Aquaporin 1 contributes to chondrocyte apoptosis in a rat model of osteoarthritis

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Abstract. Aquaporins (AQPs) have been found to be associated with a number of diseases. However, the role of AQP-1 in the pathogenesis of osteoarthritis remains unclear. We previously found that AQP-1 expression was upregulated in osteoarthritic cartilage and strongly correlated with caspase-3 expression and activity. The aim of this study was to further investigate the association of AQP-1 expression with chondrocyte apoptosis in a rat model of osteoarthritis, using RNA interference to knock down AQP-1. For this purspose, 72 male Sprague-Dawley rats were randomly assigned to 3 groups as follows: the control group not treated surgically (n=24), the sham-operated group (n=24), and the osteoarthritis group (n=24). Osteoarthritis was induced by amputating the anterior cruciate ligament and medial collateral ligament and partially excising the medial meniscus. Chondrocytes from the rats with osteoarthritis were isolated and cultured. shRNAs were used to knock down AQP-1 expression in the cultured chondrocytes. The expression of AQP-1 and caspase-3 was determined by reverse transcription-quantitative polymerase chain reaction. Caspase-3 activity was measured using a caspase-3 colorimetric assay. The rats in our model of osteoarthritis exhibited severe cartilage damage. The knockdown of AQP-1 decreased caspase-3 expression and activity in the cultured chondrocytes. In addition, the expression of AQP-1 positively correlated with caspase-3 expression and activity. Thus, the findings of our study, suggest that AQP-1 promotes caspase-3 activation and thereby contributes to chondrocyte apoptosis and to the development of osteoarthritis.

Introduction

Osteoarthritis is a degenerative disease with an irreversible course and serious consequences that affects millions of individuals worldwide (1). Increasing age is one of the most common risk factors for osteoarthritis (2). With an increase in life expectancy, more elderly patients are likely to develop osteoarthritis (3), and osteoarthritis is estimated to become the fourth most disabling disease by the year 2020 (1). However, the pathogenesis of the disease remains poorly understood.

Although chondrocytes, which are mainly responsible for the anabolic-catabolic balance in cartilage, account for only 1-2% of the total cartilage volume, they play an important role in regulating the function of articular cartilage by synthesizing the structural components of the extracellular matrix (ECM) and matrix-degrading proteases (3). Chondrocytes have been found to play a pivotal role in the pathology of osteoarthritis through chondrocyte apoptosis and cartilage matrix degradation (4-6). However, the molecular mechanisms underlying the development of osteoarthritis due to chondrocyte apoptosis have not yet been clearly elucidated.

Aquaporins (AQPs) are specific transmembrane proteins responsible for water transport and are expressed in articular chondrocytes (7). It has been reported that the expression levels of AQPs are associated with apoptosis in many types of cells. However, the role of AQPs in the pathogenesis of osteoarthritis remains unclear (7,8). It remains to be determined whether AQP-1 expression is altered in chondrocytes in osteoarthritis and whether the expression levels of AQP-1 are associated with chondrocyte apoptosis.

We have previously reported that AQP-1 mRNA expression is increased in a rat model of osteoarthritis and positively correlates with the mRNA expression and activity of the the apoptotic marker, caspase-3 (9). In the present study, we further performed RNA interference (RNAi) experiments to knock down AQP-1 and investigated the association between the expression of AQP-1 and the expression and activity of caspase-3. The aim of this study was to further determine the role of AQP-1 expression in chondrocyte apoptosis and to further explore the role of AQP-1 in the pathogenesis of osteoarthritis.

Materials and methods

Animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University, Nanjing, China. All procedures were carried

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out in accordance with the Institutional Animal Care and Use Committee Guide at Merck Research Laboratories (Germany). A total of 72 male Sprague-Dawley (SD) rats (8 weeks old, weighing 286-320 g) were obtained from the Animal Center of Nanjing Medical University (Nanjing, China). The animals were housed at room temperature (25˚C) with 60% humidity and a 12-h light/dark cycle. The animals were fed standard rat chow and were provided with water *ad libitum*. The rats were randomly assigned to 3 groups as follows: the control group not treated surgically (n=24), the sham-operated group (n=24), and the osteoarthritis group (n=24).

Establishment of rat model of osteoarthritis. The rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (1-2 ml/kg). The anterior cruciate ligament and medial collateral ligament were cut, and the anterior horn of the medial meniscus was partially removed, via the medial parapatellar approach, as previously described (10). The anterior drawer test and the lateral stress test were used to confirm the dissection of the anterior cruciate ligament and medial collateral ligament. The articular cavity was flushed with iodine and saline. The wounds were sutured, and penicillin (80,000 U; Shanghai Nuotai Chemical Co., Ltd., China) was administered for 3 days. For the rats in the the sham-operated group, the articular cavity was exposed, but the ligaments and anterior horn of the medical meniscus were not removed. For the rats in the the control group, no treatment/surgery was administered. The rats in the each group were forced to move for 2 h each day by the squirrel wheel method. The general condition of the articular cartilage, based on the color, cracking, softening and osteophyte formation, was observed. The rats were sacrificed by CO_2 inhalation, and the knee joints were harvested at 1, 2, 4 and 8 weeks after the osteoarthritis model was established. The samples were stored in 4% formaldehyde solution at -80˚C until use.

Isolation and culture of chondrocytes from rats with osteoarthritis. The cartilage was removed from the rats in the osteoarthritis group at 8 weeks post-surgery and used for chondrocyte isolation and culture. Chondrocytes were isolated from the cartilage matrix by serial digestion with trypsin (Amresco, Solon, OH, USA) and collagenase II (Sigma, St. Louis, MO, USA) and cultured as previously described (11). The survival conditions of the cultured cells were examined under an optical microscope (A11.1535; Opto-Edu, Beijing, China).

Cultured chondrocytes in the logarithmic growth phase were seeded in a 6-well plate, supplemented with 2 ml Dulbecco's modified Eagle's medium/F-12 medium containing 15% fetal bovine serum with 200,000 units penicillin. The cells were cultured for 3, 5 days and 1 week in an incubator at 37˚C with 5% CO_2. The medium was changed every other day. Transfection was performed when the cells reached 80% confluence.

Hematoxylin and eosin (H&E) and Alcian blue staining. H&E and Alcian blue staining was used to assess chondrocyte morphology. For H&E staining (AR1180-100; Boster, Wuhan, China), the cells were stained with H&E for 5 min. For Alcian blue staining, the cells were stained with Alcian blue, using the Alcian Blue pH 2.5 Stain kit (American MasterTech, Lodi, CA, USA).

Immunofluorescence staining. For immunofluorescence staining, the cells were grown on glass coverslips, rinsed with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde for 30 min at room temperature. The cells were then permeabilized with 1% Triton X-100 for 10 min. Following 3 washes with PBS, the cells were incubated with primary antibody against type II collagen (rabbit anti-rat type II collagen, 1:100 dilution; Cat no. 70R-CR008; Fitzgerald Industries International, Acton, MA, USA) at 4˚C overnight. PBS without primary antibody was used as a negative control. After the primary antibody was removed by washing in PBS, immunoreactivity was detected by incubation with fluorescein-isothiocyanate-conjugated secondary antibody (goat anti-rabbit IgG, 1:100 dilution; 111-005-144; Jackson Immuno Research, West Grove, PA, USA) at room temperature for 45 min. After the coverslips were washed with PBS, the cells were counterstained with DAPI (Sigma) and examined and photographed under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

RNAi and cell transfection. The rat cDNA sequence (GenBank NM-012778; https://www.ncbi.nlm.nih.gov/nuccore/NM_012778) was analyzed for potential small interfering RNA (siRNA) target sequences for AQP-1. The oligonucleotide was designed to have a hairpin loop and cloned into the pGenesil-1 plasmid containing the U6 promoter and green fluorescent protein (GFP) (Wuhan Cell Marker Biotechnology Co., Ltd., Wuhan, China). As previously described (12), the AQP-1-shRNA pGenesil-1 plasmid named AQP-l-pGenesil was used for RNAi to knock down AQP-1. The following oligonucleotide was used for AQPl-2 (19 nt): 5'-TTCTCAAA CCACTGGATT-3'. The oligonucleotide used for scrambled shRNA was 5'-GACTTCATAAGGCGCATGC-3'. Chondrocytes at 80% confluence were transfected with AQP-l-pGenesil using the transfection reagent, Lipofectamine® 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Untransfected cells were used as empty controls, and cells transfected with Lipofectamine 2000 were used as the empty Lipofectamie 2000 group. At 48 h post-transfection, the transfected cells showing GFP expression were sorted using flow cytometry (Guava® easyCyte 8, Merck Millipore, Billerica, MA, USA) and used in the subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the articular cartilage using the TRIzol RNA extraction kit (Invitrogen Life Technologies). RNA was reverse transcribed into complementary DNA (cDNA) using the reverse transcription system (Toyobo Co., Ltd., Osaka, Japan). Quantitative (qPCR) was performed with a 20-μl mixture containing 2.5 μl cDNA, 0.4 μl of each primer, and 10 μl SYBR-Green (Toyobo Co., Ltd.). The following primers were used: 5'-CATTGGCTTGTCTG TGGC-3' (forward) and 5'-TTTGAGAAGTTGCGGGTG-3' (reverse) for AQP-1, 5'-CTGGACTGCGGTATTGAG-3' (forward) and 5'-GGGTGCGGTAGAGTAAGC-3' (reverse) for caspase-3, and 5'-CAAGTTCAACGGCACGTCAA-3' (forward) and 5'-TGGTGAAGACGCCAGAGACTC-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control. The

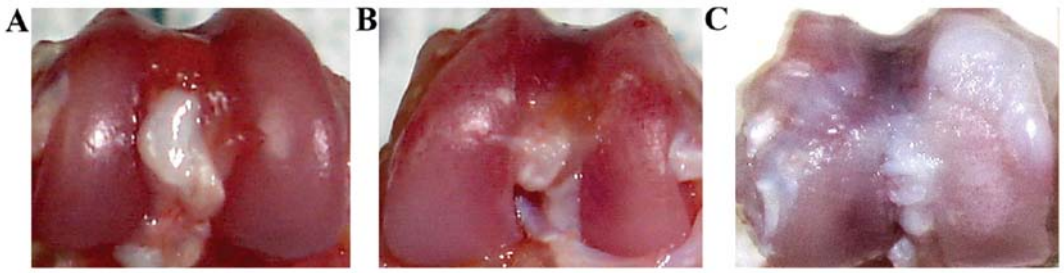

Figure 1. Cartilage damage in rats in (A) the control, (B) sham-operated, and (C) osteoarthritis groups at 8 weeks post-surgery. In the control and sham-operated groups, the surface of the cartilage was resilient and smooth, with no cracks, softening, or osteophyte formation. However, the cartilage of the rats in the osteoarthritis group had lost the original luster and showed obvious roughness, osteophyte formation and cracking. The articular surface also appeared opaque.

reaction conditions were as follows: 95˚C for 20 sec; 50.4˚C for 20 min; 95˚C for 60 sec; 95˚C for 15 sec with 40 cycles of 55˚C for 15 sec and 74˚C for 45 sec. The relative expression levels of AQP-1 and caspase-3 were calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described (13).

Determination of caspase-3 activity. Total protein was extracted from the articular cartilage of the rats in each group or the cultured chondrocytes. Protein concentrations were determined using the BCA Protein Assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Caspase-3 activity was measured using the Caspase-3 Colorimetric Assay kit (Nanjing KeyGen Biotech Co., Ltd.). The plates were read at 405 nm using a microplate spectrophotometer (Model 680; Bio-Rad, Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data are presented as the means ± standard deviation. One-way analysis of variance (ANOVA) was used to compare the differences among groups, followed by the post hoc Student-Newman-Keuls tests. Pearson's correlation analysis was used to evaluate the association between the expression of AQP-1 and caspase-3 expression or activity. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cartilage damage in our rat model of osteoarthritis. In the control and sham-operated groups, the surface of the cartilage was resilient and smooth, showing no cracks, softening, or osteophyte formation at 8 weeks group (Fig. 1A and B). At 8 weeks post-surgery, synovial hyperplasia was observed in the osteoarthritis group. The cartilage of the rats in the osteoarthritis group had lost the original luster and showed obvious roughness, osteophyte formation and cracking. The articular surface also appeared opaque (Fig. 1C).

Morphology of cultured chondrocytes from rats in our model of osteoarthritis. The chondrocytes isolated from the rats in our model of osteoarthritis were spherical in shape with strong refractivity and became triangular or polygonal in shape following adherence to the culture surfaces. The cell nuclei were round or oval with 1-3 nucleoli and located in the center of the cells. Following culture for 3 days, the cells were clustered and

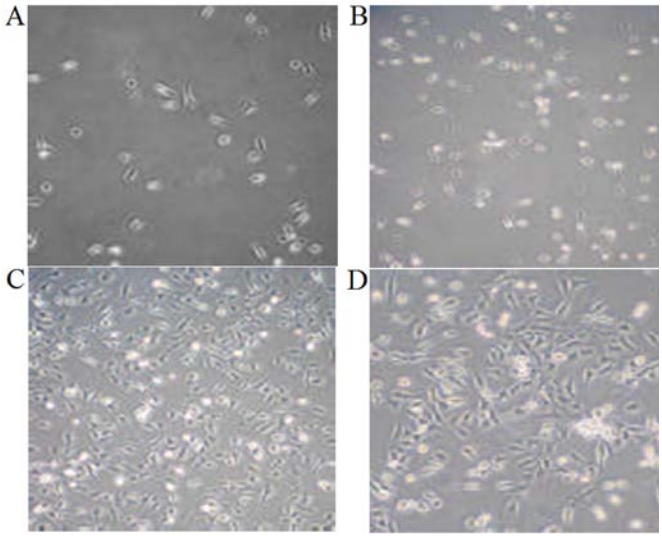

Figure 2. Morphology of cartilage cells at (A) after 1 day of culture, (B) after 3 days of culture, (C) after 5 days of culture, and (D) after 7 days of culture, as shown under an optical microscope (magnification, x100).

grew in a round or oval shape. Following culture for 5 days, the cells overlapped and proliferated significantly to form connections between cells. Matrix materials were deposited around the cells. The cells grew in a monolayer and covered the bottom of the culture bottle following culture for 1 week (Fig. 2).

H&E staining indicated that the cartilage cells at the 4th passage were triangular or polygonal. The nuclei had double or multiple nucleoli. The ECM was stained red (Fig. 3A and B). Upon Alcian blue staining, the cytoplasm and cell membrane were stained dark blue, suggesting that the cultured chondrocytes synthesized and secreted proteoglycans (Fig. 3C and D). In addition, oositive immunofluorescence staining for type II collagen indicated that chondrocyte-specific type II collagen was mainly distributed in the cytoplasm and cell membrane (Fig. 4A-C).

Knockdown of AQP-1 decreases the expression of caspase-3 in cultured chondrocytes. At 48 h post-transfection, green fluorescence was clearly observed in the transfected cells (Fig. 4D), indicating the success of the transfection. Flow cytometry revealed that fluorescent cells represented 41.9% of all cells (Fig. 5B). The expression of AQP-1 was significantly decreased in the cells transfected with AQPl-1-pGenesil-1

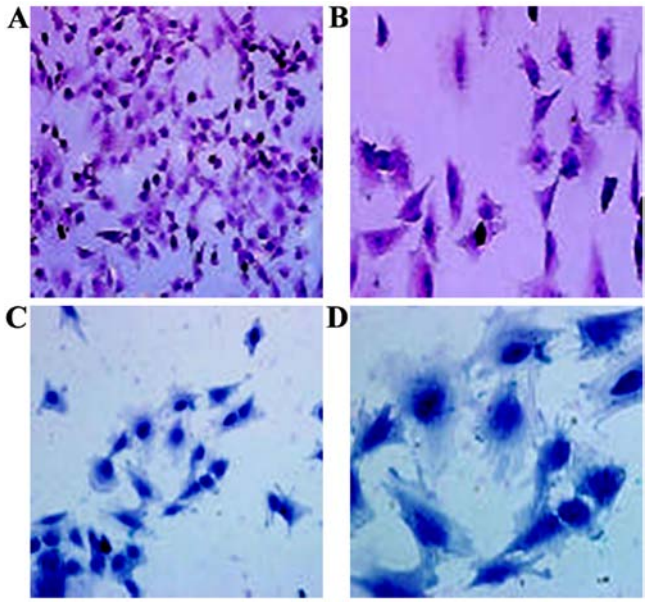

Figure 3. (A and B) Representative images of hematoxylin and eosin staining of cultured chondrocytes from rats with osteoarthritis. (A) Magnification, x200; (B) magnification, x400. (C and D) Representative images of Alcian blue staining of cultured chondrocytes from rats with osteoarthritis. (C) Magnification, x200; (D) magnification, x400.

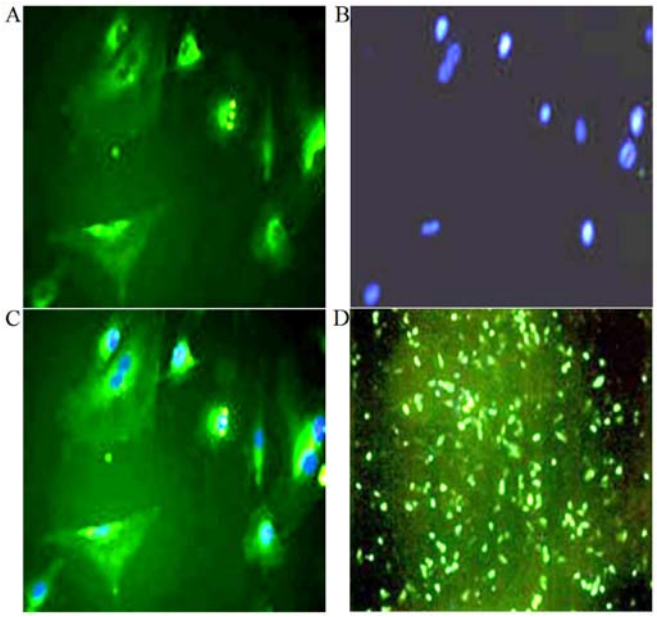

Figure 4. (A-C) Representative images of immunofluorescence staining of type II collagen in cartilage cells (magnification, x200). (A) Type II collagen; (B) DAPI; and (C) merged image. (D) Green fluorescent protein detected by fluorescence microscopy in cultured chondrocytes transfected with AQP-1-pGenesil.

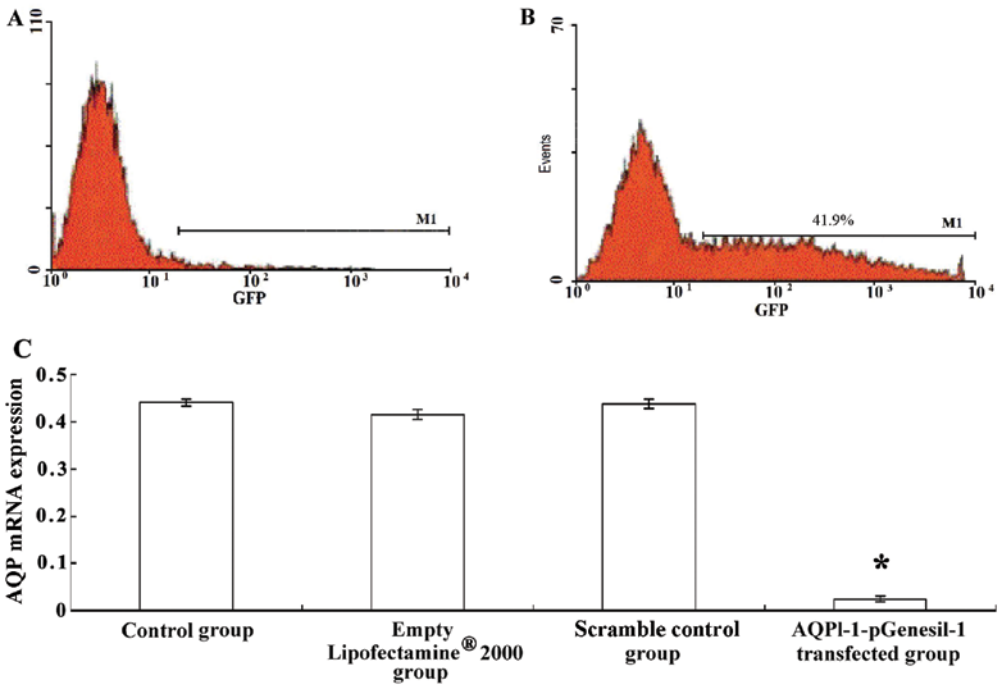

Figure 5. Positive transfection rate detected by flow cytometry. (A) Transfected cartilage cells with (A) no GFP expression; (B) transfected cartilage cells with GFP expression. (C) AQP-1 mRNA expression in control cells, cells treated with Lipofectamine® 2000 alone, cells transfected with scrambled shRNA, and cells transfected with AQPl-1-pGenesil-1. *P<0.01 vs normal control group, Lipofectamine® 2000 group, and scrambled shRNA group. GFP, green fluorescent protein; AQP, aquaporin.

compared with that in untransfected control cells, cells treated with Lipofectamine® 2000 alone, and cells transfected with scrambled shRNA (P<0.01, Fig. 5C). In addition, the expression and activity of caspase-3 were significantly decreased in the cells transfected with AQPl-1-pGenesil-1 (P<0.05, Fig. 6A and B).

Correlation between the expression of AQP-1 and caspase-3 expression and activity. Pearson's correlation analysis was used to evaluate the association between the expression of AQP-1 and caspase-3 expression or activity. The expression of AQP-1 positively correlated with the expression of caspase-3 (Fig. 6C,

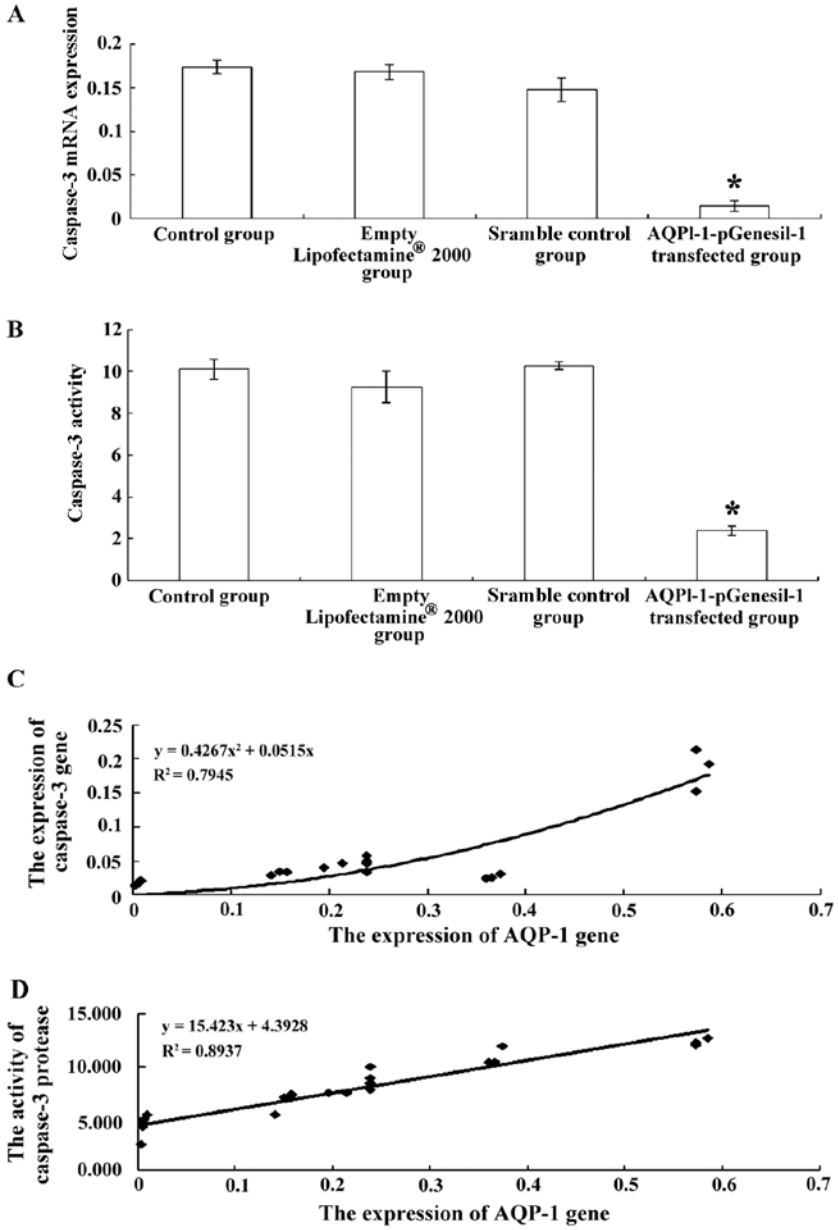

Figure 6. (A) Caspase-3 mRNA expression in control cells, cells treated with Lipofectamine® 2000 alone, cells transfected with scrambled shRNA, and cells transfected with AQPl-1-pGenesil-1. *P<0.01 vs. normal control group, Lipofectamine® 2000 group, and scrambled shRNA group. (B) Caspase-3 activity in control cells, cells treated with Lipofectamine® 2000 alone, cells transfected with scrambled shRNA, and cells transfected with AQPl-1-pGenesil-1. *P<0.01 vs. normal control group, Lipofectamine® 2000 group, and scrambled shRNA group. (C) Correlation between the expression of AQP-1 and the expression of caspase-3 and, (D) correlation between the expression of AQP-1 and caspase-3 activity in cultured chondrocytes. (C) Curve fitting was carried out regression analysis using the following equation: $y=0.4267x^2+0.0515x$ ($R^2=0.7945$, P<0.001). (D) Curve fitting was carried out regression analysis using the following equation: $y=15.423x+4.3928$ ($R^2=0.8937$, P<0.001).

r=0.817; P<0.001) and with caspase-3 activity (Fig. 6C, r=0.945; P<0.001).

Discussion

Osteoarthritis is regarded as a pathological condition resulting from the degeneration or destruction of the articular cartilage that covers and protects the moving joints. However, the role of chondrocytes in osteoarthritis remains unclear. We have previously demonstrated that the expression of AQP-1 is upregulated in a rat model of osteoarthritis, accompanied by an increase in caspase-3 expression and activity (9). In the present study, we found that the knockdown of AQP-1 in chondrocytes from rats with osteoarthritis decreased caspase-3 expression and activity, and that the expression of AQP-1 positively correlated with caspase-3 expression and activity, suggesting that AQP-1 contributes to chondrocyte apoptosis and to the development of osteoarthritis.

Chondrocytes are the major regulators of the process of matrix anabolism and catabolism and, thus, are essential to maintain the homeostasis of the cartilage matrix. Chondrocytes not only synthesize the ECM, but also play a direct role in the degradation process termed as 'chondrocytic chondrolysis'. Injured chondrocytes fail to degrade the damaged matrix in osteoarthritic cartilage and, thus, contribute to the irreversible pathological process of osteoarthritis (3). Chondrocyte apoptosis

has been found to be a major contributor to the progression of osteoarthritis (14-17). Caspase-3 protease is considered to be a killer protease and a key apoptosis mediator that mediates the terminal phase of apoptosis induced by death receptors or through the mitochondrial pathway (18). Several lines of evidence have indicated that activated caspase-3 protease will lead to irreversible apoptosis, and thus, the activation of caspase-3 is regarded as a molecular marker of apoptosis (19-22). In the present study, the significantly upregulated expression of caspase-3 was found in chondrocytes from rats with osteoarthritis, suggesting that chondrocyte apoptosis plays an important role in osteoarthritis.

It has been reported that the apoptotic volume decrease (AVD) is a common apoptotic pathway in various cells of many species and is a common reaction of cells to apoptosis inducers (23). AVD is mainly caused by the flow of monovalent cations though cation channels and alterations in water permeability via AQPs (24-28). It has been demonstrated that water and ion channels play a certain role in cell apoptosis in the central nervous system, and the expression levels of AQPs, potassium channels and chloride channels contribute to the initiation and progression of apoptosis (27). The outflow of water molecules mediated by AQPs has been reported to be one of the preconditions of AVD (25). In addition, the altered expression of AQPs in the mitochondria has been found during the apoptotic process (28). Furthermore, the activation of AQPs followed by mitochondrial swelling has been reported to induce the release of cytochrome *c* and the activation of caspase enzymes (28). It has also been reported that the overexpression of AQP-1 activates intracellular caspase-3 and induces apoptosis *in vitro* (24,25). In addition, a decrease in the expression of AQP-1 in hepatocellular carcinoma cells has been shown to be associated with resistance to apoptosis (29). Li *et al* reported that α-melanocyte-stimulating hormone reduced renal tubular epithelial cell apoptosis and prevented the downregulation of AQPs and Na^+-K^+ ATP enzymes in rats with bilateral ureteral obstruction (30). The inhibition of AQP-1 by mercuric chloride ($HgCl_2$) has been reported to induce a decrease in AVD and caspase-3 activity (27). Consistent with the literature, in this study, we found that AQP-1 was upregulated in rats with osteoarthritis, and that the expression of AQP-1 positively correlated with caspase-3 expression and activation in chondrocytes, suggesting that AQP-1 promotes chondrocyte apoptosis via the activation of caspase-3.

The findings that the knockdown of AQP-1 significantly decreased the expression of caspase-3 and activity further confirmed that the upregulation of AQP-1 expression activated caspase-3, and thus contributed to chondrocyte apoptosis and to the development of osteoarthritis.

In conclusion, we previously found that the expression of AQP-1 was upregulated in the osteoarthritic cartilage and that it strongly correlated with caspase-3 expression and activity (9). In the present study, we further found that the inhibition of AQP-1 expression using shRNA decreased the expression of caspase-3 in chondrocytes from rats with osteoarthritis, suggesting that AQP-1 participates in the process of chondrocyte apoptosis, and thereby contributes to the development of osteoarthritis.

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