Introduction

Osteoarthritis (OA) is a disease in which the joint cartilage breaks down, leading to joint changes (1) and chronic pain. Both are targets of treatment. Although acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and opiates are used for the treatment of OA pain in accordance with Osteoarthritis Research Society International, American Academy of Orthopaedic Surgeons, and National Institute for Clinical Excellence guidelines, they all are associated with some safety risks (2, 3). Damaged cartilage tissue is difficult to repair and surgical treatment does not provide permanent cure and carries the risk of infection. Methods to promote the regeneration of cartilage tissue for the treatment of disease-related deformation and cartilage tissue destruction have recently attracted attention.

Rho-associated coiled-coil kinase (ROCK) is a low molecular weight GTP-binding serine/threonine kinase that acts downstream of Rho (4, 5). Its involvement in various physiological functions such as cell motility, cytoskeleton control, vasoconstriction, and inflammation has been suggested (6). The ROCK inhibitor Y27632 has been reported to enhance bone formation by bone morphogenetic protein (BMP) (7) and to induce in vitro differentiation of chondrocyte progenitor cells to chondrocytes (8). Some studies have investigated the relationship between ROCK and chondrocytes (9 – 11). Moreover, Rho signal transduction has been reported to be involved in initiation of neuropathic pain (12) and ROCK inhibitors have also been reported to act as analgesics: Y27632 had antihyperalgesic effects in mice (13) and H-1152, a commercially available ROCK inhibitor, suppressed nociceptive and neurogenic pain (14). However, efficacy or the therapeutic effect of ROCK inhibitors in in vivo models of cartilage degeneration or in clinical settings has not been reported.

As for analgesics, NSAIDs are mainly used to treat OA pain but they are ineffective in many patients and have insufficient efficacy. In addition, prolonged treatment with these drugs may cause adverse drug reactions such as gastrointestinal disorders and an increase in the
risk of cardiovascular events. Accordingly, pain remedies that are highly effective and have fewer adverse drug reactions are needed. We have found a potent and selective ROCK inhibitor, AS1892802, that reduces both inflammatory and non-inflammatory pain in rat models (15). Furthermore, drugs that suppress destruction of cartilage or promote its regeneration and thereby slow disease progression is highly desirable.

In the present study, we investigated effects of AS1892802 (orally and intraarticularly administered) on knee cartilage damage and pain in a monoiodoacetate (MIA)-induced arthritis model. Effects of the compound on differentiation of mouse ATDC5 chondrogenic cells (16) to chondrocytes and prostaglandin E₂ (PGE₂) production in rabbit HIG-82 synovial cells were examined. We provide the first evidence that AS1892802, a ROCK inhibitor, can be used to treat OA.

**Materials and Methods**

**Animals**

Six-week-old female Sprague-Dawley (SD) rats were obtained from Charles River Japan, Inc. (Yokohama). Rats were housed under conditions that included a controlled light cycle (light/dark: 12 h each) and controlled temperature (23 ± 1°C). Tap water and standard laboratory chow were available ad libitum. Rats were acclimated for one week before use. All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma, Inc.

**Induction of MIA-induced arthritis**

This MIA model was prepared as described by Gutingamp et al. (17). Female SD rats were anesthetized with halothane (Takeda Pharmaceutical Company, Limited, Tokyo) and given a single intraarticular injection of 1 or 3 mg of MIA (n = 15 per group, one group per dose; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) through the intrapatellar ligament of the right knee. MIA was dissolved in physiological saline and administered in a volume of 50 μl with a 26-gauge, 0.5-inch needle. Rats were sacrificed at 2, 3, and 4 weeks (n = 5) after MIA injection under ether anesthesia, and macroscopic lesions were graded. Expressions of ROCKs mRNAs were also examined in the distal end of the femur at 1 and 3 weeks after injection of 1 mg of MIA. One milligram of MIA was used for evaluation of the compound AS1892802.

**Treatments**

AS1892802 (0.03, 0.3, or 3 μg/site) or vehicle alone was injected intraarticularly into the MIA-injected knee twice a week for three weeks starting the day of the MIA injection. In another experiment, AS1892802 was administered orally (1, 3.2, 10 mg/kg) once a day for 3 weeks. The vehicle control and AS1892802-treated groups consisted of 8 animals each. At the end of the study, the right knee was removed and carefully dissected to evaluate the severity of chondral alterations and the tibia was fixed in 10% formalin neutral buffered solution (Wako Pure Chemical, Osaka) (18). There were no signs of toxicity in any of the animals whether injected with vehicle or the compound.

**Macroscopic scoring of the articular cartilage lesion**

A photograph of the proximal end of the tibial cartilage was taken with a digital camera (Olympus). Two independent observers scored cartilage damage on the tibial surface in a blinded manner on a scale of 0 – 4, with severity increasing from 0 to 4 (0 = normal appearance, 1 = rough surface, 2 = moderate lesion of cartilage surface, 3 = severe lesion of subchondral bone, 4 = appearance of osteophyte formation with severe lesion of subchondral bone; modification of reference 18). The mean score was determined for each group.

**Evaluation of hind paw weight bearing**

Weight bearing was measured by an incapacitance meter (Linton Instruments, Norfolk, UK) and assumed to be a behavioral measure of musculoskeletal discomfort (19). Briefly, rats were placed in an angled plastic chamber positioned so that each hind paw rested on a separate force plate and were allowed to acclimate for about 5 min. The weight (g) measured by each hind paw was averaged for 5 s. The change in hind paw weight distribution was calculated by determining the difference in the weight between the left (contralateral control) and the right (osteoarthritic) limbs. The weight distribution was utilized as an index of joint discomfort in the MIA model. Analgesic tests of the AS1892802-treated groups were conducted before sacrifice 24 h after the last injection of the compound.

**Bradykinin-induced joint pain model**

Joint pain was induced and assessed based on the methods of Gotoh et al. (20). Female SD rats (6 – 7-week-old; Charles River Laboratories Japan, Inc.) were given AS1892802 (3.2, 10 mg/kg) orally. At 1 h after administration of the compound, a physiological saline solution of bradykinin was injected into the knee joint cavity of the hindlimb (3 μM/site in 50 μl), and then a pain response was observed beginning in 5 – 10 s and lasting until about 2 min after injection. The level of pain was scored on a 5-point scale (0 – 4) as follows: 0 = no lameness to lameness for 10 s, 1 = lameness for 10 – 30 s, 2 = lifting of the limb within 10 s or lameness for 31 or
more s, 3 = three-legged gait within 10 s followed by lameness, and 4 = three-legged gait for 10 or more s followed by lameness. Ten rats were used in each experimental group.

Chondrogenesis assays

ATDC5 cells, a chondrogenic mouse embryonic cartilage cell line, were obtained from the Riken Cell Bank (Tsukuba). Cells were cultured in a medium consisting of a 1:1 mixture of d-MEM/F-12 (Sigma-Aldrich Chemical Co.) containing 5% fetal calf serum (Intergen, Purchase, NY, USA) as previously described (21). Briefly, all cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 – 95% air. Cells (1 × 10^5 cells/well) were placed in 12-well tissue culture plates. After the cells reached confluence, the medium was replaced with a differentiation medium containing with 10 μg/ml insulin (Sigma-Aldrich Chemical Co.). Then the cells were treated with AS1892802, cultured for 2 weeks with medium replacement every 2 or 3 days, rinsed with cold phosphate-buffered saline twice, fixed with methanol (2 min, −20°C), rinsed with double-distilled water once, stained with 0.1% Alcian blue (Sigma-Aldrich Chemical Co.) in 0.1 N hydrochloric acid overnight at room temperature, washed off with double distilled water 3 times to remove excess stain, and then photographed. The staining intensity was quantified by solubilizing the stain in 6 M guanidine hydrochloride for 8 h at room temperature and measuring absorbance with a spectrophotometer at 620 nm.

In a time-course experiment, cells were maintained as mentioned above and were isolated after 4, 7, and 14 days in culture.

Synovial fibroblast assays

Rabbit synovial fibroblasts, HIG-82 cells (American Type Culture Collection, Manassas, VA, USA), were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum (Moregate Biotech, Blimba, Australia) at 37°C in 5% CO2 – 95% air. Cells (1 × 10^5 cells/well) were placed in 24-well tissue culture plates. Next day, the medium was replaced with serum-free Ham’s F-12 medium containing 0.1% bovine serum albumin and test compounds. One hour after the preincubation, recombinant human IL-1β (1 ng/ml; R&D Systems Inc., Minneapolis, MN, USA) or bradykinin (100 nM; Peptide Institute Inc., Osaka) was added to the cultured cells. The supernatants were collected after 24-h incubation and assayed for PGE2 by use of a commercial enzyme-linked immunosorbent assay kit (Cayman Chemicals, Ann Arbor, MI, USA).

Reverse transcription PCR

RNA was extracted using the RNaseasy kit (Qiagen) according to the manufacturer’s instructions. The ATDC5 cell layers were scraped and homogenized with a pipette, or femoral condyles from MIA-injected rat knees were homogenized with a polytron homogenizer. Purified total RNA was reverse-transcribed using both random hexamers pg (N)6 and poly(dt) (Amersham Biosciences). Primers were designed for GAPDH (Rat: upper 5’-accacagtcctatgccatatc, lower 5’-tcacccctggtcgtgtgtgta; Mouse: upper 5’-caccatggagaagcgccgg, lower 5’-gaaggccagctgta); ROCK I (Rat/Mouse: upper 5’-gcacatgtatgaatgtagaat, lower 5’-cataattttctgtagtagttcacaag); ROCK II (Rat/Mouse: upper 5’-gcacatgtatgaatgtagaat, lower 5’-cataattttctgtagtagttcacaag); and Type II collagen (Mouse upper 5’-ttagaagggaacagcctc, Lower 5’-taca tgcctgtagaacatgg). The PCR conditions were 94°C for 30 s, 59°C for 30 s, 72°C for 20 s with 30 cycles, and final extension at 72°C for 7 min.

Drugs

AS1892802 was synthesized by Astellas Pharma, Inc. (Tsukuba). The compound was dissolved in 10% PEG400 in saline for intraarticular injection and in distilled water containing an equimolar concentration of hydrochloric acid for oral administration. MIA was dissolved in saline.

Statistical analyses

All data are expressed as the mean ± S.E.M. Significance of differences in mean scores was assessed by Kruskal-Wallis multiple-comparisons ANOVA, and the significance of other differences was assessed using the two-tailed unpaired Student’s t-test or Dunnett’s multiple comparisons. Analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). Dose dependency was examined by the Jonckheere (nonparametric) test or the linear regression (parametric) test. A difference between experimental groups was considered significant when the P values were less than 0.05.

Results

Time course of tibial cartilage damage in MIA-injected rats

A single intraarticular injection of MIA (1 or 3 mg) into the knee joint induced cartilage damage at the proximal end of the tibia, and the severity was monitored from 2 to 4 weeks (Fig. 1A). A 1-mg dose of MIA resulted in mild cartilage damage at 2 weeks and the severity of this damage increased time dependently. Cartilage damage was already severe at 2 weeks after injection of 3 mg of MIA (3.2 ± 0.2, n = 5) and this level of severity persisted
The severity of macroscopic cartilage lesions was related to both the time elapsed after injection and dose of MIA. From these results, a dose of 1 mg of MIA for 3 weeks duration (2.8 ± 0.2, n = 5) was selected to evaluate the effect of AS1892802. ROCK was reported to be related with bone formation (7), while the expression level of ROCK in the osteoarthritic cartilage is unknown. We examined expression levels of ROCKs to clarify roles of ROCKs in cartilage lesion of OA-like pathology in rat knees and found that high levels of both ROCK I and ROCK II mRNAs persisted up to 3 weeks post injection in the distal ends of femur condyles from MIA-injected rats (Fig.1B).

**Effects of AS1892802 on cartilage lesions and weight bearing deficit in MIA-induced OA**

Intraarticular injection of AS1892802 dose dependently prevented the formation of tibial cartilage lesions due to MIA induction of OA. There was a 30% decrease in cartilage loss ($P < 0.001$) at the dose of 3 μg (Fig. 2A) and a significant decrease in hind paw weight-bearing deficit at doses of 0.3 μg ($P < 0.001$) and 3 μg ($P < 0.001$) (Fig. 2B). The effects of orally administered AS1892802 were similar. There was a 20% decrease in cartilage loss ($P < 0.01$) at the dose of 10 mg/kg (Fig. 2C) and a significant decrease in hind paw weight-bearing deficit at the doses of 1, 3, 2, and 10 mg/kg ($P < 0.001$) (Fig. 2D).

**Effects of AS1892802 on chondrogenic differentiation**

Alcian blue positive staining was detected 2 weeks after culturing ATDC5 cells in differentiation medium. In addition, the intensity of staining was greater in AS1892802-treated ATDC5 cells than control cells at the dose of 10 μM (Fig. 4A). AS1892802 significantly increased staining intensity 1.2-fold ($P < 0.001$) and 1.7-fold ($P < 0.001$) above control levels at 1 and 10 μM, respectively (Fig. 4B). Under experimental conditions, Alcian blue–positive staining increased in intensity after 1 – 2 weeks of treatment. In addition, the level of Type II collagen mRNA continued to increase in cells treated with AS1892802 (Fig. 4C).

**Effects of AS1892802 on PGE2 production in synovial cells**

IL-1β (1 μg/ml) significantly ($P < 0.001$) induced PGE2 production in HIG82 cells cultured for 24 h. AS1892802 dose-dependently inhibited IL-1β–induced PGE2 production with 10 μM achieving about 80% inhibition (Fig. 5A). In addition, 100 nM bradykinin significantly induced PGE2 production ($P < 0.05$) and 0.1 μM AS1892802 completely inhibited PGE2 production (Fig. 5B).
A Rho Kinase Inhibitor Prevents OA Effects of AS1892802 on bradykinin-induced pain behavior

A pain response (score, about 3) to an injection of bradykinin into the right knee was confirmed. Oral administration of AS1892802 significantly and dose-dependently improved indices of the pain response, especially the pain score ($P < 0.001$), which decreased from 3.1 to 1.3 following administration at 10 mg/kg (Fig. 6).

Discussion

There are many experimental animal models of OA in humans including spontaneous models in both guinea-pig (22, 23) and mouse (24); models of surgically-induced OA in dogs (25), rabbits (26) and guinea pigs (27, 28); and models of OA induced by intraarticular injection of collagenase (29), IL-1 (30), or MIA (17, 18), and so on. An MIA-induced rat model was selected in the present study because it can be used to evaluate clinical symptoms (31, 32), especially OA pain. This in vivo model permits simple and reproducible evaluation of many chemical compounds within a short time period. The pain severity and cartilage damage produced in our study were similar to those previously reported. We found that macroscopic evaluation of the tibial surface is an easy initial screening method for examining the chondroprotective effects of compounds. Figure 1A shows mild tibial cartilage erosion (and progression of erosion up to...
3 weeks) in the knee 2 weeks after a single injection of 1 mg of MIA. In so far as the cartilage erosion was already severe at 2 weeks after injection of 3 mg of MIA, we believed that drug efficacy would be difficult to evaluate under such circumstances. Therefore, we selected 3 weeks after injection of 1 mg of MIA for determination of drug efficacy. Further experiments are needed to characterize potentially useful compounds.

We have found that expressions of both ROCK I and ROCK II mRNA increased in the knee joint of the MIA model and hypothesized that the increase in ROCKs might be related to progression of cartilage damage. In our present study, AS1892802, a selective ROCK inhibitor, reduced both cartilage erosion and pain in the MIA-induced OA model. In particular, our study showed for the first time that a ROCK inhibitor could prevent cartilage erosion in vivo. The mechanism of action might involve induction of chondrocyte differentiation and protection from cartilage destruction. Beier et al. reported that RhoA/Rock signaling suppresses chondrogenesis through the control of Sox9 expression and actin organization (33). Rho–ROCK signal transduction is involved in inhibition of chondrocyte differentiation as well as modulation of chondrocyte metabolism (34). Our data showing enhancement of proteoglycan staining in ATDC5 cells treated with AS1892802 supports their results. In addition, expression of type II collagen mRNA continues to increase until the end of the 2-week period of treatment with AS1892802 in ATDC5 cells. In our previous studies, AS1892802 exhibited potent inhibitory activities for human ROCK I (IC_{50} = 122 nM), human ROCK II (IC_{50} = 52 nM), and rat ROCK II (IC_{50} = 57 nM) (15), and 1 μM of AS1892802 (effective concentration in the ATDC5 cell studies) was enough high to inhibit ROCK activities in the neurite outgrowth assay using SH-SY5Y human neuroblastoma cells (15). Therefore, the ROCK activity was considered to be inhibited at 1 μM AS1892802 in cells.

IL-1β plays a very important role as a mediator of joint destruction (35) and is reported to activate synoviocytes (36). We have demonstrated that AS1892802 inhibited IL-1β–induced PGE2 production in a synovial cell line, suggesting that AS1892802 might inhibit cartilage destruction mediated by cytokines from activated synoviocytes. As for a relationship between IL-1β and the Rho-mediated transduction pathways, it was reported that IL-1β stimulated actin stress fiber formation in a Rho-dependent manner (37) and IL-1β enhanced ROCK mRNA levels (38) in cultured human coronary vascular smooth muscle cells. Our results show that AS1892802 inhibits ROCK to protect against cartilage damage in part by inhibiting IL-1β signal transduction. Pralnacasan, an inhibitor of IL-1β–converting enzyme, was reported to reduce joint damage in OA models (39). The treatment reduced joint damage by 13% – 22% in both a collagenase-induced OA model and in STR/1N mice prone to
OA. AS1892802 treatment also improved cartilage erosion by about 20%–30% at the dose of 3 μg (intraarticularly) or 10 mg/kg (orally) in our experimental model. WF536, a Y27632 derivative and selective ROCK inhibitor (40) that differs structurally from AS1892802, dose-dependently and significantly enhanced proteoglycan staining of ATDC5 cells 1.2-fold ($P < 0.001$) and 2.0-fold ($P < 0.001$) compared to the control at 1 and 10 μM, respectively (personal observation). These results also suggest that a ROCK inhibitor might have chondroprotective effects in OA. Although enhancement of ROCK activities appears to be involved in progression of cartilage damage, the relationship between cartilage destruction and activation of ROCKs will need to be examined further in the MIA model.

The effective dose for pain reduction was lower than that for cartilage damage reduction. We have recently reported that a single dose of AS1892802 reduces both inflammatory and non-inflammatory pain (15) in rat models. The analgesic efficacy depended on blood concentration of the compound and maximum efficacy was obtained at the dose of 1 mg/kg (orally) 1 h after administration, but the activity disappeared within 24 h. In addition, analgesic activity was maintained by the repeated administration of the compound (41). The present results (Fig. 2: B, D) support our previous data. The analgesic efficacy of AS1892802 might be due to direct anti-nociceptive effects mediated through inhibition of ROCK as well as cartilage protective effects.

Many factors (including IL-1β and cartilage destruction) are involved in mediating OA pain but the main one is unknown. Bradykinin levels in the joint synovium and the severity of synovitis are reported to be increased in OA patients (42). In our in vitro studies using rabbit HIG82 cells, the ROCK inhibitor AS1892802 inhibited both IL-1β and bradykinin-induced PGE2 production. According to the previous report, the amino acid sequence of rabbit ROCK I showed over 95% homologies with those of rat, mouse, and human (43). In addition, the amino acid sequences of the ATP-binding region and serine-threonine kinase region were completely conserved among those species including rabbit. Therefore, AS1892802 is assumed to exhibit inhibitory effects on PGE2 production of rabbit HIG82 cells via ROCK inhibition. The compound does not bind to bradykinin B2 receptor (15). It is also reported to be an inhibitor of Rho-
attenuated bradykinin-induced cellular functions (44). Our results (in agreement with previous findings) demonstrated that inhibition of ROCK prevented IL-1β- or bradykinin-induced inflammatory conditions. In our previous study, a single dose of AS1892802 had no effect on PGE2 levels in rat paws after induction of inflammation by Complete Freund’s Adjuvant (CFA). As CFA injection can elevate levels of several cytokines and induce severe rheumatoid-like inflammation, significant change in the PGE2 level might not be detectable. Therefore, IL-1β- or bradykinin-induced PGE2 production in particular might be involved in Rho–ROCK signal transduction in vivo, and repeated administration of AS1892802 might inhibit production.

AS1892802 reduced knee pain induced by bradykinin injection. Intraarticular injections of hyaluronate have generally been effective for treatment of moderate to severe knee pain in OA subjects and bradykinin has been reported to have a role in this knee pain (45). Since hyaluronate can reduce bradykinin-induced pain (46), bradykinin-induced pain models might be useful for evaluating the effects of these compounds (ROCK inhibitors) on OA pain. Intraarticular injection of WF536 was also effective against bradykinin-induced pain (our unpublished data). The use of ROCK inhibitors to treat OA pain should therefore be considered. Knee cartilage improved to almost the same extent regardless of the route of drug administration (3 μg via intraarticular injection or 10 mg/kg via oral administration). The plasma concentration of AS1892802 (1 mg/kg, orally administered) is enough to inhibit the ROCK activity (15). The Cmax of AS1892802 in the knee joint was estimated to be about half the plasma concentration and the rate of disappearance was slower from the knee joint than from plasma (our unpublished data). Therefore, maintenance of a higher AS1892802 concentration may be needed to prevent cartilage destruction. The intraarticular dose of 0.3 μg showed just a tendency to reduce cartilage damage. As low molecular weight compounds pass easily from the knee joint fluid into the synovial membrane, a low concentration of AS1892802 might be effective if maintained in the joint space. We presume that a higher dose will have to be injected twice a week intraarticularly to maintain ROCK inhibition. We have reported that the efficacy of the compound against pain could be prolonged by repeated administration in rat models, suggesting that these prolonged pharmacodynamic effects are due to the compound alone.

In summary, we found that our ROCK inhibitor AS1892802 has both pain reducing and chondroprotective effects in an MIA-induced OA model. We postulate that the mechanism of action is through ROCK inhibition. Compounds with both antinociceptive and chondroprotective effects can be useful for the treatment of OA. The role of ROCK inhibitors in cartilage protection and analgesia will need further clarification.

Acknowledgments

We thank Dr. K. Itoh and Dr. K. Yoshioka of Osaka Medical Center for Cancer and Cardiovascular Diseases for providing useful discussions.

References

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