Effects of Cyclopamine on the Viability of Articular Chondrocytes in Rats with Adjuvant Arthritis In Vitro and Part Mechanisms

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Abstract

The aim of the present study was to investigate the effect of cyclopamine, a hedgehog signaling pathway inhibitor, on Adjuvant Arthritis (AA), rat articular cartilage viability and part mechanisms in vitro. In this study, an AA rat model was established by Freund’s Complete Adjuvant (FCA), and the Arthritis Index (AI), secondary paw swelling degree and HE staining was used to evaluate whether the model was successfully established. Chondrocytes of the ankle joint of AA rats were cultured and identified. Cyclopamine (0, 0.03, 0.1, 0.3, 1, 3, 10 and 30 mg/L) was administered to determine chondrocyte viability. Chondrocyte apoptosis was detected by Annexin V-FITC/PI double dye. The expression of hedgehog signaling pathway-related proteins Shh, Ptc1 and Gli1 in chondrocytes was detected by western blotting. The results showed that AA was successfully induced by FCA, since the AI of AA rats and secondary paw swelling degree increased, the cartilage tissue of the rats’ ankle joint was damaged, and the chondrocytes were successfully cultured in vitro following the identification of toluidine blue and type II collagen. Cyclopamine (0.03, 0.1, 0.3, 1, 3, 10 and 30 mg/L) could increase the viability of chondrocytes in vitro and reduce the apoptotic rate of chondrocytes.

Keywords: Adjuvant arthritis; Articular chondrocytes; Cyclopamine; Hedgehog; Viability; Western blotting

Introduction

Synovial cell viability and inflammatory cell infiltration are common in patients with Rheumatic Arthritis (RA), RA is an autoimmune disease, which can damage subsynovial cartilage and bone tissue, and cause joint deformity and dysfunction. The main cause of disability is the destruction of articular cartilage and bone tissue [1]. The hedgehog signaling pathway is a conserved evolution from lower animals such as fruit flies to higher animals as evolution from lower animals such as fruit flies to higher animals as well as mammals. Studies have shown that the hedgehog signaling pathway is involved in regulating bone and cartilage development and growth. The hedgehog signaling pathway has been considered to be an important pathway in the development and function of articular cartilage. The hedgehog signaling pathway is involved in regulating bone and cartilage development and growth. In recent years, the role of hedgehog signaling in regulating bone and cartilage development and growth has attracted considerable attention. Studies have shown that the hedgehog signaling pathway is involved in regulating bone and cartilage development and growth. In recent years, the role of hedgehog signaling in regulating bone and cartilage development and growth has attracted considerable attention. Studies have shown that the hedgehog signaling pathway is involved in regulating bone and cartilage development and growth. The role of hedgehog signaling in regulating bone and cartilage development and growth has attracted considerable attention.

Main reagents and instruments

The following reagents and instruments were used: Tuberculin derivation protein (Beijing Xiangrui Biological Products Co., Ltd., Beijing, China); Anhydrous lanolin (Xianbao Co., Ltd., Changchun, China); FCA, cyclopamine, anti-Smo, anti-Ptch1 and anti-Gli1 polyclonal antibodies (Merck KGaA, Darmstadt, Germany); total protein extraction kit (Invent Biotechnologies, Inc., Plymouth, MN, USA); nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Enzyme-linked immunosorbent assay plate reader (EON, USA); YLS-

Materials and Methods

Experimental animals

Twenty male Wistar rats (6-8 weeks) weighing (140 ± 10) g were purchased from Biological Engineering Co., LTD. Nanjing Junke (Animal Approval Number 001). Rats were raised in temperature (22.5 ± 1.5)°C and humidity (60.0 ± 2.0)% controlled rooms for 1 week. Rats had free access to standard food and tap water. The light cycle is 14 hours of light and 10 hours of darkness. The animals were used in the experiments according to the guidelines of the Animal Experimental Ethics Committee of Anhui Medical University (reference no. 2017001; Hefei, China). Twenty Wistar rats were randomly divided into two groups; the control and model groups. 100 μL Freund’s Complete Adjuvant (FCA) was injected into the right hind toe of rats for 28 days (It’s a one-time injection and then fed for 28 days). The control group rats were injected with the same amount of physiological saline.

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Paw swelling degree and arthritis index (AI) score

The parameters of non-injected swelling (left hind paw of rats) and polyarthritis index of the AA rats were evaluated every 4 days, from day 12 to 28 following injection. The degree of AI was determined using a scoring protocol [5,6] where by severity was scored on a scale of 0–4, where 0=absent, 1=minimal, 2=mild, 3=moderate, and 4=severe. With the development of rheumatoid arthritis, the degree of secondary joint swelling is an important index to evaluate rheumatoid arthritis. The left hind paw volume (ankle joint) was tested using the YLS-7C Toes Volume Measurement Instrument. Paw swelling degree (Δ mL)=paw volume after injection-paw volume before injection.

**HE staining**

Ankle joints of rats were harvested, fixed in 4% paraformaldehyde solution for 24 hours at 20°C, and immersed in 10% with EDTA decalcification solution for 8 weeks. Ankle joints were cut into 4 μm sized slices and mounted on a glass slide. The sections were stained with HE at 20°C (hematoxylin stain for 5 min and eosin stain for 30 s. Images were measured at 570 nm on an enzyme standard instrument (Figure 2).

**Identification of chondrocytes**

Collagen type II immunocytochemistry:Cells were washed with PBS for 3 times, incubated with 4% paraformaldehyde for 1 hour at room temperature and were permeabilized with 0.1% Triton X-100. The slides were blocked with Immuno Staining Blocking Buffer (Beyotime, Shanghai, China) for 1 min. After centrifugation at 13,000×g for 15 min at 4°C, the supernatant was removed, and the formazan crystals were dissolved in 150 μL DMSO. 96-well plates were shaken evenly resulting in complete dissolution of formazan. Absorbance was measured at 570 nm on an enzyme standard instrument (Figure 1).

**Chondrocyte viability assay**

Chondrocytes were subcultured in 96-well plates at a density of 5x10^4/mL. Following treatment with cyclopamine (0.03, 0.1, 0.3, 1, 3, 10 and 30 mg/L) for 44 h at 37°C with 5% CO_2, 10 μL of 5 mg/mL MTT solution was added to each well and incubated for 4 hour at 37°C. Subsequently, the supernatant was removed, and the formazan crystals were dissolved in 150 μL DMSO. 96-well plates were washed 2 times, evenly resulting in complete dissolution of formazan. Absorbance was measured at 570 nm on an enzyme standard instrument (Figure 2).

**Apoptotic rate of articular chondrocytes detected in AA rats**

The apoptotic rate of chondrocytes was detected by flow cytometry using an Annexin V-FITC/PI staining kit (BestBio, Shanghai, China). Chondrocytes were seeded in 6-well plates at a density of 5x10^4/mL, and treated with cyclopamine (0.3, 3 and 10 mg/L). After 48 hours, the cells were harvested. Following the protocol: Cells were washed twice in cold PBS and 400 μL Annexin V binding buffer, 5 μL FITC and 10 μL PI were then added for 5 min at 4°C in the dark. Finally, cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). Images were obtained using Flowjo 7.6 software (USA). Each of the experiments was repeated 3 times, n=3.

**Western blotting detected the protein expression of Shh, Gli1 and Ptch1 in chondrocytes**

Cells were seeded in 6-well plates and treated with cyclopamine (0.3, 3 and 10 mg/L) at 37°C with 5% CO_2 for 24 hours. Cells were washed 3 times with cold PBS, then suspended in 200 μL of RIPA Lysis buffer (containing 1 mmol/L protease inhibitor, PMSF). After centrifugation at 13,000×g for 15 min at 4°C, the protein concentration was determined by BCA assay kit. An equal quantity of proteins (50 μg) were separated using 10% and 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After the membranes were blocked with 5% skim milk for 1 hour at room temperature, they were incubated with the anti-Shh (1 : 1,000, S8321, Sigma), anti-Gli (1 : 1,500, SAB4501219, Sigma), anti-Ptch (1 : 1,500, SAB2508835, Sigma), anti-GAPDH (1 : 1,500, SAB3500350, Sigma) primary antibodies overnight at 4°C. The next day, membranes were washed 3 times with TBST for 5 min and incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG secondary anti-bodies (1 : 1,000, 43413, Sigma) for 1 hour at room temperature. The slides were washed twice with PBS. Images of the stained cells were obtained using light microscope (Leica, Germany). Toluol blue staining: cells were washed with PBS for 3 times, fixed with 4% paramethylal at room temperature for 30 min, stained with 1% toluidine blue at room temperature for 2 hour, rinsed with anhydrous ethanol for 2 times, and sealed with neutral gum after drying.

**Statistical analysis**

Data are expressed as the mean ± standard deviation using SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA) to determine significant differences between the groups. Statistical analysis was performed using Student’s t-test or one–way ANOVA followed by...
SNK-q test, where appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Secondary AI score and paw swelling degree

Paw arthritis severity was evaluated by the AI in the non-injection side (secondary) of rats. The results showed that, following injection for 12, 15, 18 and 21 days, the AI of AA rats had gradually increased, as compared with the control group (p<0.05). The paw swelling degree was also found to have gradually increased (p<0.05).

Morphological structure of cartilage tissue

HE staining revealed a thin layer of synovial tissue on the surface of chondrocytes in the normal control group, and showed that chondrocytes had gradually grown from the margin to the center, revealing a group of homologous cells. Abnormal hyperplasia of the synovial tissue was observed in the ankle joint of the model group, invading the cartilage tissue, and chondrocytes were disordered (Figure 3).

Chondrocyte identification and culture

Primary chondrocytes were spherical, and many spindle and polygonal chondrocytes were shown to be attached to the wall at ~10 hours in, completely adhering to the wall in ~3 days. Approximately a week later, the cells were connected into sheets and could be sub-cultured. Toluidine blue staining showed that the cytoplasm of chondrocytes was light blue and their nucleus blue. A few heterochromatic particles were identified in the cytoplasm (Figure 4). Type II collagen immunohistochemical staining showed that the cytoplasm of chondrocytes exhibited a brown-yellow type II collagen expression (Figure 5).

AA rat chondrocyte viability

As shown in Figure 5, chondrocyte viability was significantly increased following treatment with cyclopamine (0.03, 0.3, 1, 3, 10 and 30 mg/L), and had dose dependent. Considering that 0.03 mg/L cyclopamine had no obvious effect on chondrocytes, the final concentrations of cyclopamine used in subsequent experiments were 0.3, 3 and 10 mg/L.

Apoptotic rate of chondrocytes in AA rats

Annexin V-FITC/PI staining was used to detect chondrocyte apoptosis as shown in Figure 6, the apoptotic rate of chondrocytes gradually decreased with the increase in cyclopamine concentration (p<0.05). The rates of early apoptosis in chondrocytes were (5.6 ± 0.5)%, (4.4 ± 0.6)% and (3.1 ± 0.4)%, respectively, significantly lower than those in the model group (10.6 ± 0.8)% (p<0.05).

Effect of cyclopamine on the expression of hedgehog pathway-related proteins in chondrocytes

As shown in Figure 7, the expression of hedgehog pathway-related proteins Shh, Gli1 and Ptch1 were detected by western blotting in the chondrocytes of AA rats. Compared with the control group, the expression of these proteins was significantly increased in chondrocytes,
following treatment with different concentrations of cyclopamine (0, 0.3, 3 and 10 mg/L) for 24 hours.

**Discussion**

RA is an autoimmune disease caused by abnormal viability in the synovial tissue, progressive destruction of the cartilage, and bone and joint dysfunction [1,8]. In the present study, an AA model of Wistar rats was established by FCA, which is a classic animal model of RA pathogenesis [5,9]. Compared with normal rats, the AA model rats exhibited a quick onset, with the hind legs affected before the forelimbs, ankle suffered most easily, in addition to the injection paw, secondary parapodium paws underwent inflammatory changes [10]. In order to evaluate secondary paw arthritis, semi-quantitative scoring was used to score secondary side inflammation and calculate the AI and joint swelling degree in this study [11,12]. The research showed that secondary AI and secondary joint swelling were obvious in AA rats, indicating that the AA rat model was successfully established. HE staining showed synovial tissue hyperplasia in the ankle joint of the model group, invasion of cartilage tissue, and disordered arrangement of chondrocytes, which indicated the successful establishment of the model.

The main pathological features and clinical manifestations of RA are cartilage, bone and joint destruction, and its pathogenesis is not completely clear [13]. Articular cartilage tissue covers the surface of joint bone, mainly including chondrocytes and extracellular matrix [14]. Chondrocytes have an active metabolism, which is crucial to chondrogenesis, metabolism and repair [15]. During RA, chondrocyte apoptosis leads to decreased cartilage density, impaired matrix metabolism balance, and articular cartilage damage [7]. Type II collagen is an important component of cartilage matrix. Toluidine blue staining results showed that the nuclei of chondrocytes stained blue and the cytoplasm light blue, confirming that the culture of AA rat chondrocytes was successful.

Currently, there is a lack of clinically specific drugs for RA treatment. Cyclopamine is an isostereoid alkaloid isolated from veratrol plants, which can inhibit the growth of tumor cells [4]. In the present study, MTT assay showed that cyclopamine could increase the viability of joint chondrocytes in AA rats in a dose-dependent manner in vitro.
To study this effect, we used Annexin V, which can be sensitive to detecting cell surface phosphatidylserine of the probe, the principle of PI staining can observe the apoptosis [16], the results showed that chondrocyte apoptosis gradually decreased with the increase in cyclopamine concentration, and the result was significant, as compared with the model group. It has been suggested that cyclopamine administration could effectively reduce apoptosis in AA articular chondrocytes in vitro. Limitedly, our experiments only demonstrated the effect of cyclopamine on chondrocytes in AA rats, and no study was conducted on normal chondrocytes. We will use of sham chondrocytes constitutes an important control for further comparisons of the effect of cyclopamine. The experiment is now under way.

Studies have shown that the activated hedgehog signaling pathway is involved in bone and joint destruction during the progression of osteoarthritis [17]. The hedgehog signaling pathway has four main aspects: Hedgehog signaling molecules, Dhh, Shh and Ihh; transmembrane receptors, Smo and Ptc; Glis family of nuclear transcription factors, and downstream target genes [18,19]. In general, Ptc inhibits Smo activity following its interaction with Smo, leading to the inhibition of hedgehog signaling and decreased cell viability. In inflammation diseases, Hh protein binds to Ptc, weakening Ptc's inhibition of Smo, activating the downstream nuclear transcription factor Glis family, thus inducing the expression of target genes and aggravating inflammation. As an important pathway regulating the growth and development of bone and cartilage [20,21], the role of abnormal hedgehog signaling has been linked to the development of bone and joint [22]. In this study, western blotting results showed that, as compared with the control group, the protein level of Shh, Ptc and Glil in articular chondrocytes from AA rats significantly decreased following treatment with cyclopamine (0.3, 3 and 10 mg/L) for 24 hour, suggesting that the hedgehog signaling pathway in AA rat articular chondrocytes had been abnormally activated. Cyclopaemin drug delivery in vitro could lead to a significant reduction in the protein expression pathway, which might be closely associated with the inhibition of viability and decline of apoptosis in articular chondrocytes of AA rats following treatment with cyclopamine.

Conclusion

In conclusion, in the present study, an AA rat model was successfully established by FCA, and the articular chondrocytes of AA rats were isolated, cultured and identified. The cyclopamine pathway inhibitor cyclopamine could significantly improve the viability of articular chondrocytes from AA rats and inhibit their apoptosis. This effect may be associated with the inhibition of the abnormal activation of the hedgehog signaling pathway.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ Contributions

Xianbin Song and Xiaoyu Chen designed the study. Bao Zhang, Yanping Huang, Ye Zhang and Meimei Liu performed the experiments. Taorong Wang and Xiaoyu Chen analyzed the data and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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