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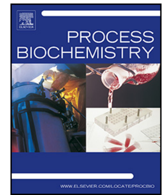

L-glutamine regulates the expression of matrix proteins, pro-inflammatory cytokines and catabolic enzymes in interleukin-1beta-stimulated human chondrocytes

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ABSTRACT

Osteoarthritis (OA) of the knee is a major cause of mobility impairment. A number of studies demonstrated the therapeutic effect of L-glutamine (Gln) on different cells and tissues. However, little research has addressed the potential use of Gln on the treatment of OA. In this study, the regulation of Gln on OA-related mRNA expression and cytokine production in chondrocytes was evaluated. Human chondrocytes were stimulated with interleukin-1β (IL-1β) and subsequently cultured in Gln-supplemented medium. The results showed that Gln prevented the hypertrophic transformation and promoted the proliferation rate of IL-1β-stimulated chondrocytes. Gln also downregulated the mRNA expressions of type I collagen, IL-1β, and tumor necrosis factor-alpha (TNF-α) in stimulated cells. In addition, Gln restored the aggrecan and upregulated the TIMP-1 expressions. However, the matrix metalloproteinase-3 (MMP-3) and MMP-9 levels were highly expressed in IL-1β-stimulated chondrocytes and Gln treatment did not show any effects. Although Gln decreased the protein productions of IL-1β and TNF-α, the MMP-3 still highly produced. However, the production of tissue inhibitor of metalloproteinase-1 significantly increased. Our results suggest that Gln possesses anti-inflammatory and structure-protective properties in terms of preventing the hypertrophic transformation, regulating the productions of matrix proteins and catabolic enzymes in human chondrocytes.

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1. Introduction

Osteoarthritis (OA) is the leading cause of chronic pain and physical disability among adults [1]. Because of the limited capacity for self-regeneration, injured articular cartilage may lead to progressive degeneration which represented in narrowed joint space and could be observed under radiology frequently. Currently, conservative treatments for OA include weight loss, therapeutic exercise, activity modification, and assistive devices. Oral medications such as acetaminophen, non-steroidal anti-inflammatory drugs, and opioids are used [2,3]. Intra-articular injection of corticosteroid has also been widely accepted [4]. However, the clinical practice of corticosteroid is limited because of articular cartilage damage, secondary infection, and systemic side effects with repeated injections. Even though other treatments including analgesics, glucocorticoids, and exogenous viscosupplementations are available, none of them has been shown to reverse progression of OA [5].

L-Glutamine (Gln) is synthesized by the enzyme glutamine synthetase from glutamate and ammonia [6]. In human blood, Gln

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is the most abundant free amino acid with a concentration of about 500–900 μmol/L, which plays an important role in regulating various metabolic activities, genes expression, and protein activations [7,8]. Recent studies indicated that Gln has therapeutic effects on various cells and tissues. Erbil et al. found that Gln can preserve intestinal mucosal structure and decrease pancreatic inflammation in rats that received abdominal irradiation [9]. Evans et al. reported that Gln prevents human colonic epithelial cells from tumor necrosis factor-alpha (TNF-α) induced apoptosis [10]. Moreover Peng et al. observed that Gln maintains immunological function in severely burned patients [11].

A few studies also found that Gln has chondroprotective effects. An animal study revealed that Gln protects chondrocytes from apoptosis which induced by heat stress and nitric oxide [12]. Another interesting study found that intra-articular injection of a nutritive mixture solution (including Gln and other amino acids) protects articular cartilage from osteoarthritic progression in rabbits [13]. However, most of these studies employed animal models or immortalized cell-lines. The effects of Gln on human subject are still unclear. In this study, we hypothesize that Gln may prevent OA progression through anti-inflammation effects. The purpose of this study is to test the regulations of Gln to OA-related cytokines in human chondrocytes culture.

2. Materials and methods

2.1. Patient selection and cartilage sample collection

Nine patients (five females and four males) with OA knees were enrolled in this study. The average age of patients was 67.5 years (range, 58–74). In all patients, OA severity was grade 4 at the medial compartment and grade 2 or less at the lateral compartment of the knee (Kellgren–Lawrence grading scale) [14]. All patients underwent total knee replacement surgery (Fig. S1A and B). Articular cartilage samples were collected from the lateral compartment of the knee (Fig. S1C and D). Ethical approval was provided by the institution's ethics committee (DMR100-IRB-270 and ECKIRB1030502).

2.2. Isolation, culture, and identification of human chondrocytes

The harvested cartilages were stored in a container at 4 °C and immediately transported to the laboratory (ischemia time <1 h). The tissues were sectioned from the cartilages and minced as 1 mm × 1 mm fragments using a scalpel. The tissues were treated with 1% type II collagenase (C6885, Sigma–Aldrich) overnight in an incubator at 37 °C and 5% CO_2. Digested tissues were then collected, washed with phosphate-buffered saline (PBS), and filtered through a nylon cell strainer to obtain single-cell suspensions. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, SH30003.01, Hyclone, UT) supplemented with 50 μg/mL L-ascorbic acid (A5960, Sigma–Aldrich), 10% fetal bovine serum (FBS, 100–106, Gemini Bio-Products, USA), and 1% antibiotic (P4083, Sigma–Aldrich, MO). After 3 days, non-adherent cells were washed out with PBS, and the adherent cells were cultured for cell proliferation. The culture medium was changed every 3 days.

The proliferative chondrocytes (P1–P3) were identified by immunohistochemical (IHC) staining before further experiments. The cells obtained from each donor were used independently. Cells were fixed with ice-cold methanol for 10 min, washed twice with PBS, and blocked with 1% bovine serum albumin for 30 min at room temperature to block non-specific binding. The samples were incubated with anti-type II collagen antibody (ab34712, Abcam, Cambridge, UK) in a humidified chamber at 4 °C overnight. Antigen was detected with Chromeo™ 488 (ab60314, Abcam, Cambridge, UK). Negative controls were processed in the same manner, except that rabbit IgG isotype antibody was used instead of the primary antibody.

2.3. Cytotoxicity assays of glutamine

The cytotoxicity of Gln was determined by water-soluble tetrazolium salt-1 assay (Cell Proliferation Reagent WST-1, Roche, Mannheim, Germany) and lactate dehydrogenase (LDH, CytoTox 96® NonRadioactive Cytotoxicity Assay, G1780, Promega, WI, USA) assays. Chondrocytes were seeded in 96-well culture plates at a density of 5×10^3 cells/well and cultured in normal DMEM for 24 h. After incubation with various concentrations of Gln (0, 1, 3, 5 and 10 mM, 25030-081, Gibco) for additional 24 h, the culture media were removed and cells were washed with PBS. Finally, chondrocytes were cultured in serum-free DMEM containing 10% WST-1 reagent for 4 h to evaluate viability. The result of WST-1 assay was measured by a spectrophotometer (Multiskan™ GO Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 460 nm. For the LDH assay, the removed media were centrifuged at 10,000 rpm at 4 °C, and the supernatant was reacted with the LDH assay. The results of the LDH assay were determined using a spectrophotometer at a wavelength of 490 nm.

2.4. IL-1β stimulation and medium supplementation with glutamine

Chondrocytes were seeded in six-well culture plates at a density of 1×10^5 cells per well and cultured in normal DMEM. When the confluence of the cells achieve 60–70%, cells were stimulated with 1 ng/mL interleukin-1beta (IL-1β, 4128-10, BioVision, CA) for 24 h. The medium was then removed, washed twice with PBS, and subsequently cultured in normal medium or containing 3 mM Gln (based on the results of cytotoxicity of Gln) for 3 days. Non-stimulated cells were also cultured in normal DMEM or Gln-supplemented medium for the same period.

2.5. Morphology, proliferation, and GAG production of chondrocytes

The morphology of IL-1β treated and untreated chondrocytes cultured in normal and Gln-supplemented media was examined using an optical microscope. The cells were then detached, and cell number was counted at day 3. Total DNA of the cells was extracted using a DNeasy Blood and Tissue kit (69504, QIAGEN, Germany). The amount of DNA was determined using a nanodrop (NanoQ, Optizen, Korea) spectrophotometer.

Toluidine blue staining was used to detect the production of GAGs after IL-1β stimulation and culture in Gln-supplemented medium. Chondrocytes on culture plates were washed with PBS, fixed in neutral buffered 10% formalin solution, stained with toluidine blue, and examined under an optical microscope. The GAG content was also evaluated quantitatively by using a 1,9-dimethyl-methylene blue (DMMB, 341088, Sigma–Aldrich) assay. Cells cultured under different conditions were digested in papain solution (P4762, Sigma–Aldrich) at 60 °C for 16 h, and the digested sample was reacted with DMMB reagent. The chondroitin sulfate sodium salt (C4384, Sigma–Aldrich) was used to establish a standard curve with sequential dilution. Absorbance was detected at a wavelength of 595 nm by a microplate reader. The GAG content was normalized based on the cell number for each group.

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