Promotion of G1/S Transition and Inhibition of Inflammatory Cytokine Production by Hydroxypyridinone-Coumarin in Osteoarthritis Rats

Kai Yang, Gang Chen, Xiongxun Wang

Background: Osteoarthritis is a joint disorder characterized by articular cartilage degradation leading to joint stiffness and pain. The present study investigated the effect of hydroxypyridinone-coumarin on proliferation of chondrocytes.

Material/Methods: Chondrocyte proliferation was assessed by MTT assay, and distribution of cells in various phases of the cell cycle was determined using flow cytometry. RT-PCR and Western blot assays were used for assessment of mRNA and protein levels, respectively. Osteoarthritis was induced in the rats by injecting monosodium iodoacetate (5 mg/kg) by the intra-articular route. The rats in the treatment groups were intraperitoneally injected with 5, 10, or 15 mg/kg doses of hydroxypyridinone-coumarin alternately for 1 month.

Results: The proliferation of chondrocytes was increased significantly (P<0.05) by treatment with hydroxypyridinone-coumarin in a concentration-based manner. The increase in chondrocyte proliferation by hydroxypyridinone-coumarin was maximum at 50 µM. Treatment with hydroxypyridinone-coumarin markedly increased chondrocyte population in S and G2/M phases, with subsequent reduction in G0/G1 phase. The cyclin D1, CDK4, and CDK6 levels in the chondrocytes were increased by treatment with hydroxypyridinone-coumarin. The production of IL-6, TNF-α, and IL-1β in the osteoarthritis rats was markedly suppressed by hydroxypyridinone-coumarin. Treatment of the OA rats with hydroxypyridinone-coumarin markedly reduced the expression of IκB-α and NF-κB p65.

Conclusions: The present study revealed that the proliferative potential of chondrocytes is increased by hydroxypyridinone-coumarin through acceleration of G1/S transition. Moreover, hydroxypyridinone-coumarin treatment reduced inflammatory cytokine production in the osteoarthritis rats. Therefore, hydroxypyridinone-coumarin should be evaluated further for possible use in the treatment of osteoarthritis.

MeSH Keywords: Anti-Inflammatory Agents • Chondrocytes • Cytokines

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Background

Osteoarthritis is a disorder of joints characterized by the degeneration of articular cartilage leading to stiffness of joints and acute pain [1,2]. Various factors, including age, trauma, obesity, and genetics, have been found to be responsible for the development of osteoarthritis [3]. The pathogenesis of osteoarthritis is not fully understood [3]. The clinical symptoms of early-stage osteoarthritis include inflammation, edema, joint stiffness, and severe pain [4]. The secondary stage of osteoarthritis leads to difficulty in movement and severely affects quality of life [4]. The small percentage of non-proliferating chondrocytes present in the adult articular cartilage secretes extracellular matrix [5]. The 2 major components of extracellular matrix are collagens and proteoglycans. Tissue homeostasis in cartilage is maintained by chondrocytes through synthesis and breakdown of extracellular matrix [5]. Thus, higher proliferation of chondrocytes plays a vital role in regulating chondrocyte integrity and cellular function [5]. The main inducer of osteoarthritis is interleukin (IL)-1β cytokine, which produces higher levels of catabolic enzymes, leading to metabolic imbalance. The increased production of catabolic enzymes degrades cartilage matrix and interferes with the normal physiological functioning of cartilage [6–8]. Chondrocytes of osteoarthritis patients have been shown to secrete higher levels of IL-1β, TNF-α, and IL-6 [9]. The secretion of inflammatory cytokines is also higher in synovial membranes [10]. These cytokines penetrate into the cartilage, damage its integrity, and lead to the development of osteoarthritis [10].

The cell cycle consists of a sequence of properly regulated events during which DNA is synthesized and cells undergo reproduction. At the initial stage of the cell cycle, replication of DNA and duplication of the chromosomes takes place [11]. In S phase, the DNA replication involves opening of the double-stranded structure, exposing the individual helices to enzymes for the synthesis of new strands [11]. In the M phase, chromosomes segregate, providing a template for the synthesis of more strands [12,13]. The G1 phase of cell cycle is the decisive period during which cells either divide or not [14,15]. Several factors, such as Ser/Thr protein kinases (CDK4 and CDK6), regulate cell cycle progression [16]. Cyclin D1 upregulates the transition from G1 phase to S phase on combination with CDK4 or CDK6 [16]. Heterocyclic compounds exhibit a wide range of biological activities, including anticancer, anti-inflammatory, and immune-modulator effects. The present study investigated the effect of hydroxyypyridinone-coumarin on the proliferative potential and cell cycle progression of chondrocytes in vitro and on inflammatory cytokine production and NF-κB signalling pathway activation in vitro in osteoarthritis rats in vivo.

Material and Methods

Chondrocyte isolation from rats and culture

We obtained 4-week-old Sprague-Dawley rats (weight 180–200 g) from the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China, which were used for isolating the chondrocytes. The articular cartilage was carefully excised from the knee joint of rats using isoflurane anaesthesia. The cartilage was washed 3 times in cold PBS and then sliced into thin 1-mm³ sections. The slices were digested in 0.2% type II collagenase, after which isolation of chondrocytes was performed in an incubator at 37°C. The supernatant obtained from the digested slices after every 3 h was centrifuged for 10 min at 1000 g to isolate the cell pellet. Stainless-steel filters with mesh size 200 were used to filter the cells, which were seeded at 2×10^5 cells/ml density in 6-well plates. The cells were cultured at 37°C in DMEM supplemented with 10% FBS under 5% CO₂. The cultured primary cells were monitored using an inverted microscope.

MTT assay

The chondrocytes were distributed at 1.0×10⁴ cells/well density in 96-well plates containing 10% FBS/DMEM. Hydroxypyridinone-coumarin at 5, 10, 15, 20, 25, 30, 40, and 50 μM concentrations was added to the plates and incubation was performed for 48 h. The medium was replaced by fresh medium, and then 20 μl of MTT solution (0.5%) was put into each well of the plates. Following incubation for 4 h at 37°C, 120 μl dimethyl sulfoxide (DMSO) was put into each well of the plate. Absorbance was recorded for each well of the plate at 487 nm using an ELISA reader (ELx800™, BioTek Instruments, Inc., Winooski, VT, USA).

Analysis of cell cycle

The chondrocytes were distributed in 35-mm petri dishes at 2×10^4 cells/ml density. Incubation of the chondrocytes was performed for 48 h with 5, 30, 40, and 50 μM of hydroxyypyridinone-coumarin. Then, chondrocytes were digested and subsequently re-suspended in ice-cold PBS. The cell suspension was centrifuged at 1000xg for 10 min at room temperature, after which the concentration of cells was adjusted to 2×10⁴ cells/ml. The cell plates were fixed using 70% ethyl alcohol for 12 h at 4°C, followed by incubation with DNease-free RNaseA and propidium iodide according to the manufacturer’s instructions. The cells were analyzed by a flow cytometer (BD Accuri™ C6; BD Biosciences, Franklin Lakes, NJ, USA).

RT-PCR analysis

The chondrocytes at 2×10^4 cells/well density were distributed in 6-well plates containing 2 ml of medium and incubated...
for 48 h with 5, 30, 40, and 50 μM of hydroxypyridinone-coumarin. The cells were treated with TRizol reagent (Invitrogen) in accordance with the manual protocol for isolation of total RNA. The 3-μg RNA samples were used as templates for reverse transcription into cDNA using oligo(dT) primers and SuperScript III RT (Invitrogen). The gene expression was quantified using SYBR Green Master mix according to manufacturer’s instructions. The sequence of events involves denaturation at 93°C for 5 min, then 40 cycles of amplification at 93°C for 10 s, followed by quantification at 58°C for 1 min. Data were assessed for relative gene expression using the 2−ΔΔCt method.

Western blot assay

The chondrocytes cultured in culture flasks were treated with 5, 30, 40, and 50 μM of hydroxypyridinone-coumarin for 48 h. The cells were then scrapped from the medium, washed 2 times with PBS, and subsequently suspended in RIPA buffer (30 μl). The lysate was centrifuged to collect the supernatant, in which the protein concentration was determined by bicinchoninic acid (BCA) protein assay kit in accordance with the manufacturer’s instructions. The 20-μg protein samples were subjected to electrophoresis using 8–12% SDS-polyacrylamide gels and subsequently transferred to the PVDF membranes. The membranes were blocked by incubation with 5% skimmed milk in TBST solution. Incubation of the membranes was performed overnight with primary antibodies against CDK6, CDK4, cyclin D1, lxBα, and NF-xB p65 at 4˚C. After washing with PBS, the membranes were incubated with secondary antibodies conjugated to HRP for 2 h. Enhanced chemiluminescence detection was used for visualization of the protein bands.

Animals

Twenty-five male Wistar rats (age 10–12 weeks, weight 285–405 g) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China. The rats were housed individually in plastic boxes under a 12-h light/dark cycle. The temperature in the animal house was maintained 23±2°C and humidity was controlled at 55±10%. All the rats were provided free access to food and water ad libitum. The study was approved by the Animal Ethics Committee, Zhengzhou University, Henan China [ZU/08/16]. The experimental protocols involving animals were conducted in accordance with the guidelines of the Animal Ethics Committee, Chinese Academy of Science.

Establishment of osteoarthritis rat model

The rats were divided randomly into 5 groups of 5 each and then anesthetized using 2% isoflurane anaesthesia. The rats in 4 groups (untreated control group and 3 treatment groups) were administered a single dose of monosodium iodoacetate (5 mg/kg) through the intra-articular route. The rats in the treatment groups were intraperitoneally injected with 5, 10, and 15 mg/kg doses of hydroxypyridinone-coumarin alternately for 1 month starting on day 2 of MIA administration. The normal control and untreated groups received equal volumes of normal saline.

Determination of cytokine levels

The rats were anaesthetized on day 45th of monosodium iodoacetate administration using mebunal sodium (1.1% solution). Blood was collected from rat carotid arteries and subsequently centrifuged for 20 min at 3500×g. The obtained supernatant was frozen at −78°C for measurement of cytokine production. Commercially available ELISA kits supplied by Santa Cruz Biotechnology were used for measurement of IL-6, TNF-α, and IL-1β content in the supernatant in accordance with the manufacturer’s instructions.

Statistical analysis

The expressed data are the mean±SD of 3 experiments performed independently. The data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test. Statistical analysis of the data was performed using SPSS 16.0 software. The differences were regarded as statistically significant at P<0.05.

Results

Hydroxypyridinone-coumarin promotes chondrocyte viability

The chondrocyte viability was determined after 48-h treatment with 5–50 μM hydroxypyridinone-coumarin (Figure 1A). The hydroxypyridinone-coumarin treatment significantly (P<0.05) increased the viability of chondrocytes in a dose-dependent manner. Treatment of the chondrocytes with 5, 10, 15, 20, 25, 30, 40, and 50 μM of hydroxypyridinone-coumarin increased viability by 5.63%, 8.23%, 13.47%, 17.45%, 20.59%, 24.33%, 32.72%, and 39.54%, respectively. The effect of 50 μM hydroxypyridinone-coumarin on chondrocyte viability was also determined at 12 h, 24 h, 48 h, and 72 h (Figure 1B). The chondrocyte viability increased significantly (P<0.05) with the increase in hydroxypyridinone-coumarin treatment duration from 12 h to 48 h.

Hydroxypyridinone-coumarin promotes chondrocyte cell cycle progression

The chondrocytes were treated with hydroxypyridinone-coumarin for 48 h and cell cycle distribution was analyzed by flow cytometry (Figure 2). Treatment with 5, 30, 40, and 50 μM
hydroxypyridinone-coumarin significantly (P<0.05) reduced the population of chondrocytes in G0/G1 phase. The percentage of chondrocytes in S phase was increased by treatment with hydroxypyridinone-coumarin. The percentage of chondrocytes was also increased by hydroxypyridinone-coumarin treatment in the G2/M phase.

**Hydroxypyridinone-coumarin increased cyclin protein expression in chondrocytes**

The chondrocytes were exposed to hydroxypyridinone-coumarin for 48 h and cyclin protein levels were determined by Western blotting (Figure 3A). Treatment with 5, 30, 40, and 50 μM of hydroxypyridinone-coumarin markedly promoted the...
expression of CDK6, CDK4, and cyclin D1 proteins in chondrocytes. The RT-PCR assay also showed that hydroxypyridinone-coumarin treatment of chondrocytes significantly increased the levels of CDK6, CDK4, and cyclin D1 mRNA (Figure 3B).

Hydroxypyridinone-coumarin inhibits level of cytokines in OA rat serum

The level of inflammatory cytokines in OA rats treated with hydroxypyridinone-coumarin was markedly lower in comparison to the untreated rats (Figure 4). Treatment of the OA rats with 5, 10, and 15 mg/kg doses of hydroxypyridinone-coumarin markedly suppressed IL-6, TNF-α, and IL-1β levels in the serum.

Hydroxypyridinone-coumarin inhibits NF-κB signalling pathway activation

Treatment of the OA rats with hydroxypyridinone-coumarin markedly suppressed the levels of IκBα and NF-κB p65 (Figure 5). The expression of activated IκBα and NF-κB p65 was also suppressed in the OA rats by treatment with hydroxypyridinone-coumarin, whereas the levels of IκBα and NF-κB p65 were markedly higher in the untreated OA rats.

Discussion

Osteoarthritis (OA) is the most commonly detected joint disorder in aged people caused by the degeneration of cartilage [17,18]. The loss of articular cartilage integrity is associated with extracellular matrix breakdown and chondrocyte death [17–19]. The present study investigated the effect of hydroxypyridinone-coumarin on chondrocyte viability and cell.
cycle progression in vitro and inflammatory cytokine level in vivo in an OA rat model.

The proliferative activity of chondrocytes has been found to be very low during osteoarthritis; therefore, promotion of proliferation is believed to play a vital role in the treatment of osteoarthritis [20]. The present study investigated changes in chondrocyte viability after treatment with hydroxyppyrindone-coumarin. The study showed that hydroxyppyrindone-coumarin treatment significantly increased the proliferation of chondrocytes in vitro compared to untreated cells. The DNA is synthesized in the S phase of the cell cycle and becomes 4N during the G2/M phases [21,22]. The increase in S to G2/M phase transition rate facilitates cell cycle progression and promotes proliferation activity [22]. In the present study, hydroxyppyrindone-coumarin treatment increased the population of chondrocytes in S to G2/M phases, with a subsequent decrease in the population of chondrocytes in the G1/G0 phases. Therefore, hydroxyppyrindone-coumarin promoted G1/G0 to S phase transition in the chondrocytes. The 2 major check-points for regulation of interphase transition in the cell cycle are G1/S and G2/M. The activity of cyclins is positively regulated by their interaction with DKS [23,24]. The increased expression of CDK6, CDK4, and cyclin D1 plays a vital role in up-regulation of G1/S transition [23,24]. In the present study, hydroxyppyrindone-coumarin treatment markedly upregulated the expression of CDK6, CDK4, and cyclin D1 proteins and mRNA in chondrocytes. Thus, hydroxyppyrindone-coumarin increased proliferation and promoted G1/G0 to S phase transition in chondrocytes. Osteoarthritis is characterized by joint inflammation, articular cartilage degeneration, and synovial tissue hyperplasia [25]. The inflammation leads to joint pain through neuronal activation [25]. Inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are overexpressed in chondrocytes obtained from osteoarthritis patients [9]. The present study showed that treatment of osteoarthritis rats with hydroxyppyrindone-coumarin suppressed the production of IL-1β, TNF-α, and IL-6 cytokines. The NF-κB pathway is considered to be an important target for the treatment of various inflammation-associated disorders [26]. It is reported that stimulation of chondrocytes with IL-1β leads to activation of the NF-κB pathway [26–28]. The results from the present study revealed that hydroxyppyrindone-coumarin suppressed the levels of IκB-α and NF-κB p65 expression in osteoarthritis rats. The levels of phosphorylated IκBα and NF-κB p65 were also reduced in osteoarthritis rats after treatment with hydroxyppyrindone-coumarin.

Conclusions

In summary, hydroxyppyrindone-coumarin increases chondrocyte viability by promoting the G1/S transition in cell cycle progression of chondrocytes in vitro. Moreover, the production of cytokines and activation of NF-κB were downregulated in the osteoarthritis rats by treatment with hydroxyppyrindone-coumarin. Therefore, hydroxyppyrindone-coumarin appears to be a promising treatment of osteoarthritis.

Conflict of interest

None.

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