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Methylene blue relieves the development of osteoarthritis by upregulating lncRNA MEG3

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Authors: Xinyi Li, Chaoliang Tang, Jin Wang, Peipei Guo, Chengyao Wang, Yanlin Wang, Zongze Zhang, Huisheng Wu

View Affiliations

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Abstract

Methylene blue (MB) is a long-term inhibitor of peripheral nerve axons, thereby alleviating or permanently eliminating pain. However, it remains unknown whether MB

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joint following injection of 1 mg/kg MB at 1, 4, 8 and 24 weeks post-injection. Compared with the vehicle control, MB treatment significantly enhanced the weight distribution and significantly decreased the swelling ratio of the rabbits. Additionally, levels of long non-coding RNA (lncRNA) maternally expressed 3 (MEG3) mRNA were significantly increased following treatment with MB, but the protein expression of P2X purinoceptor 3 (P2X3) was significantly suppressed compared with the vehicle control. The levels of interleukin (IL) 6, tumor necrosis factor (TNF)α, IL-1β and IL-8 were significantly suppressed following MB treatment, indicating that MB protects against OA progression. It was also revealed that MEG3 overexpression significantly suppresses levels of P2X3 protein. ELISA indicated that the MEG3-induced reduction of IL-6, TNFα, IL-1β and IL-8 expression was significantly reversed following P2X3 overexpression. Therefore, the results of the present study demonstrated that MB is an effective method of treating OA-associated pain by upregulating lncRNA MEG3 levels. Additionally, lncRNA MEG3 relieves the OA-associated pain and inflammation in a rabbit model of OA by inhibiting P2X3 expression.

Introduction

Osteoarthritis is the most common type of joint disease; patients may develop stiff joints which are painful to move (1,2). Chronic pain is a major problem for millions of patients with OA (3) and the key focus of OA treatment is to reduce pain and improve joint function. However, for older patients, relieving pain is considered to be more important than improving joint function (4,5). For moderate and severe OA, long-term oral medication is not effective (6). Joint replacement therapy may be effective at treating OA in certain cases; however there are a number of problems, including trauma, high cost and the risks associated with surgery (7). To improve the therapeutic options available for patients with OA, it is necessary to explore alternative safe and effective treatment methods (8–10).

Methylene blue (MB) is an anti-oxidative and anti-inflammatory agent, which is used to treat clinical pain syndromes, malaria and psychotic disorders (11,12). Previous studies have demonstrated that MB exhibits a strong affinity for nerve tissue and may used as a long-term inhibitor of peripheral nerve axons, thus alleviating pain in patients with OA (13–15) Neuropathic pain is a type of chronic pain caused by nervous system damage and dysfunction (16). The pathogenesis of chronic pain is complicated; recent studies have suggested that the activation of P2X purinoceptor 3 (P2X3) receptors serve a key role during the progression of chronic pain conditions (17,18).

Long non-coding RNAs (lncRNAs) are long transcription RNAs containing >200 nucleotides (19). It has been demonstrated that the pathogenesis of OA is closely associated with aberrantly expressed lncRNAs, including HOX transcript antisense RNA (HOTAIR), lncRNA-co-repressor interacting with RBPJ, 1 (CIR), lncRNA-H19, imprinted maternally expressed transcript (H19) and lncRNA-maternally expressed 3 (MEG3) (19–21). It has been suggested that there is a negative correlation between decreased lncRNA MEG3 and vascular endothelial growth factor levels in patients with OA (22). However, to the best of our knowledge, whether MB treatment regulates the expression of MEG3 in the progression of OA has never been explored.

In the present study, the effects of MB on the expression of lncRNA MEG3 in the articular cavity were evaluated. The results revealed that the expression of MEG3 was increased following MB treatment and further investigation demonstrated that the enhanced expression of lncRNA MEG3 inhibited the expression of P2X3, thereby suppressing pain and inflammation in a rabbit model of OA.

Materials and methods

Animal model of OA

A total of 120, specific pathogen free, male New Zealand white rabbits (11–12 weeks; weighing 2.1–2.3 kg) were used to investigate the effects of MB on the pathogenesis of OA. All animals were purchased from the animal center of the Zhongnan Hospital of Wuhan University (Wuhan, China). Rabbits were kept in a controlled environment with a

approved by the Ethics Committee of the Zhongnan Hospital of Wuhan University.

Animals were anesthetized with intravenous pentobarbital (32.4 mg/kg) prior to 1–4% isoflurane administered via inhalation to maintain anesthesia, followed by subcutaneous infusion of lidocaine (~3 ml) in the right leg during surgery. The rabbits were under general anesthesia during this process. The meniscus of the right leg was completely removed. Briefly, the boundary between the patellar ligament and the articular capsule of the right hind leg and the lateral-collateral ligament were dissected. The articular capsule was subsequently removed to expose the interior meniscus and the meniscus was removed. Following total meniscectomy of the right knee joint, rabbits were randomly divided into either a vehicle- or MB-treated group (n=10/group). Intra-articular treatment was initiated 7 days post-surgery. To evaluate the safety of MB in the articular cavity, different concentrations of MB [0.5, 1 and 2 mg/kg; cat. no. CAS:122965-43-9; Meryer (Shanghai) Chemical Technology Co., Ltd., Shanghai, China] or the same volume of vehicle control (saline) were injected into the articular cavity and the rabbits were euthanized after 1, 2, 4, 8, 12 or 24 weeks as a preliminary investigation performed prior to the other procedures. There were 30 rabbits in each group. For all subsequent studies, rabbits in the MB-treated group were administered with 1 mg/kg MB. The rabbits used for the preliminary experiment were the same ones as used in the primary study.

Measurement of pain

Changes in hind paw weight distribution between the right (OA model) and left (contralateral control) limbs were measured as a pain index using the previously described method (23). Hind-paw weight distribution was measured after 1, 2, 4, 8, 12 and 24 weeks. The percentage of weight distribution of the right hind paw was calculated using the following equation: Weight distribution of right hind paw (%)=[(weight of the right leg)/(weights of both legs)] ×100.

Measurement of swelling

At 42 days following meniscectomy, articular swelling was measured using a digital vernier caliper. The maximum widths of the right and left hind paws were measured and recorded. The percentage of swelling was calculated as follows: Swelling ratio (%)=[(width of the right knee-width of the left knee)/(width of the right knee+width of the left knee)] ×100.

Histopathological examination

Histopathological examination was performed as previously described (24). Rabbits were anesthetized and euthanized at 24 weeks post MB or saline treatment and the left paw of all rabbits in each group was cut above and below the 0.5 cm of the joints. To leave the synovial membrane intact, the muscle and skin of the joints were trimmed away. All tissues were treated with 3% hydrochloric acid (HCl) solution for 5–7 days and HCl was replaced every 24 h to allow complete decalcification of joints. Joints were subsequently fixed in 10% neutral buffered formalin for 2 days at room temperature. Decalcified joints of the rabbits were then dehydrated in an ascending series of alcohol and embedded in liquid paraffin. Embedded sections were sliced into 5-µm-thick sections and tissues were stained with hematoxylin and eosin (H&E) at room temperature for 3 min. Slides were the evaluated using a light microscope at magnification of ×40 (DXIT 1200; Nikon Corporation, Tokyo, Japan). H&E stained joint slides were examined for bone and cartilage destruction by synovial hyperplasia. For the estimation of synovial proliferation, the following scoring system was used: No change, 0; mild proliferation with 2–4 layers of synoviocytes, 1; moderate proliferation with ≥4 layers of synoviocytes and absent synovial cell invasion of adjacent connective tissue and bone with enhanced mitotic activity, 2; proliferation distinguished by adjacent cartilage and effacement of joint space, connective tissue and bone, 3.

Cell culture

Cell culture was performed as previously described (25). Human chondrogenic SW1353 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences,

atmosphere with 5% CO_2.

Western blot analysis

Total protein was isolated from articular tissues or SW1353 cells using a total protein extraction kit (cat no. KGP2100; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 20 µg protein was loaded per lane and separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were then blocked with 5% fat-free milk at room temperature for 2 h. The membrane was incubated with primary antibodies against P2X3 (cat. no, ab140870; 1:1,000; Abcam, Cambridge, UK) and ß-actin (cat. no. 4970; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA), which was used as a control at 4°C overnight. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (1:5,000; ZB-2301; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature and then washed three times using Tris-buffered saline and Tween-20. The signals were detected using a Super ECL Plus kit (Nanjing KeyGen Biotech Co., Ltd.) and quantified using a GelDoc-It® TS2 310 Imager with UVP software (UVP, LLC, Upland, CA, USA). The relative contents of protein were normalized against GAPDH and ImageJ software version 1.43b (National Institutes of Health, Bethesda, MD, USA) was used for densitometry analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from articular tissues or SW1353 cells using RNA TRIzol reagent (Life Sciences; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Total RNA was subsequently reverse transcribed to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The primers for RT-qPCR were designed using Primer3 version 0.4.0 (bioinfo.ut.ee/primer3-0.4.0/) and the Basic Local Alignment Search Tool was run for online specificity, which eliminated nonspecific amplification of PCR (ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). qPCR was performed using SYBR Green PCR Master mix (Roche Diagnostics, Basel, Switzerland) on an Applied Biosystems ViiA 7 Real-time PCR system (Thermo Fisher Scientific, Inc.). The final reaction volume was 10 µl and contained 5 µl SYBR Green PCR Master mix (2X), 0.5 ml forward and 0.5 µl reverse primers (10 mM), 2 ml cDNA and 2 µl double-distilled water. The sequences of all primers used are presented in Table I. The thermocycling conditions of qPCR were: Denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. The results were normalized to GAPDH expression to obtain ΔCq values and fold changes in expression were calculated using the $2^{-\Delta\Delta Cq}$ method (26).

Table I.

Primers used for reverse transcription-quantitative polymerase chain reaction.

ELISA

ELISA was performed out as previously described (27). In brief, frozen articular tissues (~100 mg) were homogenized in lysis buffer (50 mmol/l Tris-HCl, 300 mmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100 and 0.02% sodium azide) containing a protease inhibitor cocktail (Roche Diagnostics). Lysates were centrifuged at 16,000 × g for 15 min at 4°C and levels of TNF-α (cat no. DY5670), IL-6 (cat. no. DY7948) (both R&D Systems, Inc., Minneapolis, MN, USA), IL-1ß (cat. no. JEB-14488) and IL-8 (cat. no. JEB-14519) (both Nanjing Jin Yibai Biotechnology Company Ltd., Nanjing, China) in the supernatants were quantified using ELISA assays following the manufacturer's protocols. Samples were read at 450 nm using a microplate reader.

Transfection

Small interfering (si)RNA against lncRNA MEG3 (forward, 5′-GCUUGGGUUGUAUUGUGAAUGGUUU-3′ and reverse

were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Transfection of siMEG3 or NC was performed using the HiPerFect transfection reagent (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Briefly, 6×10^5 SW1353 cells/well were seeded in 6-well plates with 2 ml DMEM containing serum and antibiotics. A total of 5 µl siMEG3 (4 µM) and 5 µl non-specific siRNA (NC; 4 µM) for each well were mixed with the HiPerFect transfection reagent and incubated at room temperature for 10 min. The complex was subsequently added to the wells and the cells were incubated for a further 48 h prior to investigation.

Promoter reporter analysis

A promoter reporter assay was performed as previously described (28). The PGL3 promoter vector and the PRL-TK vector were purchased from Promega Corporation (Madison, WI, USA) and used as the internal control. The promoter region of P2X3 was amplified from the genomic DNA of rabbit articular cartilages. The amplification was performed using a TrasnDirect Animal Tissue PCR kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). In brief, 4 µl unpurified lysate was mixed with 0.4 µl forward primer, 0.4 µl reverse primer, 10 µl 2xTransDirect PCR SuperMix, and 5.2 µl ddH_2O. The PCR conditions were 94°C for 10 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 5 min. The primers for P2X3 were as follows: P2X3 forward, GGAATACCGCTGACACCCAA; and P2X3 reverse, GGAGCGGGCGAATCCATTAT. The PGL3 promoter vector and amplified fragments were digested by *Xho*I and *Kpn*I and purified by 2% agarose gel electrophoresis. The digested fragment was then inserted into the PGL3 vector upstream of the simian vacuolating virus 40 promoter. 293T cells (American Type Culture Collection) were co-transfected with the PGL3 vector and the PRL-TK vector using the VigoFect Transfection reagent (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China). Cells were harvested and lysed 48 h post-transfection. The relative light units were determined using the Dual-luciferase reporter assay system (Promega Corporation) according to the manufacturer's protocol. Normalized luciferase data (firefly/renilla) was compared with the empty PGL3-promoter vector. The primers for amplification were as follows: P2X3, forward, 5′-GGGTACCAAAGCCACAGGCAGAAACTACTA and reverse, 5′-CCTCGAGAGGAGGTAGGTGGTGGTCGT. The restriction sites for *Kpn*I and *Xho*I are underlined.

Construction of plasmid (pc)MEG3 and pcP2X3

To upregulate MEG3 in SW1353 cells, pcP2X3, pcMEG3 or blank pcDNA vectors were constructed by GenChem&GenPharm (Changzhou) Co., Ltd., Changzhou, China). In brief, 6×10^5 SW1353 cells/well were seeded in 6-well plates with 2 ml DMEM containing serum and antibiotics. After 24 h, the pcMEG3, blank pcDNA or pcP2X3 vectors were transfected into SW1353 cells for 48 h at a final concentration of 25 nM using the VigoFect Transfection reagent according to the manufacturer's protocol. The cells underwent transfection for 48 h and the cells were then collected for further study.

Statistical analysis

Comparisons were performed using Origin version 6.1 software (OriginLab Corporation, Northampton, MA, USA). A 2-tailed t-test was used to compare paired data and one-way analysis of variance followed by Tukey's post hoc analysis was used to compare multiple groups. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed a minimum of three times.

Results

Intra-articular injection of MB is safe

Rabbits were treated with 0.5, 1 or 2 mg/kg MB to evaluate its safety. The results revealed that MB treatment did not induce any changes in articular cartilage and the level of synovial hyperplasia (data not shown). In subsequent studies, 1 mg/kg MB was selected to evaluate the effects of MB on a rabbit model of OA. A total of 1 mg/kg MB was injected into the joint cavity of the rabbit knees. No notable morphological changes were observed in the rabbit articular cartilage at 1, 2, 4, 8, 12 and 24 weeks post-injection (Fig. 1A). The cartilage was intact and the chondrocytes and matrix were

following injection (Fig. 1C). At 24 weeks following injection, no lesions of the cruciate ligament or the meniscus were observed (Fig. 1D) and no notable damage was identified in the important histological structures of the knee joint. In addition, no histological characteristics of articular cartilage sections were observed in the MB group or the control group 24 weeks following injection (Fig. 1E). According to the synovial hyperplasia scoring system, no significant changes in synovial hyperplasia scores were identified between the MB and the control groups at 24 weeks following the injection of 1 mg/kg MB (Fig. 1F). These results suggest that the intra-articular injection of MB is safe in rabbits.

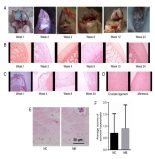


Figure 1.

Intra-articular injection of methylene blue is safe in rabbits. (A) No notable morphological changes were observed in rabbit articular cartilage at 1, 2, 4, 8, 12 and 24 weeks following MB injection. (B) The rabbit cartilage was intact and the chondrocytes and matrix were normal at all time points, as indicated by hematoxylin and eosin staining at magnification, ×100. (C) No synovial hyperplasia was observed at 1, 4, 8 or 24 weeks following injection of 1 mg/kg MB into the rabbit knee joint cavities at magnification, ×100. (D) No lesions of the cruciate ligament or meniscus were observed 24 weeks following injection of 1 mg/kg MB into the rabbit knee joint cavities at magnification, ×100. (E) No histological characteristics of articular cartilage sections were observed in the MB or control groups 24 weeks following the injection of 1 mg/kg MB. (F) There were no significant differences between the synovial hyperplasia scores of the control group and the MB group. MB, methylene blue; NC, normal control.

MB reduces the pain and inflammation induced by OA

The right paw weight distribution of the rabbits was investigated from weeks 0–24 (Fig. 2A). Rabbits in the vehicle and MB groups recovered from the surgical stress observed at week 1. Following week 2, the weight distribution of the right paw in the vehicle-treated group gradually decreased over time, suggesting that OA was induced by the meniscectomy. By contrast, weight distribution was significantly higher in the MB-treated group compared with the vehicle-treated group from week 4 onwards. These results indicate that MB decreases pain in a rabbit model of OA. The swelling ratio between the vehicle and MB-treated groups was also investigated. Compared with the vehicle group (13.5±6.3), the MB group exhibited a significantly reduced swelling ratio (2.4±3.5) at week 24 (Fig. 2B). These results indicate that MB suppresses the swelling caused by OA inflammation in a rabbit meniscectomy OA model and suggest that MB may be an effective method of treating pain and inflammation induced by OA.

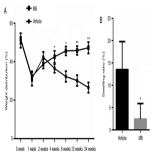


Figure 2.

MB reduces the pain and inflammation induced by OA. (A) Compared with the vehicle control, MB treatment significantly increased the weight distribution of the rabbits. (B) Compared with the vehicle group (13.5±6.3), MB significantly reduced the swelling ratio (2.4±3.5) in the rabbit model of OA after 24 weeks. *P<0.05 and **P<0.01 vs. vehicle control. MB, methylene blue; OA, osteoarthritis.

HOTAIR, lncRNA-CIR and H19 levels do not change following MB treatment in a rabbit model of OA

HOTAIR, CIR and H19 are associated with OA (19,21,29) and in the present study, it was evaluated whether MB treatment altered the expression of these lncRNAs. RT-qPCR was performed and the results revealed that the levels of HOTAIR, lncRNA-CIR and H19 were not significantly altered following MB treatment in a rabbit model of OA compared with the non-treated control group (Fig. 3).

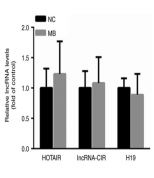


Figure 3.

Reverse transcription-quantitative polymerase chain reaction revealed that levels of HOTAIR, lncRNA-CIR and H19 are not significantly altered following treatment with MB in a rabbit model of osteoarthritis. HOTAIR, HOX transcript antisense RNA; CIR, long non coding RNA-co-repressor interacting with RBPJ, 1; H19, lncRNA-H19, imprinted maternally expressed transcript; MB, methylene blue; NC, normal control.

P2X3 expression is reduced and MEG3 mRNA expression is increased in MB-

The results of RT-qPCR revealed that the expression of lncRNA MEG3 was significantly increased in the MB group compared with the vehicle group (Fig. 4A). By contrast, western blot analysis indicated that the protein expression of P2X3 was significantly reduced in the MB-treated group compared with the vehicle treated group (Fig. 4B). Additionally, it was revealed that levels of the inflammatory factors IL-6, TNFα, IL-1β and IL-8 were all significantly reduced in the MB treated group compared with the vehicle treated group (Fig. 4C).

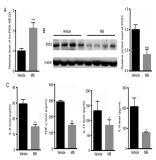


Figure 4.

MB treatment affects the cytokine expression in rabbit articular cartilage. (A) Reverse transcription-quantitative polymerase chain reaction indicated that levels of lncRNA MEG3 were significantly increased following MB treatment compared with the vehicle control. (B) Western blot analysis revealed that the expression of P2X3 was significantly suppressed in MB-treated rabbit articular cartilage compared with vehicle controls. (C) ELISA analysis demonstrated that the levels of IL-6, TNFα, IL-1β and IL-8 were significantly decreased following treatment with MB. *P<0.05, **P<0.01 and ***P<0.01 vs. the vehicle control. MEG3, maternally expressed 3; P2X3, P2X purinoceptor 3; MB, methylene blue; IL, interleukin; TNF, tumor necrosis factor; lncRNA, long non-coding RNA.

The upregulation of MEG3-reduced inflammation cytokine expression may be reversed by P2X3 in SW1353 cells

To further evaluate whether MB alleviates pain and inflammation by regulating MEG3 and P2X3, SW1353 cells were treated with pcMEG3 to upregulate MEG3 expression. Transfection with pcMEG3 for 48 h significantly increased the mRNA level of MEG3 in SW1353 cells compared with the control (Fig. 5A). The overexpression of P2X3 induced by the administration of pcP2X3 eliminated the significant reduction in P2X3 expression induced by MEG3 overexpression in SW1353 cells (Fig. 5B). ELISA indicated that the MEG3-reduced expression of IL-6, TNFα and IL-1β were reversed following P2X3 overexpression (Fig. 5C-E). It was also determined whether MEG3 and P2X3 reverse the effects of MB on the expression of IL-6, TNFα, IL-1β and IL-8. A specific siRNA targeting MEG3 was selected and RT-qPCR revealed that transfection with si-MEG3 significantly suppressed MEG3 mRNA expression in SW1353 cells (Fig. 5F). Furthermore, knockdown of lncRNA MEG3 and overexpression of P2X3 significantly reversed the MB-induced reductions of IL-6, TNFα, IL-1β and IL-8 levels in SW1353 cells (Fig. 5G and H). These results indicate that MB attenuates the progression of OA by enhancing lncRNA MEG3 and suppressing P2X3 expression.

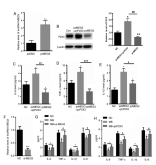
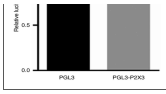


Figure 5.

MEG3-reduced inflammatory cytokine expression was notably reversed by P2X3 overexpression in SW1353 cells. (A) RT-qPCR revealed that transfection with pcMEG3 significantly enhanced levels of MEG3 lncRNA. (B) Western blot analysis demonstrated that P2X3 overexpression reversed the MEG3-induced reduction of P2X3 expression in SW1353 cells. RT-qPCR indicated that the MEG3-reduced expression of (C) IL-6, (D) TNFα and (E) IL-1β may be largely reversed by the overexpression of P2X3. (F) The relative expression of lncRNA MEG3 was determined following transfection with siMEG3. Levels of IL-6, TNF-α, IL-1β and IL-8 in SW1353 cells were measured following (G) the knockdown of MEG3 and (H) the overexpression of P2X3 in SW1353 cells. *P<0.05, **P<0.01 and ***P<0.001 vs. the NC; #P<0.05, ##P<0.01 and ###P<0.001. lnc, long non-coding; MEG3, maternally expressed 3; P2X3, P2X purinoceptor 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IL, interleukin; TNF, tumor necrosis factor; NC, normal control; siRNA, small interfering RNA; MB, methylene blue; pc, plasmid.

P2X3 is not a downstream target of MEG3

To elucidate the potential mechanism by which MEG3 modulates the expression of P2X3, the promoter region of P2X3 was cloned into a luciferase reporter vector. PGL3-P2X3 plasmids and the PRL-TK vector were transfected into 293T cells for 48 h. A dual luciferase reporter assay did not reveal any significant changes between the blank vector or the PGL3-P2X3 vector in 293T cells (Fig. 6). These results suggest that MEG3 does not directly regulate the transcription of P2X3; therefore further studies are required to explore the underlying mechanism by which MEG3 alters P2X3 expression.



The dual luciferase reporter assay did not reveal any significant changes between the blank vector or the PGL3-P2X3 vector in 293T cells. P2X3, P2X purinoceptor 3.

Discussion

MB induces long-term analgesic effects and is extensively applied in the clinic. MB may be locally applied in the perianal area where it suppresses peripheral nerve conduction, thereby relieving the long-term pain caused by surgery (30,31). Local injection of 0.2% MB suppresses long-term peripheral nerve medulla pain to the treat neurodermatitis (11,13). It has also been revealed that the injection of MB into the fracture space of patients with pelvic fractures who were treated with in-screw augmented sacroiliac screw fixation, provided an analgesic effect for ~3 weeks (32). However, to the best of our knowledge, it has not yet been explored whether MB may be applied to treat OA-associated pain.

Patients with OA can experience chronic pain, which has an impact on their quality of life (33,34). Pain signals are conveyed through the signal transduction and transmission of primary afferent neurons, spinal dorsal horn neurons and the central nervous system, including the cerebral cortex (35). In peripheral tissues, noxious stimulation may directly activate ion channels on sensory nerve endings, thus evoking receptor potentials (36). In addition, noxious stimulation may be caused by mediators released by epithelial and endocrine immune cells, thereby indirectly activating afferent nerve endings (37). Adenosine triphosphate is one of the inflammatory mediators that may cause pain via the activation of P2X receptors (38,39). P2X3 receptor activation is closely correlated with inflammatory pain (40). It has been reported that the downregulation of P2X3 receptor expression may be involved in acupuncture analgesia in the spinal cord of rats with chronic constriction injuries (41), suggesting that the P2X3 receptor serves an essential role in the regulation of peripheral and central nervous system pain.

In the present study, no significant damage was observed in important histological structures of the knee joint following injection of 1 mg/kg MB for 1–24 weeks. Additionally, no significant histological changes in the articular cartilage were identified following treatment with MB in a rabbit model of OA and the level of synovial hyperplasia was not altered. These results suggest that MB is safe to use to treat OA.

Furthermore, MB treatment significantly enhanced the weight distribution and significantly decreased the swelling ratio of the rabbits compared with the vehicle group, indicating that MB may potentially serve a protective role in spinal cord injury. Furthermore, lncRNAs may serve an important role during OA progression; HOTAIR, lncRNA-CIR, H19 and MEG3 are all associated with OA (19–21). In the current study, RT-qPCR was performed to measure levels of HOTAIR, lncRNA-CIR and H19 mRNA, and it was demonstrated that they were not significantly altered following MB treatment. However, MB treatment significantly increased MEG3 levels. Therefore, the present study primarily focused on lncRNA MEG3, which is decreased in patients with OA (22). The results revealed that MB treatment significantly enhanced lncRNA MEG3 expression in rabbits; however, the expression of P2X3 protein was significantly decreased in MB-treated rabbits compared with the vehicle controls. Further investigations were performed to evaluate the potential association between lncRNA MEG3 and P2X3. It was observed that the overexpression of lncRNA MEG3 significantly suppressed the expression of P2X3. One potential mechanism by which lncRNA MEG3 regulates P2X3 expression is via proteasomal degradation or an RNA binding protein, which is involved in altering mRNA and protein stability; however, further investigations are required to confirm this. To further evaluate whether MEG3 alleviates pain and inflammation by regulating P2X3, SW1353 cells were treated with a plasmid overexpressing P2X3. This upregulation of P2X3 expression attenuated the MEG3-induced reduction of IL-6, TNFα, IL-1β and IL-8 levels in SW1353 cells.

The potential mechanism by which lncRNA MEG3 regulates the expression of P2X3 was also investigated. A dual luciferase reporter assay did not reveal any significant changes between the blank vector and the PGL3-P2X3 vector following lncRNA MEG3 overexpression in 293T cells, indicating that MEG3 does not exert a direct effect on P2X3

and inflammation.

In conclusion, to the best of our knowledge, the present study is the first to report that MB suppresses OA-associated pain and inflammation by enhancing lncRNA MEG3 expression and suppressing the expression of P2X3. These results may provide an insight into potential methods of alleviating pain in patients with OA.

Competing interests

The authors declare that they have no competing interests.

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