African Journal of Biotechnology Vol. 11(27), pp. 7094-7097, 3 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3385
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

L-Monomethyl-arginine decreases apoptosis of chondrocytes by altering *Bax* and *Bcl-2* expression in osteoarthritis of rabbit knee

Zong-bao Wang[1,2], Qing-you Lu[3]*, Zeng-chun Li[3], Zhao-hui Chen[2], Wei-ming Liao[4], Guang-zhi Kuang[1] and Zhuo-peng Wu[1]

[1]Science Research Base of Kaiping Central Hospital, Postdoctoral Mobile Station of Sun Yat-sen University, Kaiping 529300, People's Republic of China.
[2]Clinical School of Acupuncture and Orthopaedics, Anhui University of Traditional Chinese Medicine, Hefei 230038, People's Republic of China.
[3]Department of Traumatic Surgery, East Hospital Affiliated to Tongji University, Shanghai 200120, People's Republic of China.
[4]Department of Orthopedic, the First Hospital Affiliated to Sun Yat-sen University, Guangzhou 510700, People's Republic of China.

Accepted 27 January, 2012

Previous studies found that NG-monomethyl-L-arginine (L-NMMA) treatment inhibits progression of osteoarthritis. Here, we aimed to explore the effects of L-NMMA on chondrocyte apoptosis and *Bax* and *Bcl-2* mRNA expression in rabbits with knee osteoarthritis. Knee osteoarthritis was induced in 24 healthy rabbits by Hulth method, and rabbits were randomly divided into control (*n* = 12) and experimental (*n* = 12) groups. Once weekly, knee joints of control rabbits were injected with saline solution, while knees of experimental rabbits were injected with L-NMMA. Knee joint samples were collected after 6 weeks of treatment. Apoptosis of chondrocytes was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and *Bax* and *Bcl-2* mRNA expression by *in situ* hybridization. The results show that the mean rate of chondrocyte apoptosis in knees of the experimental rabbits was significantly lower than that of the control rabbits (*P*<0.05). Additionally, Bax expression decreased and *Bcl-2* expression increased in the experimental group (*P*<0.05). In brief, L-NMMA can inhibit apoptosis of joint chondrocytes through changes in the expression of apoptosis-related genes. Thus, this molecule offers the potential for treating osteoarthritis.

Key words: NG-Monomethyl-L-arginine, knee osteoarthritis, chondrocyte, apoptosis-related regulatory gene.

INTRODUCTION

Osteoarthritis (OA) is a chronic, progressive osteoarthropathy that poses a health hazard in aging individuals. OA pathology includes articular cartilage degeneration, characterized by degradation of cartilage matrix and reduction in chondrocytes (Lories and Luyten, 2011). One report analyzing the ultrastructure of cartilage in OA (Weiss and Mirow, 1972) linked an increased number of degraded chondrocytes to disease severity. A subsequent study of a rabbit knee OA model by Hashimoto et al. (1998), reported an increase in apoptosis of chondrocytes with accompanying pannus formation; specifically, 28.7% of chondrocytes were apoptotic in OA-affected articular cartilage. Further, apoptotic cells were located in the cartilage surface and middle layer. In contrast, only 6.7% of chondrocytes were apoptotic in normal cartilage, located only in the cartilage surface. Similarly, using TUNEL and flow cytometry, Blanco et al. (1998) determined that apoptotic cells accounted for 11 to 22% of OA articular cartilage, but only

*Corresponding author. E-mail: zongbao_wang@126.com. Tel: +86-021-38804518.

2 to 4% of normal cartilage. Staining of cartilage sections confirmed the presence of apoptotic cells in both the surface and middle layer, as well as identifying proteoglycan depletion. Indeed, the number of apoptotic cells was significantly and positively correlated with OA severity.

In a follow-up study, the same group induced apoptosis of rabbit articular chondrocytes using varying concentrations of sodium nitroprusside over different time periods. They found that the exogenous nitric oxide (NO) produced by sodium nitroprusside induced apoptosis of cultured chondrocytes, indicating that NO plays an important role in chondrocyte apoptosis (Blanco et al., 1995). Injecting collagenase into upper compartments of the bilateral temporomandibular joints, Gao et al. (2003) established a goat OA model. They then injected the joints with 0.5 ml of 0.5% L-monomethyl-arginine (L-NMMA), an NO synthase inhibitor, in 3-day intervals. After one week OA progression was inhibited in L-NMMA-treated joints, likely by indirect inhibition of NO synthesis. However, additional studies are needed to confirm the inhibitive effect of L-NMMA on chondrocyte apoptosis. Here, we investigated the mechanisms behind the therapeutic effects of L-NMMA on OA in rabbit knees by assessing apoptosis and *Bax* and *Bcl-2* expression in chondrocytes.

MATERIALS AND METHODS

Experimental animals

Twenty-four healthy adult New Zealand rabbits of both sexes were obtained from the Experimental Animal Center of Wuhan University. Rabbits weighed from 2.8 to 3.5 kg, with a mean body weight of 3.2 ± 0.2 kg. These were caged for one week before experimentation to assure no obvious abnormalities were observed before inclusion. Laboratory temperature was 22 ± 2°C with relative humidity of 60%. Rabbits were fed regular diets with free access to water.

Rabbit knee OA models were established as in previous studies (Hulth et al., 1970). Briefly, skin on the right knee was shaved one day before modeling. Prior to surgery, rabbits were anesthetized with 3% amyl sodium pentobarbital (30 mg/kg) via ear vein, and then incised from the patella medial site. The medial collateral ligament was cut to open the knee joint cavity, then the anterior and posterior cruciate ligaments were cut with ophthalmic scissors and the medial meniscus was resected. Surgery did not damage the articular cartilage surface. After complete hemostasis, the wound was sutured by layer. Limb fixation was not needed after surgery. Rabbits were randomized to either the control or experimental groups, with 12 in each group. One week after surgery, rabbits were treated with either 0.3 ml saline (control) or 0.3 ml 0.5% L-NMMA (experimental; Sigma Chemical Co., St. Louis, MO) via cavity puncture of the right knee joint once per week. After 6 weeks of treatment, rabbits were killed by air embolism, the right knees were opened, and the lateral tibial plateau of the right upper end was removed. Samples were fixed and paraffin embedded for serially sectioning with thickness of 4 μM.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL reactions were performed according to the manufacturer's instructions, with some modifications (Roche, Mannheim, Germany). Briefly, sections were dewaxed prior to digestion with 20 mg/ml trypsin for 15 min at room temperature. Following treatment with 3% H_2O_2 solution for 5 min, slides were placed in 50 μL balanced fluid for 1 min, to which 15 μL reaction solution containing TDT enzyme was added. Reaction proceeded at 37°C for 1.5 h before being stopped with reaction-termination solution. Slides were rinsed with buffer solution twice for 15 min each. Slides were incubated with 20 ml/L normal goat serum for 30 min and then anti-digoxigenin alkaline phosphatase antibody in a humid chamber for 2 h. Following rinses with buffer 1 and buffer 3, freshly-prepared DAB color reagent was added to develop the color. After visualization, color development was halted, slides were washed and stained with hematoxylin and finally sealed with neutral gum.

In situ hybridization (ISH)

Bax and *Bcl-2 in situ* hybridization detection kits were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, US). Following dewaxing and treatment with 3% H_2O_2 solution for 10 min at room temperature, samples were digested for 5 min by pepsase diluted with 3% citric acid. Then 20 μL pre-hybridization solution were added and slides were placed in a humidified chamber for incubation at 37°C for 2 h. Next, 20 μL hybridization solution were then added and slides were incubated at 37°C overnight. Following incubation with blocking solution at 37°C for 30 min, slides were treated with biotinylated mouse anti-digoxigenin at 37°C for 60 min. Streptavidin--biotin complex (SABC) was incubated on slides at 37°C for 20 min, then biotinylated peroxidase was added for 20 min, and finally 3,3´-diaminobenzidine (DAB) was used to develop color for 10 min. Slides were rinsed with tap water for 10 min and then stained with hematoxylin and sealed with neutral gum. Positive staining results were interpreted as appearance of brown tinting in the cytoplasm and nucleus. Sections without probes were used as negative control. Staining was visualized under light microscope (400×) and analyzed with Image-pro Plus6.0 image processing system (Media Cybernetics, Inc. Bethesda, MD, US). Ten different fields were selected for each section. *Bax* and *Bcl-2* mRNA expression in chondrocytes was assessed by positive area and grayscale values (maximum grayscale was set as 255 for white, and minimum grayscale was set as 0 for black; smaller grayscale indicates higher intensity of positive reaction/expression).

Statistical methods

SPSS13.0 statistical software was used for statistical analysis. Results are expressed as mean± standard deviation. Independent sample χ^2 test was used to compare apoptosis of chondrocytes or *Bax* and *Bcl-2* mRNA expression between different groups. Two-sided test was used, with α level of 0.05 and $P < 0.05$ considered statistically significant.

RESULTS

Reduced apoptosis of chondrocytes following L-NMMA treatment

In both L-NMMA-treated and untreated OA rabbit knees, apoptotic nuclei appeared brown and were mainly distributed in the cartilage proliferation zone and shallow layer of hypertrophic cells. In the control group, 12.05 ± 1.17% of chondrocytes was apoptotic, primarily

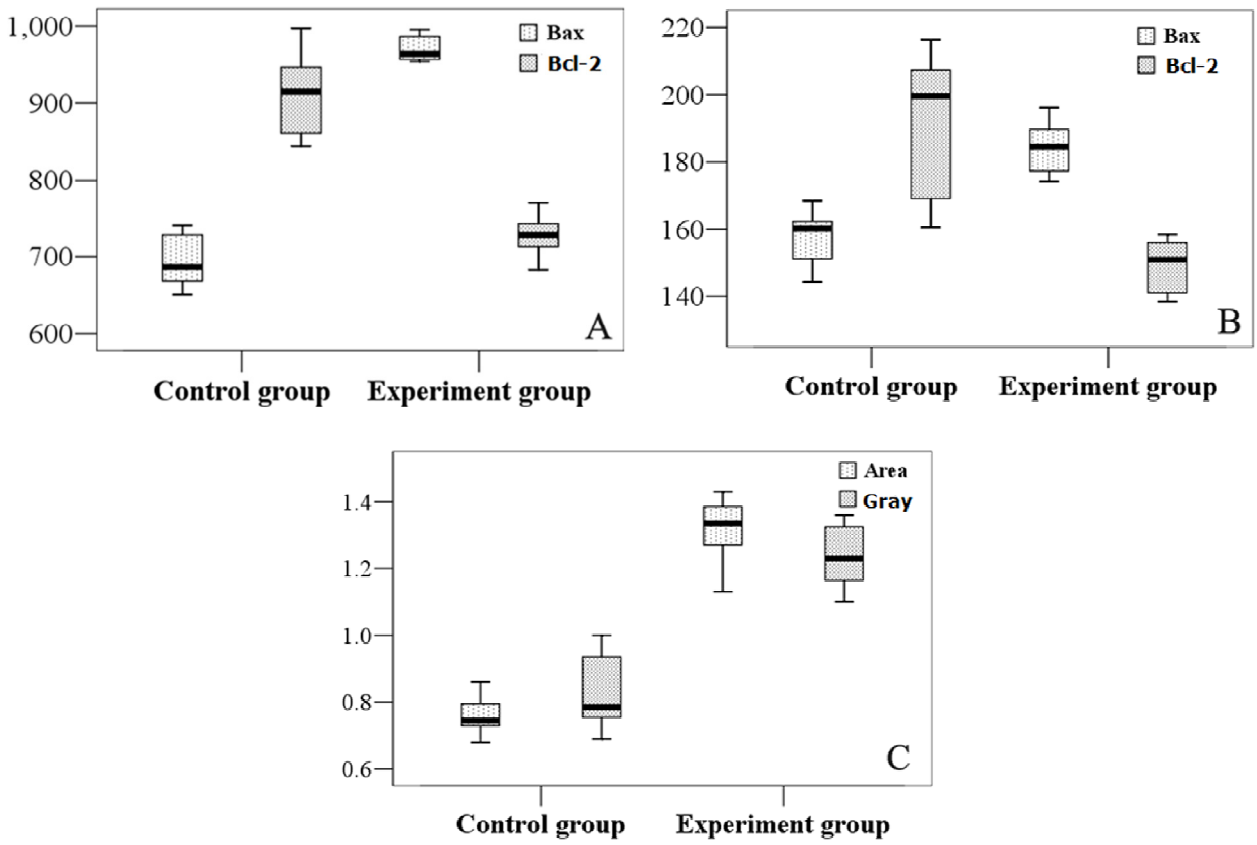

Figure 1. Altered expression of *Bax* and *Bcl-2* mRNA in chondrocytes following L-NMMA treatment. A, Expression area; B, expression grayscale; C, ratio of *Bax*/*Bcl-2*.

concentrated in the cartilage proliferation zone. However, in experimental rabbits treated with L-NMMA, the number of apoptotic cells was significantly reduced [5.09 ± 0.31%, t=19.851, *P*=0.001] and cartilage chondrocyte layers were maintained.

Altered Bax and Bcl-2 expression following L-NMMA treatment

We next explored the mechanism behind this decreased apoptosis following L-NMMA treatment. Using image analysis software to quantify ISH signals, we measured the mRNA expression area of apoptosis-related genes *Bax* (pro-apoptotic) and *Bcl-2* (anti-apoptotic). Mean *Bax* expression area was reduced following L-NMMA treatment measured as 912.22 ± 50.99 in the controls and 728.39 ± 26.00 in the experimental rabbits (Figure 1A). Mean expression areas of *Bcl-2* were 693.65 ± 32.36 and 958.23 ± 38.95 in the control and experimental groups, respectively (Figure 1B). Thus, the ratio of *Bax*/*Bcl-2* expression area was significantly altered from (0.76 ± 0.06) in the controls to (1.32 ± 0.09) in the experimental group (Figure 1C, t = 18.182, P = 0.001). Statistical analysis showed that compared with the control group, the expression area of *Bax* in experimental group was significantly reduced (t = 18.327, P = 0.001), while *Bcl-2* expression area was significantly increased (t = 11.126, P = 0.001).

Expression intensity of *Bax* and *Bcl-2* in chondrocytes was measured via ISH grayscale values (higher value = less intensity). Grayscale values for *Bax* expression were 157.88 ± 7.79 and 184.37 ± 7.36 in the control and experimental groups, respectively reflecting a significant increase in *Bax* expression in the experimental group (Figure 1B; t = 8.566, P = 0.001). Grayscale values for *Bcl-2* were (190.88 ± 20.17) and (149.13 ± 7.83), respectively for the control and experimental groups, reflecting a significant reduction in *Bcl-2* expression in experimental rabbit knees (Figure 1B; t = 6.687, P = 0.001). The ratio of *Bcl-2/Bax* grayscale intensity was therefore 1.21 ± 0.15 for the controls and 0.81 ± 0.06 for the experimental rabbits, with a statistically significant difference (Figure 1C; t = 8.555, P=0.001).

DISCUSSION

The pathogenesis of OA is characterized by degenerative changes in articular cartilage, followed by proliferation of

adjacent cartilage and ossification. Recent studies of OA pathogenesis mainly focused on apoptosis of the chondrocyte itself (Ishoguro et al., 2002; Loeser, 2006). Farrell et al. (1992) found that a large amount of the NO metabolite nitrite could be detected in synovial fluid and serum of OA patients, suggesting that NO may be involved in the pathology of OA. Meanwhile, *in vitro* studies (2005) found that high concentrations of NO cause apoptosis or necrosis of chondrocytes. Thus, inhibition of chondrocyte apoptosis by preventing NO production may offer a new approach in treating OA. In addition, two molecules, L-NMMA and L-arginine, may compete for NO synthase binding sites, thereby inhibiting NO synthase and indirectly preventing NO production. In a study by Amin et al. (2000) in which L-NMMA was used to treat arthritis, L-NMMA effectively reduced NO, ameliorating arthritis swelling and tissue damage. Similarly, administering L-NMMA to OA rat models by intraperitoneal injection resulted in reduced swelling and redness (McCartney-Francis et al., 2001).

Here, we used a rabbit knee OA model to investigate the mechanisms behind the effects of L-NMMA treatment. TUNEL staining revealed an obvious decrease in apoptosis of chondrocytes, with a corresponding increase in expression of *Bcl-2* (anti-apoptotic) and decrease in *Bax* (pro-apoptotic) expression. Thus, in inhibiting NO synthase, L-NMMA can inhibit apoptosis of knee chondrocytes, thus resulting in a protective effect whereby degeneration of articular cartilage is delayed. However, the repair of cartilage defects is a regulatory process involving multiple factors, and inhibition of excessive release of NO is only one aspect of this process. NO is a signal transmission molecule with broad functions in the body, and normal physiological concentrations of NO may be interrupted by L-NMMA administration, potentially producing serious side-effects (McCartney-Francis et al., 2003). Therefore, further studies on NO inhibitors are needed to develop more specific drugs before these molecules can be used as effective therapy for cartilage repair in OA.

REFERENCES

Amin AR, Dave M, Attur M, Abramson SB (2000).COX-2, NO, and cartilage damage and repair. Curr Rheumatol Rep. 2(6): 447-453.

Blanco FJ, Guitian R, Vázquez-Martul E, de Toro FJ, Galdo F (1998). Osteoarthritis chondrocytes die by apoptosis: a possible pathway for osteoarthritis pathology. Arthritis Rheum. 41: 284-289.

Blanco FJ, Ochs RL, Schwarz H, Lotz M (1995). Chondrocyte apoptosis induced by nitric oxide. Am. J. Pathol. 146: 75-85.

Farrell AJ, Blake DR, Palmer RM, Moncada S (1992). Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. Ann. Rheum. Dis. 51(11): 1219-1222.

Gao ZW, Hu J, Wang DZ, Li JH (2003). The effects of L-NMMA on experimental temporomandibular joint osteoarthrosis in goats. Zhonghua Kou Qiang Yi Xue Za Zhi 38(4): 295-297.

Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M (1998). Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum. 41(7): 1266-1274.

Hulth A, Lindberg L, Telhag H (1970). Experimental osteoarthritis in rabbits: preliminary report. Acta Orthop Scand. 41(5): 522-530.

Ishiguro N, Kojima T, Poole AR (2002). Mechanism of cartilage destruction in osteoarthritis. Nagoya J. Med Sci. 65(3-4): 73-84.

Kim HA, Lee KB, Bae SC(2005). The mechanism of low-concentration sodium nitroprusside-mediated protection of chondrocyte death. Arthritis Res. Ther. 7(3): R526-535.

Loeser RF (2006). Molecular mechanisms of cartilage destruction: Mechanics, inflammatory mediators, and aging collide. Arthritis Rheum. 54(5): 1357-1360.

Lories RJ, Luyten FP (2011). The bone-cartilage unit in osteoarthritis. Nat. Rev. Rheumatol. 7(1): 43-49.

McCartney-Francis NL, Song X, Mizel DE, Wahl SM (2001). Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. J Immunol .166(4): 2734-2740.

McCartney-Francis NL, Chan J, Wahl SM (2003). Inflammatory joint disease: clinical, histological, and molecular parameters of acute and chronic inflammation and tissue destruction. Methods Mol. Biol. 225: 147-159.

Weiss C, Mirow S (1972). An ultrastructural study of osteoarthritis changes in the articular cartilage of human knees. J. Bone. Joint. Surg. Am. 54(5): 954-972.