

Original Research Article

Hh pathway expression in the blood, synovial cells and chondrocytes of different rheumatoid arthritis models

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Abstract

Purpose: To investigate the effect of the hedgehog (Hh) pathway inhibitor, cyclopamine, and activator purmorphamine on articular cartilage cell proliferation.

Methods: Rats were subjected to AA and CIA models. Secondary paw swelling was measured at 12, 15, 18, 21, 24, 27, and 30 days. The rats were sacrificed on day 30. Tissues from the cartilage and knee joints were collected. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay while cell apoptosis was determined by annexin V-fluorescein isothiocyanate/propidium iodide assay. Protein expression levels of Shh, Ptch1 and Gli1 were determined by Western blotting.

Results: Compared with the control group, arthritis index and secondary foot swelling of the adjuvant arthritis (AA) and collagen-induced arthritis (CIA) groups deteriorated significantly ($p < 0.05$). MTT data revealed that cyclopamine promoted articular cartilage cell proliferation of the AA and CIA groups. The cell proliferation rates of AA and CIA groups were significantly higher than that of control group ($p < 0.05$). Flow cytometry showed that the cell apoptosis rates of AA and CIA groups were significantly lower than that of control group ($p < 0.05$). Compared with control, cyclopamine decreased the protein expression levels of sonic Hh, patched homologue 1 and glioma-associated oncogene homologue, but the effect of purmorphamine was the reverse.

Conclusion: Hh pathway inhibitor (cyclopamine) and activator (purmorphamine) affect the expression of Hh pathway. Disruption of the Hh pathway may be of potential therapeutic significance in protecting articular cartilage from rheumatoid arthritis.

Keywords: Adjuvant arthritis, Cyclopamine, Purmorphamine, Synovial cells, Chondrocytes, Hedgehog signaling pathway

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease leading to the symptoms like synovial inflammation, bone erosion, and cartilage destruction [1]. Almost all RA patients eventually

develop varying degrees of joint dysfunction that poses great threat to human health [2]. Globally, the incidence of RA is approximately 1 %. Although the pathogenesis of RA is largely unknown, it is recognized that synoviocyte proliferation and inflammatory cell infiltration

cause destruction of subsynovial cartilage and bone tissue [3].

Increasing evidence demonstrated that hedgehog (Hh) signaling regulates cartilage cell growth and differentiation, and is very important for maintaining the biological function of cartilage cells [4,5]. The Hh protein family consists of sonic Hh (Shh), glioma-associated oncogene homologue (Gli1) and patched homologue 1 (Ptch1). The expression of Gli1 and Ptch1 are regarded as markers of Hh pathway activity [6].

The pathological role of Hh signaling in chondrocyte proliferation and differentiation as well as bone formation and metabolism have been verified in osteoarthritis (OA) [7-9]. Activation of the Hh signaling pathway was evident in the articular chondrocytes of patients with OA, and inhibition of the Hh pathway by a specific blocker or gene silencing could significantly alleviate the condition of patients with OA [10,11]. These findings provide a new idea for the study and prevention of osteoarticular diseases. Some studies have shown that progressive cartilage destruction is a main feature of RA and OA. However, no study has reported the specific mechanism of the Hh signal pathway in RA. The effect of Hh signaling on synovial cells and articular chondrocytes in different rat models of RA *in vitro* has not been investigated.

The present study investigated the expression of the Hh signal pathway in the blood, synovial cells, and chondrocytes of an adjuvant arthritis (AA) rat model and a collagen-induced arthritis (CIA) rat model. The effects of Hh pathway inhibition or activation on the activation of synovial cells and chondrocytes in AA rats and CIA rats were observed to provide a new experimental basis for RA.

EXPERIMENTAL

Experimental animals

Sprague-Dawley (SD) rats weighing 220 - 250 g were purchased from Changzhou Cavens Experimental Animal Company, China. The rats were fed under standard laboratory conditions. In addition, the housing conditions of mice were controlled at a temperature of 22 ± 2 °C, a relative humidity of 55 ± 5 %, and a 12-h light/12-h dark cycle. Standard chow and water were given in the laboratory. The use of animals in the laboratory was conducted in accordance with international guidelines [12]. In addition, the animal experimental protocols designed in this study were approved by the University Ethics

Committee on Animal Research (approval no. LL170607Z). The SD rats were randomly divided into three groups as follows: Control, AA group, and CIA group. AA and CIA models were created as previously described [13,14].

Preparation of peripheral blood mononuclear cells (PBMCs)

Fresh whole-blood samples of SD rats were used to isolate PBMCs, which were purified by density centrifugation using Lymphoprep (Gunma, Japan) according to the manufacturer's instructions. The PBMCs were cultured in cell culture medium (RPMI 1640) supplemented with 10 % heat-inactivated (HI)-fetal bovine serum (FBS). Next, the PBMCs were activated with 10 µg/mL lipopolysaccharide.

Isolation and culture of synoviocytes and chondrocytes

The rat tissues were obtained under aseptic conditions, were washed with D-Hanks solution (without Ca^{2+} and Mg^{2+}), and then were cut into 1-mm pieces. Synoviocytes and chondrocytes were isolated with 5 mg/mL of pronase (Tokyo, Japan) at 37 °C for 1 h, followed by 2 mg/mL of collagenase (MO, USA) at 37 °C for 6 h in cell culture medium (Kyoto, Japan) with 10 % HI-FBS (Tulare, CA, USA) in a humidified atmosphere of 5 % CO_2 and 95 % air. To remove non adherent lymphoid cells, the cell culture medium was changed every 3 days. At confluence, the chondrocytes and synoviocytes were separated and passaged once. Next, they were seeded at high density and allowed to grow in cell culture medium as previously described [15-17].

Drug treatment

The treatment groups were as follows: blank control group, AA model negative control group, CIA model negative control group, AA model + cyclopamine group (0.5, 5, and 20 µmol/L), CIA model + cyclopamine groups (0.5, 5, and 20 µmol/L), AA model + pumorphamine (0.15, 1.5, and 6 µmol/L), and CIA model + pumorphamine (0.15, 1.5, and 6 µmol/L).

MTT assay

Synoviocytes and chondrocytes in the logarithmic phase were collected, and 10×10^4 cells were added to each well in 96-well plates. Next, 50 µL of cyclopamine (0.5, 5, and 20 µmol/L) or pumorphamine (0.15, 1.5, and 6 µmol/L) was added to the AA and CIA groups. In addition, 50 µL of nutrient fluid was applied to each well. The 96-well plate was maintained at

37 °C for 48 h with 5 % CO₂ air. Thereafter, each well was added with 20 µL of MTT. After 4 h, the reaction was stopped by acidified DMSO. The optical density value (OD) at 490 nm of each well was measured by MK3 Enzyme Sign (Dutch Thunder Suddenly Company). In addition, cell proliferation was measured as described previously [15].

Flow cytometry

Synoviocytes and chondrocytes were seeded on plates for 12 h. Next, the cells were treated with 0.25 % trypsin for 24 h then harvested and washed twice with phosphate-buffered saline (PBS). The cell suspension was evaluated for apoptosis by double staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) in binding buffer for 30 min at 37 °C. Apoptotic cells were detected using a FACS Calibur flow cytometer (BD Biosciences, CA). Flow cytometry analysis was repeated at least three times.

Western blot analysis

Isolated synoviocytes and chondrocytes were incubated at 37 °C in 5 % CO₂ for 24 h in 60-mm dishes. Cells were then washed three times with PBS and were lysed in 1X sodium dodecyl sulfate. Next, total protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto PVDF membranes. Thereafter, the membranes were blocked with 5 % skim milk overnight at 4 °C. After washing with TBS three times, the membranes were incubated with anti-caspase-3 and mouse monoclonal anti-β-actin (at a dilution of 1:2000) at 37 °C for 1 h. Next, the protein bands were developed with electrochemiluminescence reagents, and images were acquired using the ChemiDoc Imaging system. Western blot analysis was repeated at least three times.

Statistical analysis

Data are expressed as mean ± standard deviation and were analyzed using one-way analysis of variance. Significance threshold was set at $p < 0.05$. SPSS 17.0 was used for statistical analyses.

RESULTS

Comparison of histopathological changes in AA and CIA rats

To confirm the establishment of the rat model, the degree of foot swelling and histopathological

changes of the knee joint were evaluated. As shown in Figure 1 (A and B), the secondary side of AA and CIA rats showed obvious swelling and the score of the secondary arthritis index and degree of foot swelling were significantly higher than those of the normal group ($p < 0.05$), suggesting that the rat model was established.

After successful modeling, five rats were selected for each group, and blood was collected by retro-orbital venous plexus puncture. After sacrificing rats by cervical dislocation, the expression of Ptch1, Gli1, and Shh in articular chondrocytes and synovial cells were evaluated by Western blotting. Compared with the control group, the expression of Shh, Gli1, and Ptch1 protein in PBMCs, articular chondrocytes, and synoviocytes of AA and collagen-induced (CIA) rats were significantly increased ($p < 0.05$) (Figure 1 C, D and E).

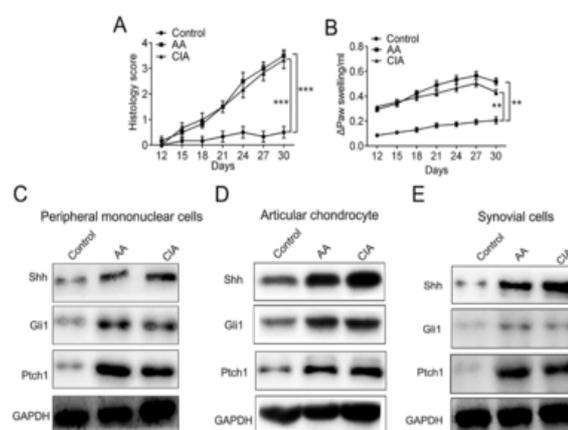


Figure 1: Comparison of histopathological changes in the knee joint (A) and degree of foot swelling (B); Comparison of the expression of Shh, Gli1, and Ptch1 protein in peripheral mononuclear cells (C), chondrocytes (D) and synoviocytes (E) in normal rats (control group), CIA rats, and AA rats

Effect of cyclopamine on proliferation and apoptosis of articular chondrocytes

The effect of the Hh pathway inhibitor cyclopamine on the proliferation of articular chondrocytes is shown in Figure 2 A. Cell proliferation rates of the AA and CIA groups were significantly higher than that of the control group ($p < 0.05$). In AA and CIA groups, 20 µmol/L of cyclopamine induced the highest level of cell proliferation (1.24 ± 0.11 , $p < 0.05$) and (1.14 ± 0.11 , $p < 0.05$), respectively. The cell proliferation increased by cyclopamine in a dose-dependent manner ($p < 0.05$). Next, the effect of cyclopamine on apoptosis in articular chondrocytes was evaluated by Annexin V-FITC/PI double staining. Following treatment with different concentrations of cyclopamine (0.5, 5,

and 20 μmol/L), the percentages of apoptotic cells in the AA and CIA groups were found to gradually decrease compared with that in the control group (Figure 2 B). In the AA and CIA groups, 20 μmol/L of cyclopamine caused the lowest level of cell apoptosis (0.24 ± 0.03 , $p < 0.05$) and (0.17 ± 0.02 , $p < 0.05$), respectively. Compared with the control group, the Shh, Gli1, and Ptch1 protein expression in cartilage cells of AA and CIA rats were significantly decreased ($p < 0.05$) (Figure 2 C).

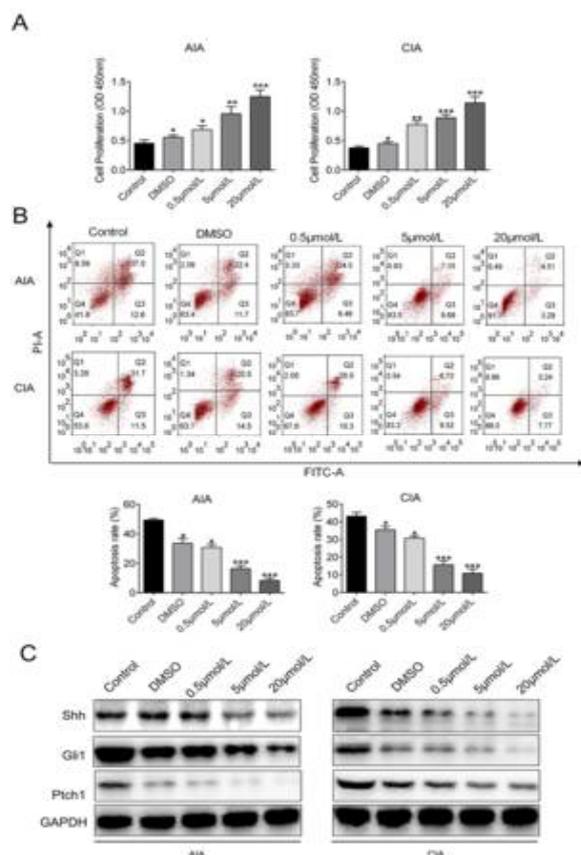


Figure 2: Effect of cyclopamine on the proliferation (A) and apoptosis (B) of articular chondrocytes and Hh pathway-related protein expression (C) in the cartilage cells of AA rats and CIA rats

Effect of purmorphamine on proliferation of articular chondrocytes

The effect of the Hh pathway activator purmorphamine on the proliferation articular chondrocytes is shown in Figure 3 A. The cell proliferation rates of AA and CIA were significantly lower than that in the control group ($p < 0.05$). In the AA and CIA groups, the cell proliferation rate with 6 μmol/L of purmorphamine were the lowest (8.31 ± 1.74 , $p < 0.05$) and (10.54 ± 2.45 , $p < 0.05$), respectively. Cell proliferation rate was decreased with increase in the concentration of purmorphamine ($p < 0.05$). To quantitatively examine the effect of

purmorphamine on apoptosis in articular chondrocytes, Annexin V-FITC/PI double staining was measured via flow cytometry. Following treatment with different concentrations of purmorphamine (0.15, 1.5, 6 μmol/L), the percentages of apoptotic cells of the AA and CIA groups were found to gradually increase when compared with that in the control group (Figure 3 B). In both AA and CIA groups, 6 μmol/L purmorphamine induced the highest level of cell apoptosis [41.3 ± 1.83 ($p < 0.05$) and 43.36 ± 0.07 ($p < 0.05$), respectively]. Compared with the control group, the Shh, Gli1, and Ptch1 protein expression levels in the cartilage cells of AA and CIA rats were significantly increased ($p < 0.05$, Figure 3 C).

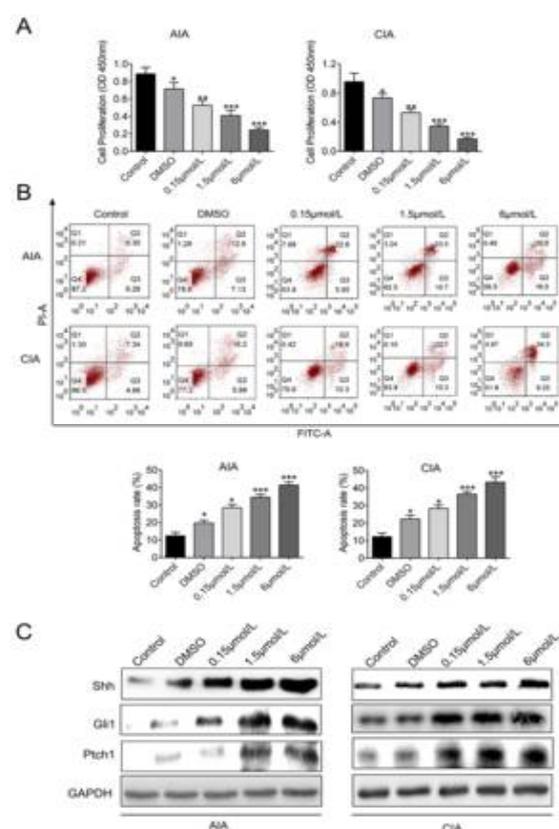


Figure 3: Effect of purmorphamine on the proliferation (A) and apoptosis (B) of articular chondrocytes and Hh pathway-related protein expression (C) in articular cartilage of AA rats and CIA rats

DISCUSSION

Rheumatoid arthritis is a chronic, and systemic inflammatory disease which is characterized by persistent inflammatory synovitis, affecting mainly the peripheral joints [18]. In China, the mortality rate of RA is 0.32 - 0.36 %, which is lower than the global average [19]. Wang *et al.* reported that in the absence of effective and timely treatment, more than 75 % of patients with RA would be disabled within 3 years [20].

The Hh pathway plays an important role in the process of controlling cell migration, cell differentiation, and cell differentiation of embryonic cells [21]. It has been reported that the Hh pathway plays a complex role in the physiological and pathological processes of chondrocyte proliferation and differentiation, bone formation, and bone metabolism. In the present study, the Hh signal pathway was found to play a crucial role in the regulation of differentiation, development, and tumor formation in embryonic tissues.

AA and CIA models are typical RA models [22]. The pathological mechanisms of the AA and CIA models are similar to that of human RA, but some differences exist between the models. The expression of the Hh signaling pathway in different RA models was investigated in the present study. Agonists and inhibitors were used to verify whether the Hh signaling pathway might play a role in the development of RA. In the present study, abnormal activation of the Hh signaling pathway in articular cartilage and synovial cells of AA and CIA rats suggests that this pathway may be a new target for the treatment of RA. The Hh pathway inhibitor cyclopamine and activator purmorphamine can affect the protein expressions of Shh, Gli1, and Ptch1. Cyclopamine promoted the proliferation of chondrocytes and inhibited the excessive apoptosis of chondrocytes *in vitro*, suggesting that intervention of the Hh pathway in chondrocytes may be of potential therapeutic significance in protecting articular cartilage from RA.

CONCLUSION

This study, for the first time, reveals that Hh signal pathway is activated in the articular cartilage of AA and CIA rats. *In vitro* inhibition of Hh signaling by cyclopamine promote the cell proliferation rate and protein expression of Shh, Gli1, and Ptch1. However, cell apoptosis in AA and CIA groups are inhibited by cyclopamine. On the other hand, purmorphamine treatment causes the opposite effect. Thus, Hh signaling pathway contributes to the pathogenesis of cartilage destruction in RA. Further studies are needed to investigate the potential therapeutic effect of purmorphamine and cyclopamine, and their mechanisms of action in AA and CIA rats.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yingyi Wu designed all the experiments and revised the paper. Guangxia Yang and Jing Fei performed the experiments, Yang Huang wrote the paper and final approve.

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