locally-produced peptide growth factors, the net result of which is to trigger changes in gene expression in growth plate chondrocytes (Adams et al., 2007). Many transcription factors, such as Runx2 and Sox9, play an important role in the complex process of chondrogenesis (Lefebvre et al., 1998; Akiyama et al., 2002; Yoshida et al., 2004). The transcription factor Sox9, which contains an SRY-related high-mobility group box, promotes the differentiation of mesenchymal cells into chondrocytes and induces the expression of Col2α1 and Col2α2 aggrecan in the chondrocytes (Lefebvre et al., 1998; Akiyama et al., 2002). Runx2 is essential for osteoblast differentiation and is involved in chondrocyte maturation (Yoshida et al., 2004).

Since ancient times, people in Asian countries have used a variety of plant-derived substances to treat disease. In a previous study, we assessed whether Genkwadaphnin stimulates chondrogenesis in ATDC5 cells (Choi et al., 2011). Kaempferol is a flavonoid that is relatively abundant in plants, (Miean and Mohamed, 2001) including tea, cruciferous vegetables, grapefruit, Gingko biloba, and some edible berries (An et al., 2011; Hakkinen et al., 1999; Nirmala and Ramanathan, 2011)(Fig. 1). It is unknown whether kaempferol has chondrogenic effects, though previous studies have reported that it has anti-oxidative, (Lee et al., 2010) anti-inflammatory, (Crespo et al., 2008) anti-hypertensive, (Loizzo et al., 2007) lipotytic (da-silva et al., 2007) and anti-cancer effects (Kang et al., 2010; Mylonis et al., 2010; Zhang et al., 2008). Although kaempferol at high doses (⩾30 μM) exerts cytotoxic effects on several types of cancer cells, (Huang et al., 2010; Marfe et al., 2009) it has no such effect at these concentrations on a variety of normal cells (Sharma et al., 2007) which suggests that dietary intake of kaempferol does not have significant side effects.

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http://dx.doi.org/10.1016/j.fct.2013.08.034

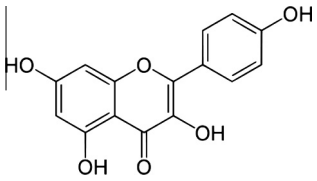

Fig. 1. Structure of Kaempferol.

In the present study, we investigated the chondrogenic-stimulatory effects of kaempferol on ATDC5 cells in vitro. We found that kaempferol induced the synthesis of matrix proteoglycans and the expression of other chondrogenic markers, such as collagen type II, collagen type X, osteocalcin (OCN), Runx2, Sox9, and the chondrogenic signaling molecules, BMP-2 and BMP-4. Kaempferol also induced MAP kinase activation, suggesting that chondrogenesis was stimulated through a BMP-2/ERK pathway in our in vitro experiment. We suggest that kaempferol could potentially be used as a therapeutic agent for the treatment of skeletal disorders.

2. Materials and methods

2.1. Reagents and chemicals

PD 98059 and SP 600125 were purchased from Calbiochem (San Diego, CA, USA). Cell culture medium and fetal bovine serum (FBS) were obtained from Invitrogen (Gaithersburg, MD, USA). PCR primers were purchased from Bioneer (Daejeon, Korea). All other chemicals were purchased from Sigma (St. Louis, MO, USA), and unless otherwise indicated, they are the same as those described elsewhere (Nepal et al., 2011; Choi et al., 2011).

2.2. Cell culture and treatment

Pre-chondrogenic ATDC5 cells were purchased from the RIKEN Cell Bank (Ibaraki, Japan). ATDC5 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (Invitrogen, Gaithersburg, MD, USA) and Ham's F-12 medium (Invitrogen) containing 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). This medium was replaced with a medium containing 10 μg/ml transferrin, 3×10^{-8} M sodium selenite, and 5 μg/ml bovine insulin to induce cell differentiation, and the cells were cultured at 37 °C for different time periods (up to 21 days) with 5% CO_2. In this study, we used kaempferol instead of bovine insulin to induce chondrogenic differentiation of ATDC5 cells. The culture medium was changed every 2–3 days.

2.3. Alcian blue staining

ATDC5 cells were cultured for up to 21 days. Cells were rinsed twice with phosphate buffered saline (PBS), fixed with 95% methanol, and then stained for 16 h with 1% Alcian blue 8GS (Sigma, St. Louis, MO, USA). Cells were washed with 3% acetic acid for 30 s three times, and then photographed. Stained cells were dissolved in 10% acetic acid for subsequent quantification of the absorbance at 650 nm (Shukunami et al., 1997).

2.4. Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNAs were isolated from cultured cells using TRIzol (Invitrogen), and cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) in accordance with the manufacturer's protocol. The primer sequences and PCR conditions used in this study are listed in Table 1. After an initial denaturation step at 95 °C for 1 min, PCR was performed for various cycles (30 s at 94 °C, 1 min at the primer-pair specific annealing temperature, and 2 min at 72 °C) using Taq polymerase (Promega, Madison, WI, USA). Reaction products (10 μl) were separated on a 0.8% agarose gel stained with ethidium bromide, and analyzed densitometrically. Band intensity was analyzed by densitometry using a phosphoimager and Quantity One version 4.3.1 software (Bio-Rad, Hercules, CA, USA).

2.5. Alkaline phosphatase (ALP) activity

Cells were harvested and re-dissolved in lysis buffer [20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 × protease inhibitor cocktail]. After centrifugation at 16000*g* for 15 min, the supernatant was collected as the cell extract. Cell extracts were assayed in a reaction mixture containing pNPP with Alkaline Phosphatase Yellow Liquid Substrate (Sigma) in accordance with the manufacturer's instructions.

2.6. MTT assay

Cells (5×10^3 cells) were seeded in a 96-well plate with medium supplemented with 10% FBS and incubated for 24 h with various concentrations of kaempferol or insulin. The cells were incubated for a further 46 h, washed with PBS, and then treated with a medium containing 100 μg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 h at 37 °C. The cells were then washed with PBS, and dissolved in 200 μl of DMSO. The resulting intracellular purple formazan was quantified using a spectrophotometer that measured the absorbance at a wavelength of 540 nm.

2.7. Immunoblot analysis

Cell extracts were separated by 8–10% SDS–PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS) containing 0.25% Tween-20 (TTBS) at room temperature for 1 h and then incubated for 16 h at 4 °C with rabbit anti-phospho ERK (Cell Signaling Technology Inc., Beverly, MA, USA),

Table 1
Primer sequences and conditions for RT-PCR.

Target genes	Primers forward/reverse	Strain	PCR condition		
			Tm (°C)	Cycles	Size (bp)
Collagen II	5′-ctgtaagaacagcatcgcctacctg-3′ 5′-caggaatttggtgtggacataggg-3′	Mouse	60	27	271
SOX9	5′-cacaagaaagaccaccccgatac-3′ 5′-ggcaaagttgatctgaagcgaga-3′	Mouse	60	32	272
Collagen X	5′-cgtctctgcttttactgtca-3′ 5′-ctcacagaaaatgaccaggt-3′	Mouse	48	35	300
Runx2	5′-actttctccaggaagactgc-3′ 5′-acagcaacagcaacaacagc-3′	Mouse	50	35	366
OCN	5′-cagcttggtgcacacctagc-3′ 5′-ggagcagtgtcacgttaacct-3′	Mouse	58	30	242
GAPDH	5′-accacagtccatgccatcac-3′ 5′-tacagcaacagggtggtgga-3′	Mouse	56	25	452
BMP2	5′-gttccacaaacgagaaaagc-3′ 5′-agcaaggggaaaaggacact-3′	Mouse	54	25	379
BMP4	5′-ctgctcttcctcctcctcct-3′ 5′-tgatacctgagaccgggaag-3′	Mouse	54	25	382
B-actin	5′-ttctacaatgagctgcgtgt-3′ 5′-ctcatagctcttctccaggg-3′	Mouse	60	25	456