J Pharmacol Sci **110**, 201 – 211 (2009)

Journal of Pharmacological Sciences

Full Paper

The Chondroprotective Agent ITZ-1 Inhibits Interleukin-1β–Induced Matrix Metalloproteinase–13 Production and Suppresses Nitric Oxide–Induced Chondrocyte Death

Haruhide Kimura[1,2,*], Hiroshi Yukitake[1], Hirobumi Suzuki[1], Yasukazu Tajima[1], Koyo Gomaibashi[1], Shinji Morimoto[1], Yasunori Funabashi[1], Kiyofumi Yamada[2,#], and Masayuki Takizawa[1]

[1]*Pharmaceutical Research Division, Takeda Pharmaceutical Co., Ltd., 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan*
[2]*Laboratory of Neuropsychopharmacology, Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan*

Received March 5, 2009; Accepted April 23, 2009

Abstract. In a screening program aimed at discovering anti-osteoarthritis (OA) drugs, we identified an imidazo[5,1-*c*][1,4]thiazine derivative, ITZ-1, that suppressed both interleukin-1β (IL-1β)-induced proteoglycan and collagen release from bovine nasal cartilage in vitro and suppressed intra-articular infusion of IL-1β–induced cartilage proteoglycan degradation in rat knee joints. ITZ-1 did not inhibit enzyme activities of various matrix metalloproteinases (MMPs), which have pivotal roles in cartilage degradation, while it selectively inhibited IL-1β–induced production of MMP-13 in human articular chondrocytes (HAC). IL-1β–induced MMP production has been shown to be mediated by extracellular signal–regulated protein kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK) of the mitogen-activated protein kinase (MAPK) family signal transduction molecules. An ERK–MAPK pathway inhibitor (U0126), but not a p38 kinase inhibitor (SB203580) or a JNK inhibitor (SP600125), also selectively inhibited IL-1β–induced MMP-13 production in HAC. Furthermore, ITZ-1 selectively inhibited IL-1β–induced ERK activation without affecting p38 kinase and JNK activation, which may account for its selective inhibition of MMP-13 production. Inhibition of nitric oxide (NO)-induced chondrocyte apoptosis has been another area of interest as a therapeutic strategy for OA, and ITZ-1 also suppressed NO-induced death in HAC. These results suggest that ITZ-1 is a promising lead compound for a disease modifying anti-OA drug program.

Keywords: ITZ-1, cartilage degradation, chondrocyte, matrix metalloproteinase (MMP)-13, cell death

Introduction

There are two major arthritides in humans, that is, osteoarthritis (OA) and rhumatoid arthritis (RA). OA is a primary disease of the cartilage characterized by deterioration of the articular cartilage caused by deleterious proteinases released from articular chondrocytes; RA is characterized by a chronic proliferative synovitis, which is caused by abnormal immunological reactions and exhibits hyperplasia of the synovial lining cells, inflammatory cell infiltration, and angiogenesis in the sublining cell layer. Although these arthritides are different in the origin of the diseases, they share destructive mechanisms of the articular cartilage by proteinases (1, 2).

OA is a degenerative joint disease occurring mainly in the elderly and is characterized by a slow but irreversible, progressive destruction of articular cartilage. Although the etiology of OA is not completely under-

*Corresponding author (affiliation #1).
Kimura_Haruhide@takeda.co.jp
#Present address: Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65, Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan
Published online in J-STAGE
doi: 10.1254/jphs.09076FP

stood, it is believed that OA is a consequence of mechanical and biochemical events that result in an imbalance between the synthesis and degradation of the articular cartilage matrix (1 – 5). Chondrocytes, which constitute the only cell type of the articular cartilage, regulate cartilage metabolism under both normal and pathological conditions. In OA, these cells initiate the production of proteolytic enzymes, mainly matrix metalloproteinases (MMPs) including collagenase and aggrecanase, in response to inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α). Among these inflammatory cytokines, the sustained production of IL-1β in the affected joints may play a key role in OA pathogenesis (3, 5 – 11).

The MMPs degrade the principal matrix macromolecules such as proteoglycan and collagen from cartilage. Especially, MMP-1 and MMP-13 have pivotal roles in the irreversible break down of cartilage matrix via digestion of type II collagen and the consequent release of matrix proteoglycan from the cartilage. The integrity of these macromolecules is vital to cartilage joint function, thus loss of these molecules result in cartilage cracking, abrasion, and ulcers (1 – 5).

Adult human articular cartilage is believed to be a post-mitotic tissue with virtually no cellular turnover. The extracellular matrix is avascular and thus no external cell supply can compensate for any sort of cell loss (5, 12). Therefore, survival of the chondrocytes is essential for the maintenance of proper articular cartilage. As apoptotic chondrocyte death is observed in clinical specimens from OA cartilage, apoptotic cell death has become a focus of interest and was suggested to be an important event in OA cartilage degeneration (12, 13). Since no therapeutic agents to prevent erosion of articular cartilage or disease progression are known, agents that interfere with cartilage destruction would be of considerable therapeutic value (1 – 5).

In our search for agents that inhibit IL-1β–induced destruction of bovine nasal septum cartilage (14, 15), we discovered *N*-{(1*Z*)-5,6-dimethyl-3-oxo-8-[(4,4,5,5,5-pentafluoropentyl)thio]-2,3-dihydro-1*H*-imidazo[5,1-*c*][1,4]thiazin-1-ylidene}-4-methylbenzenesulfonamide (ITZ-1). Here, we report the chondroprotective potency of ITZ-1: ITZ-1 suppressed IL-1β–induced destruction of bovine nasal septum cartilage in vitro, and the intra-articular infusion of IL-1β–induced proteoglycan release in rats. By analysis of mechanisms of action of ITZ-1, we discovered that ITZ-1 selectively inhibited IL-1β–induced MMP-13 production and suppressed nitric oxide (NO)-induced death of human articular chondrocytes (HAC) (16). The molecular mechanism underlying the selective inhibition of MMP-13 production by ITZ-1 was also analyzed. These results suggest that ITZ-1 is a promising lead compound with novel mechanisms of action for a disease modifying anti-OA drug program.

Materials and Methods

Materials

SP600125 was purchased from ENZO Life Sciences AG (Lausen, Switzerland). U0126 and SB203580 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Recombinant human IL-1β and oncostatin M (OSM) were purchased from R&D Systems (Minneapolis, MN, USA). Methods for ITZ-1 synthesis will be described elsewhere.

Proteoglycan degradation suppression assay

Cartilage explants prepared from bovine nasal septum (3 – 6 mg) were transferred into 96-well plates with 100 μl/well of medium consisting of a 1:1 (v/v) mixture of Dulbecco's modified Eagle's MEM (DMEM) and Ham's F-12 medium (DMEM/F-12) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 50 U/ml penicillin (Lonza Walkersville, Inc., Walkersville, MD, USA) and 50 μg/ml streptomycin (Lonza Walkersville). Proteoglycan degradation was induced by the addition of 20 ng/ml of IL-1β to the culture medium. After 4 days of cultivation at 37°C in 5% CO_2/95% humidified air in the presence or absence of test compounds, the medium was aspirated and the remaining cartilage was digested with papain (Sigma-Aldrich, St. Louis, MO, USA). The glycosaminoglycan (GAG) concentrations in the medium and the digest from each cartilage explant were determined by 1,9-dimethylmethylene blue dye assay (Sigma-Aldrich) and used as a marker of cartilage proteoglycan content (15, 17).

Collagen degradation suppression assay

The cartilage explants described above were transferred into 96-well plates with 100 μl/well of DMEM (Invitrogen Corp.). Collagen degradation was induced by the addition of 20 ng/ml of IL-1β and 50 ng/ml of OSM. After 14 days of cultivation in the presence or absence of test compounds, the medium was aspirated and the remaining cartilage was digested with papain. Hydroxyproline concentrations in the medium and the digest from each explant were measured with chloramine T (Wako) and *p*-demethylaminobenzaldehyde (Wako), and used as a marker of cartilage collagen content (14).

Intra-articular infusions of IL-1β in rats

Eight week old male Sprague-Dawley rats (CRJ:IGS; Japan Charles River, Yokohama) were used. IL-1β solution (0.2 ng/μl IL-1β and 0.5% bovine serum albumin in sterile saline) was infused intra-articularly

into the knee joint at 0.5 μl/h for 7 days by an Alzet pump (Model 1007D; DURECT Corp., Cupertino, CA, USA). ITZ-1 was orally administered daily at the indicated doses. After treatment, rats were sacrificed and their tibial articular cartilages were excised. The GAG content in the articular cartilage was evaluated as previously described and used as a marker of cartilage proteoglycan content (18). The GAG levels obtained were normalized to total wet weight of digested cartilage. Results were expressed as the percent decrease from the GAG content in intact counterparts in the same animals. The methods for care and use of animals and the experimental protocols were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Co., Ltd.

For histological examination, knee joints were dissected, fixed in 10% formalin neutral buffer solution (Wako), decalcified in EDTA, dehydrated, and then embedded in paraffin blocks. Eight-micrometer microsections were prepared and stained with hematoxylin and eosin (H&E) or with Safranin O. Apoptotic chondrocytes were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA, USA).

Bioavailability of ITZ-1

The bioavailability of ITZ-1 from 10 mg/kg oral dosing (n = 3) in rats was 63.6 ± 12.8%. The peak plasma concentration (C_{max}) and area under the curve (AUC_{0-24h}) were 2.1 ± 0.2 μg/ml and 11.1 ± 1.4 μg·h /ml, respectively.

Assay for MMP enzyme activity

Inhibitory activities of ITZ-1 against enzyme activity of human recombinant MMP-1, MMP-8, or MMP-13 were evaluated by QuantiZyme assay systems (MMP-1, MMP-8, and MMP-13 Colorimetric Assay Kit for Drug Discovery; ENZO Life Sciences International, Inc., Plymouth Meeting, PA, USA), which uses a thiopeptolide (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5) as a colorimetric substrate, according to the manufacturer's instructions. *N*-Isobutyl-*N*-[4-methoxyphenylsulfonyl]glycyl hydoxamic acid (NNGH) at 1.3 μM was used as a positive control.

Assay for MMP production

HAC (Lonza Walkersville) were maintained in Chondrocyte Growth Medium (CC-3216, Lonza Walkersville) until used. HAC were suspended in DMEM/F-12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin (Lonza Walkersville), and 0.1% fetal bovine serum (FBS; SAFC Biosciences, Inc., Lenexa, KS, USA) at a density of 5×10^4 cells/ml and cultured in 96-well plates for 1 day. MMP production was induced by the addition of 10 ng/ml of IL-1β to the culture medium. Subsequently the cells were cultured in the presence or absence of test compounds for 2 days at 37°C in 5% CO_2 / 95% humidified air. The amount of MMPs produced in the culture medium was determined with ELISA kits (GE Healthcare UK Limited, Buckinghamshire, UK).

Assay for MMP-13 mRNA induction

HAC maintained in Chondrocyte Growth Medium were suspended in DMEM/F-12 supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at a density of 2.2×10^4 cells/ml and cultured in 12-well plates at 37°C in 5% CO_2 / 95% humidified air for 1 day. After medium change, HAC were treated with 10 ng/ml of IL-1β in the presence of the indicated concentration of ITZ-1 for 1 day. Total RNA was extracted with an RNeasy 96 kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and further purified by DNase digestion using MessageClean (GenHunter Corporation, Nashville, TN, USA). Real-time quantitative PCR was carried out using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) in conjunction with a QuantiTect SYBR Green PCR Kit (QIAGEN GmbH). RNA samples were normalized by glyceraldehyde-3-phosphate dehydrogenase (Gapdh) with GAPDH Control Reagents (Applied Biosystems). Primers used for MMP-13 analysis were MMP-13-F (5'-CAAGGG ATCCAGTCTCTCTATGG-3') and MMP-13-R (5'-GGTCACATTTGTCTGGCGTTT-3').

Evaluation of cytotoxicity by the WST-8 assay

HAC were suspended in Chondrocyte Growth Medium at a density of 5×10^4 cells/ml and cultured in 96-well plates at 37°C in 5% CO_2 / 95% humidified air for 1 day. After medium change with DMEM/F-12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, and 0.1% FBS, HAC were treated with 10 ng/ml of IL-1β and the indicated concentration of ITZ-1 for 1 day. Afterwards, cell viability was evaluated with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8; Dojindo, Mashiki) (WST-8 assay) according to the manufacturer's instructions.

Assay for mitogen-activated protein kinase (MAPK) activation

HAC were pre-cultured at 1×10^5 cells/ml in 6-well

plates for 1 day and then further cultured in DMEM/F-12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml gentamicin at 37°C in 5% CO_2 / 95% humidified air for 1 day. The HAC were treated with ITZ-1 for 24 h followed by further incubation with 10 ng/ml IL-1β for 30 min. Following the culture, HAC were washed with ice-cold PBS, lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM p-APMSF, 200 μM sodium β-glycerophosphate *n*-hydrate, 20 mM NaF, 2 mM sodium diphosphate decahydrate, 1 mM sodium orthovanadate V, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Triton X-100, 0.5% Nonidet P40, and 0.1% SDS) and then subjected to western blot analysis.

Western blot analysis

Samples were subjected to SDS-PAGE; transferred onto nitrocellulose filters; and probed with the following primary antibodies: phosphorylated state– and non-phosphorylated state–specific antibodies for extracellular signal–regulated protein kinase (ERK), p38 kinase, or c-Jun N-terminal kinases (JNK); followed by a secondary antibody (anti-rabbit IgG, HRP-linked). All antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The immune complexes were visualized by conventional methods and quantified by densitometry using GS-800 (Bio-Rad Laboratories, Hercules, CA, USA).

MTT assay for evaluation of NO-induced cell death

HAC were suspended in Chondrocyte Growth Medium at a density of 4×10^4 cells/ml and cultured in 96-well plates at 37°C in 5% CO_2 / 95% humidified air for 1 day. After medium change with DMEM/F-12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml gentamicin, HAC were pretreated with test compounds together with 10 ng/ml of IL-1β for 1 day. Cell death was induced by treatment with 110 μM (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3, Dojindo), which generates NO, for 1 day. Afterwards, cell viability was evaluated with a soluble tetrazolium salt MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazorium bromide; Dojindo] (MTT assay) (19).

Statistical analyses

The proteoglycan or collagen degradation by IL-1β or IL-1β + OSM was analyzed by the Aspin-Welch test, and statistical significance of drug efficacy was examined by the one-tailed Shirley-Williams test. The proteoglycan degradation by intra-articular infusions of IL-1β was analyzed by the *t*-test, and statistical significance of drug efficacy was examined by the one-tailed Williams' test. Cell death induced by NOR3 was analyzed by the Aspin-Welch test and statistical significance of drug efficacy was examined by the one-tailed Williams' test.

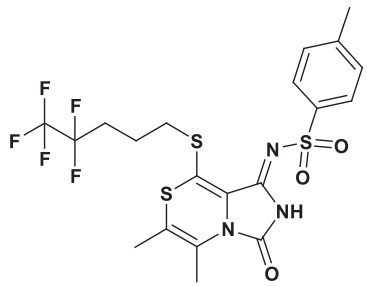

Fig. 1. Chemical structure of ITZ-1.

Results

Discovery of ITZ-1

To discover novel therapeutic drugs for OA, we established an in vitro screening assay based on the IL-1β–induced degradation of bovine nasal cartilage. In the assay, cartilage degradation was measured as the release of proteoglycan or collagen into the culture medium from cartilage explants (14, 15). Using the assay, ITZ-1 (Fig. 1) was identified from our chemical library. IL-1β induced proteoglycan release (from 11.3% to 87.7%) and IL-1β plus OSM induced collagen release (from 3.4% to 92.3%) from bovine nasal cartilage. ITZ-1 significantly inhibited both proteoglycan (Fig. 2A) and collagen (Fig. 2B) release with a half-maximal inhibitory concentration (IC_{50}) of 0.098 and 0.099 μM, respectively.

ITZ-1 suppresses IL-1β–induced cartilage proteoglycan degradation in rat synovial joints

We next evaluated the effects of ITZ-1 on cartilage proteoglycan degradation in rat synovial joints. The intra-articular infusion of IL-1β into the rat knee joint induced proteoglycan loss from articular cartilage (7, 8) (from 31.2% to 72.1%, $P = 0.013$), and oral administration of 10 mg/kg of ITZ-1 significantly inhibited the proteoglycan loss ($P = 0.019$, Fig. 3A). H&E staining of tibial condyle microsections suggested that oral administration of 10 mg/kg of ITZ-1 prevented IL-1β–induced disappearance of surface layer cells. ITZ-1 at 10 mg/kg showed a tendency to prevent IL-1β–induced reduction of proteoglycan content as measured by Safranin O staining. Interestingly, IL-1β infusion slightly increased the number of apoptotic chondrocytes, while ITZ-1 at 10 mg/kg seemed to suppress apoptosis (Fig. 3B). These data suggest that ITZ-1 is a chondroprotective agent.

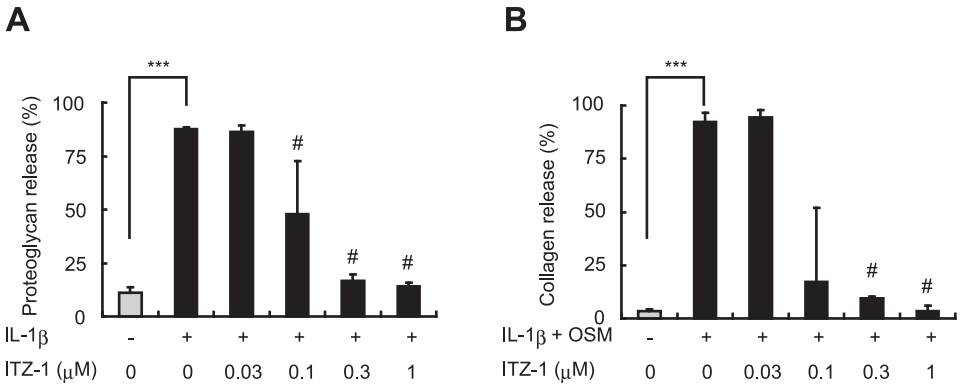

Fig. 2. ITZ-1 suppressed IL-1β–induced degradation of bovine nasal cartilage in vitro. A: Proteoglycan release was induced by IL-1β. B: Collagen release was induced by IL-1β and OSM. The GAG content was determined by the 1,9-dimethylmethylene blue assay, and hydroxyproline content was measured with chloramine T and *p*-demethylaminobenzaldehyde. The release rate of proteoglycan and collagen from cartilage to the culture medium was determined based on the GAG and hydroxyproline content in the medium and the remaining cartilage explant, respectively. Results shown are each a mean ± S.D., n = 4. ***$P \leq 0.001$. $^{\#}P \leq 0.025$: the IL-1β or IL-1β + OSM alone–treated group compared with the IL-1β + ITZ-1 or IL-1β + OSM + ITZ-1–treated group, respectively.

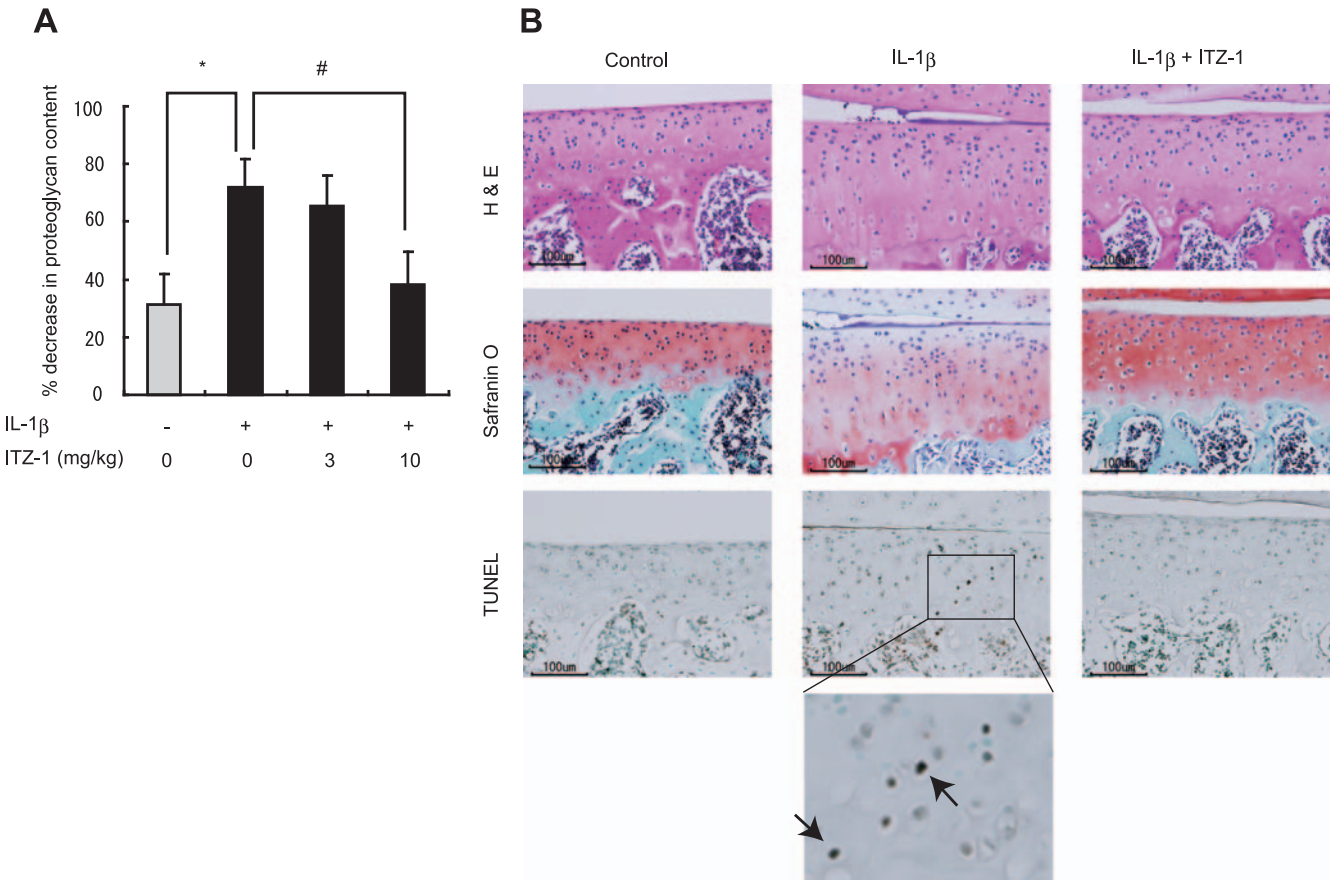

Fig. 3. ITZ-1 suppressed intra-articular infusion of IL-1β–induced proteoglycan loss from rat cartilage. A: Proteoglycan content in each articular cartilage was evaluated by the dimethylmethylene blue assay. Results are expressed as the percentage decrease from the proteoglycan content in the contralateral intact knee joints in the same animal. Each value is a mean ± S.E.M., n = 8. *$P \leq 0.05$: the IL-1β alone–treated group compared with the saline-treated control group. $^{\#}P \leq 0.025$: the IL-1β + ITZ-1 (10 mg/kg)-treated group compared with the IL-1β alone–treated group. B: For histological examination of the knee joint, H&E staining, Safranin O staining, and TUNEL assay were carried out. Arrows indicate typical apoptotic cells. ITZ-1 at 10 mg/kg prevented IL-1β–induced cartilage surface irregularity and reduction of Safranin O staining. ITZ-1 at 10 mg/kg might prevent increased expression of TUNEL-positive chondrocytes.

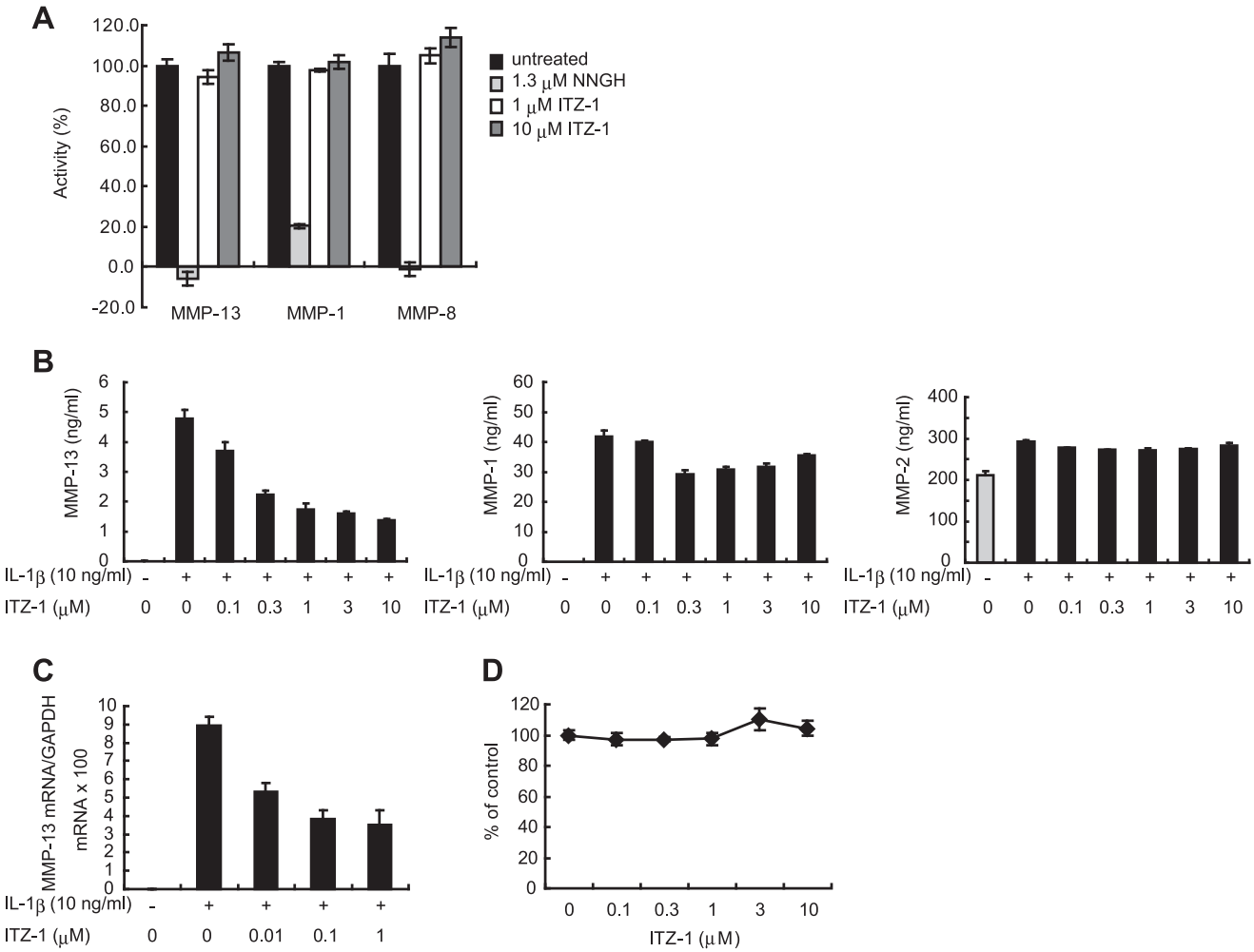

Fig. 4. ITZ-1 did not inhibit enzyme activity of MMPs, but selectively suppressed IL-1*β*–induced MMP-13 production. A: Enzyme activities of recombinant MMP-13, MMP-1, or MMP-8 were measured using a thiopeptolide as a colorimetric substrate. NNGH was used as a positive control. Results shown are each a mean ± S.D., n = 3. B: ITZ-1 selectively inhibited IL-1*β*–induced production of MMP-13 in HAC. HAC were treated with IL-1*β* for 2 days in the presence of the indicated concentration of ITZ-1. Protein levels of MMP-13, MMP-1, and MMP-2 in the culture medium were evaluated by ELISA. Results shown are each a mean ± S.D., n = 3. C: ITZ-1 inhibited IL-1*β*–induced MMP-13 mRNA expression in HAC. HAC were treated with IL-1*β* for 1 day in the presence of the indicated concentration of ITZ-1. Messenger RNA level of MMP-13 was measured by real-time quantitative PCR. Results shown are each a mean ± S.D., n = 3. D: Cytotoxicity of ITZ-1. HAC were treated with IL-1*β* and the indicated concentration of ITZ-1 for 1 day, and then cell viability was evaluated by WST-8 assay. Results shown are each a mean ± S.D., n = 4.

ITZ-1 selectively inhibits IL-1β–induced MMP-13 production

IL-1*β*–induced up-regulation of MMPs is a key event in the irreversible breakdown of the cartilage matrix. In particular, MMP-1 and MMP-13 may have a role in cartilage degradation (2, 4, 5). However, ITZ-1 did not inhibit enzyme activities of MMP-1, MMP-8, and MMP-13 at less than 10 μM (Fig. 4A). Thus, we assessed its effects on IL-1*β*–induced production of MMPs using HAC (9 – 11). HAC were treated with IL-1*β* alone or with ITZ-1 for 2 days and then the amount of each MMP in the culture medium was evaluated by ELISA. ITZ-1 inhibited IL-1*β*–induced production of MMP-13 with an IC_{50} of 0.51 μM, whereas it showed only a slight effect on the production of MMP-1 and MMP-2 (IC_{50} >10 μM, Fig. 4B). Measurement of MMP-13 mRNA levels by real-time quantitative PCR revealed that ITZ-1 inhibited IL-1*β*–induced MMP-13 mRNA expression in HAC (Fig. 4C). As ITZ-1 did not show cytotoxicity in HAC at concentrations up to 10 μM (Fig. 4D), inhibition of MMP-13 production by ITZ-1 is not a byproduct of cytotoxicity.

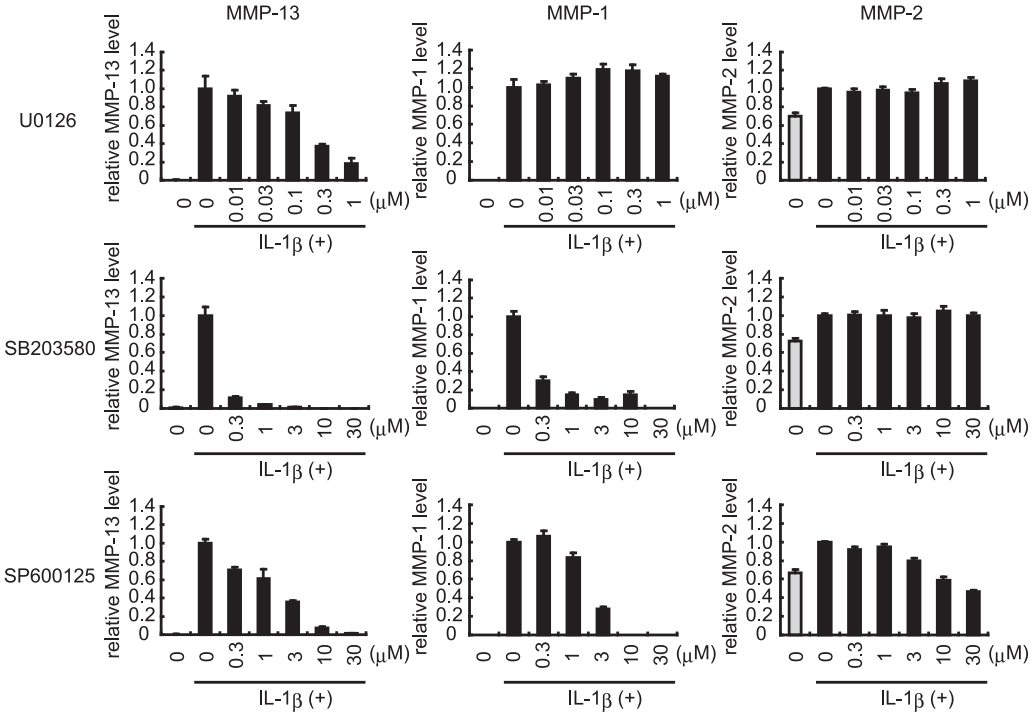

Fig. 5. Inhibition of the ERK-MAPK pathway resulted in the selective inhibition of IL-1*β*–induced MMP-13 production in HAC. HAC were treated with IL-1*β* for 2 days in the presence of the indicated concentration of an ERK-MAPK pathway inhibitor (U0126), a p38 kinase inhibitor (SB203580), or a JNK inhibitor (SP600125). Protein levels of MMPs in the culture medium were evaluated by ELISA. Results shown are each a mean ± S.D., n = 3.

ITZ-1 selectively inhibits IL-1β–induced activation of the ERK-MAPK pathway

IL-1*β*–induced production of MMPs has been shown to be mediated by ERK, p38 kinase, and JNK of the MAPK family signal transduction molecules (6, 11, 20). Thus, we investigated the effects of the MAPK-pathway inhibitors on the IL-1*β*–induced MMPs production. Interestingly, an inhibitor of the ERK–MAPK pathway (U0126) (20) selectively inhibited MMP-13 production (IC_{50} = 0.20 μM for inhibition of MMP-13 production and >1 μM for inhibition of MMP-1 and MMP-2 production), whereas a p38 kinase inhibitor (SB203580) (20) or a JNK inhibitor (SP600125) (21) inhibited both MMP-13 and MMP-1 production with IC_{50} values for SB203580 of 0.046 and 0.044 μM and for SP600125 of 1.3 and 2.0 μM, respectively (Fig. 5). SP600125, but not SB203580, also inhibited MMP-2 production (Fig. 5).

Next, we examined whether ITZ-1 could inhibit IL-1*β*–induced MAPK activation in HAC. HAC were pretreated with ITZ-1 for 24 h and then stimulated with IL-1*β* for 30 min to induce MAPK activation (11). ITZ-1 inhibited the IL-1*β*–induced ERK activation (about 60% inhibition at 3 μM), while it had little or no effect on the p38 kinase or JNK activation at less than 10 μM (Fig. 6: A and B). This result suggests that ITZ-1 selectively inhibited IL-1*β*–induced MMP-13 production by selective inhibition of the ERK–MAPK pathway.

Cytoprotective activity of ITZ-1

Recent findings suggest that chondrocyte death and survival are closely linked to cartilage matrix integrity (5, 12, 13). Furthermore, several reports suggested that intra-articular injection of IL-1*β* induces death in chondrocytes through NO production in rats (22, 23). As ITZ-1 showed a tendency to suppress IL-1*β*–induced chondrocyte apoptosis in vivo (Fig. 3B), we addressed whether ITZ-1 could suppress NO-induced death of chondrocytes in vitro. HAC were pretreated with ITZ-1 in the presence of IL-1*β* co-stimulation for 1 day, and then cell death was induced by stimulation with the NO-generator NOR3. ITZ-1 suppressed NO-induced cell death in a dose-dependent manner (Fig. 7A). As ITZ-1 suppressed IL-1*β*–induced ERK activation, the effect of U0126 on the NO-induced cell death was examined; however, U0126 did not have a remarkable effect at concentrations less than 0.3 μM (Fig. 7B).

Discussion

The irreversible destruction of cartilage, tendon, and bone that comprise synovial joints is the hallmark of OA

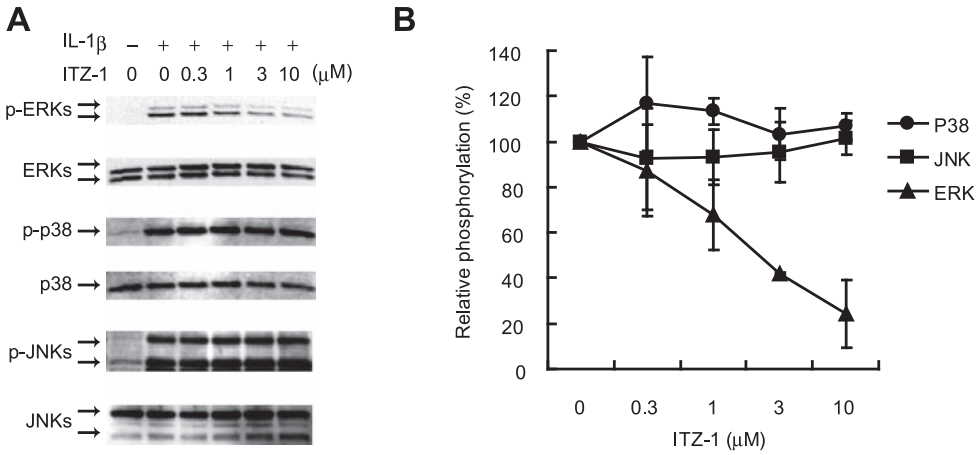

Fig. 6. ITZ-1 selectively inhibited IL-1β–induced ERK activation in HAC. A: HAC were pretreated with ITZ-1 for 24 h and then stimulated with IL-1β for 30 min to induce MAPK activation. Total and phosphorylated protein levels of each MAPK in equal amounts of whole-cell lysates were detected by western blot analysis and quantified by densitometry. B: Relative phosphorylation levels of ERK, p38 kinase, and JNK in the IL-1β stimulated HAC. Results shown are each a mean ± S.D., n = 3.

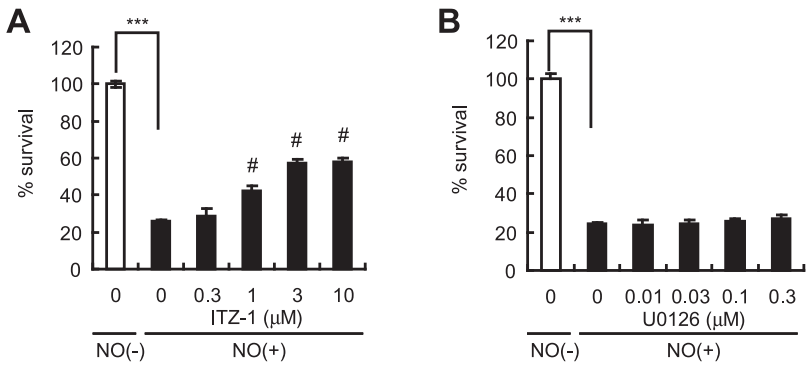

Fig. 7. Cytoprotective activity of ITZ-1 against NO-induced death of HAC. HAC were pretreated with ITZ-1 (A) or U0126 (B) together with 10 ng/ml of IL-1β for 1 day and then treated with an NO generator {NOR3: (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide} for 1 day. Afterwards, cell viability was evaluated by the MTT assay. Results shown are each a mean ± S.D., n = 4. ***$P \leq 0.001$. $^{\#}P \leq 0.025$: the IL-1β + NO–treated group compared with the IL-1β + NO + ITZ-1–treated group.

(1 – 5). IL-1, found in large amounts in joints of patients with OA, is thought to play a key role in cartilage damage by depressing the synthesis of the main extracellular matrix components and by promoting their degradation by MMPs (3, 5). In this study, we show that ITZ-1 inhibited the IL-1β–induced destruction of cartilage in vitro and in vivo: ITZ-1 at 0.3 μM significantly inhibited both IL-1β–induced proteoglycan and collagen release from bovine nasal cartilage in vitro, and it significantly suppressed IL-1β–induced cartilage proteoglycan degradation in rat synovial joints at 10 mg/kg. After oral administration of 10 mg/kg of ITZ-1, its plasma concentration reached a peak level of 3.9 ± 0.4 μM at 1 h after administration and was sustained at greater than 0.6 μM for over 7 h, concentrations adequate to suppress IL-1β–induced proteoglycan degradation in vitro. These results support the in vivo efficacy of ITZ-1. ITZ-1 could be a promising lead compound for a disease-modifying anti-OA drug program.

Cartilage is composed mainly of proteoglycans including the major proteoglycan aggrecan and other minor proteoglycans and collagens such as fibrillar type II collagen and other minor collagens. Type II collagen fibrils form an interwoven network providing tensile strength, while the proteoglycans embedded within this matrix draw water into the tissue allowing cartilage to resist compression. Depletion of proteoglycans from articular cartilage is a common initial change in OA with subsequent degradation of the collagen fibrils. Proteoglycans have a high turnover rate and are readily released from cartilage in response to proinflammatory cytokines; however, when the stimulus is removed, proteoglycans are quickly resynthesized by chondrocytes within the collagen network. In contrast, type II collagen has a low turnover rate and when collagen degradation occurs, the structural integrity of the tissue is irreversibly lost. Thus, degradation of type II collagen is a committed step in OA progression (1 – 5).

A number of proteinases including MMPs are capable of degrading proteoglycans. Within the approximately 20 member MMP-family, MMP-1, MMP-8, and MMP-13 are interstitial collagenases that degrade type II collagen (2, 3, 5). In particular, MMP-13 may have a pivotal role in cartilage degradation (2, 24) because it is expressed by chondrocytes in human OA (25), it digests type II collagen more efficiently than the other interstitial collagens (25), and its overexpression in

chondrocytes actually induces OA changes (26). As ITZ-1 suppressed IL-1β–induced collagen degradation as well as proteoglycan degradation in bovine nasal cartilage, we speculated that ITZ-1 might have some impact on the activity of MMP-13. Although ITZ-1 at concentrations up to 10 μM did not affect the enzyme activity of purified recombinant MMPs including MMP-13, it selectively suppressed IL-1β–induced MMP-13 production in HAC. It is therefore conceivable that ITZ-1 suppressed IL-1β–induced cartilage degradation via inhibition of MMP-13 production.

The selective inhibition of MMP-13 enzyme activity is important in arthritis research (2, 24), but the use of potent MMP enzyme inhibitors has been limited due to safety issues. Thus, inhibition of MMP-13 production may be a viable alternative therapeutic approach, although, with the exception of glucocorticoid hormones, no such compounds are currently in clinical use (24). ITZ-1 could be a lead compound toward a disease modifying anti-OA drug that acts by inhibiting MMP-13 production.

IL-1β–induced production of MMPs has been shown to be mediated by ERK, p38 kinase, and JNK of the MAPK family signal transduction molecules (6, 11, 20). Stimulation by IL-1β activates MAPK kinase kinases, transforming-growth-factor-β-activated kinase-1 (TAK-1), and Raf. TAK-1 then activates several MAPK kinases including MKK6, MKK4, and MKK7, which in turn activate p38 kinase and JNK, while Raf then activates MEK1, which in turn activates ERK (6). In our study, an ERK–MAPK pathway inhibitor (U0126) selectively inhibited IL-1β–induced MMP-13 production, whereas a p38 kinase inhibitor (SB203580) or a JNK inhibitor (SP600125) inhibited both IL-1β–induced MMP-13 and MMP-1 production in HAC. Furthermore, ITZ-1 inhibited IL-1β–induced ERK activation without affecting p38 kinase and JNK activation. These results indicate the possible involvement of the ERK–MAPK pathway in the selective inhibition by ITZ-1 of MMP-13 production.

Several studies have suggested that ERK can play a key role in the cartilage destruction seen in OA. A voltage-activated Ca^{2+}-channel ligand, PD-0200347, which could reduce the development of OA lesion in the dog model, reduced the levels of phosphrylated ERK, but not that of p38 kinase or JNK (27 – 29). The MEK1/2 inhibitor PD198306, which blocks ERK activation, also reduced the severity of OA lesions in a rabbit model of OA (30). Furthermore, avocado-soybean unsaponifiables (ASU), which are used to treat OA in Europe, were reported to inhibit IL-1β–induced ERK activation, but not p38 kinase or JNK activation, in chondrocytes in vitro (31). These results suggest that inhibition of ERK activation is sufficient to reduce OA lesions. However, ERK inhibitors have received little attention due to the potential toxicity of systemic ERK inhibition because the ERK–MAPK pathway is involved in a multitude of growth factor signaling pathways. Therefore, new strategies to inhibit the ERK–MAPK pathway such as non-ATP competitive inhibitors or inhibitors of downstream factors are needed (27).

Chondrocyte death and survival may be closely linked to cartilage matrix integrity (5, 12, 13). Increasing evidence indicates that NO may contribute to the pathophysiology of joint disorders including OA because increased nitrite concentrations in serum and synovial fluid from OA patients have been reported (32, 33); IL-1β has been reported to induce NO production in the synovial fluid of rats (22); IL-1β has been reported to induce death in chondrocytes through NO production (22, 23); and inhibition of NO production has been reported to suppress IL-1β–induced cartilage degradation in rats (22). Thus, inhibition of NO-induced apoptosis of chondrocytes has been a focus of interest as a therapeutic strategy for OA. Interestingly, ITZ-1 significantly suppressed NO-induced death in HAC. ITZ-1 might inhibit cartilage destruction by suppressing both IL-1β–induced MMP-13 production and cell death.

NO induces apoptosis via a mitochondria-dependent mechanism, and several factors or compounds, such as heat shock protein 70 (Hsp70) (16), the selective phoshodiesterase type III inhibitor cilostazol (34), and nonsteroidal anti-inflammatory drugs (NSAIDs) (35), have been reported to suppress NO-induced chondrocyte apoptosis. NO-induced apoptosis of articular chondrocytes has been reported to be regulated by opposite functions of MAPK subtypes, ERK and p38 kinase; NO-induced activation of ERK has been reported to show inhibitory effects on apoptosis, whereas activation of p38 kinase has been reported to induce apoptosis (36). In addition to MAPK signaling, NO was reported to cause the inhibition of protein kinase C (PKC)-α and -ζ activities (37). Both cilostazol and NSAIDs have been reported to block NO-induced p38 kinase activation, and NSAIDs have been reported to potentiate NO-induced ERK activation and block inhibition of PKC-α and -ζ (34, 35). As ITZ-1 suppressed IL-1β–induced ERK activation without affecting p38 kinase activation, the mechanisms of ITZ-1 to suppress NO-induced apoptosis might be different from those of cilostazol and NSAIDs, although ITZ-1's effect on the IL-1β– and NO-induced MAPK activation might be different. Furthermore, the ERK–MAPK pathway inhibitor U0126 showed only a slight effect on the NO-induced chondrocyte death in our experiments. These results suggest that the cell death inhibition by ITZ-1 might not depend on the blockade of

the ERK–MAPK pathway. Further studies are needed to determine the exact mechanism of action of ITZ-1. To further elucidate mechanisms of action for ITZ-1, we plan to identify ITZ-1–binding proteins by drug affinity chromatography (38).

Acknowledgments

We wish to express our sincere thanks to Drs. Seiich Tanida, Hidekazu Sawada, Masaomi Miyamoto, Hideaki Nagaya, and Takeo Wada for their helpful discussions. We also thank Takako Fuse and Tomoko Chikatsu for their technical assistance.

References

1 Loeser RF. Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. Arthritis Rheum. 2006;54:1357–1360.
2 Takaishi H, Kimura T, Dalal S, Okada Y, D'Armiento J. Joint diseases and matrix metalloproteinases: a role for MMP-13. Curr Pharm Biotechnol. 2008;9:47–54.
3 Ge Z, Hu Y, Heng BC, Yang Z, Ouyang H, Lee EH, et al. Osteoarthritis and therapy. Arthritis Rheum. 2006;55:493–500.
4 Hunter DJ, Felson DT. Osteoarthritis. Br Med J. 2006;332:639–642.
5 Aigner T, Sachse A, Gebhard PM, Roach HI. Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. Adv Drug Delivery Rev. 2006;58:128–149.
6 Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res. 2002;4:157–164.
7 Pettipher ER, Higgs GA, Henderson B. Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. Proc Natl Acad Sci U S A. 1986;83:8749–8753.
8 van de Loo AAJ, van den Berg WB. Effects of murine recombinant interleukin 1 on synovial joints in mice: measurement of patellar cartilage metabolism and joint inflammation. Ann Rheum Dis. 1990;49:238–245.
9 Mix KS, Mengshol JA, Benbow U, Vincenti MP, Sporn MB, Brinckerhoff CE. A synthetic triterpenoid selectively inhibits the induction of matrix metalloproteinases 1 and 13 by inflammatory cytokines. Arthritis Rheum. 2001;44:1096–1104.
10 Ahmed S, Wang N, Lalonde M, Goldberg VM, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1β-induced expression of matrix metalloproteinase-1 and -13 in human chondrocytes. J Pharmacol Exp Ther. 2004;308:767–773.
11 Ahmed S, Wang N, Hafeez BB, Cheruvu VK, Haqqi TM. *Punica granatum L*. extract inhibits IL-1β-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-κB in human chondrocytes in vitro. J Nutr. 2005;135:2096–2102.
12 Aiger T, Kim HA, Roach HI. Apoptosis in osteoarthritis. Rheum Dis Clin N Am. 2004;30:639–653.
13 Kühn K, D'Lima DD, Hashimoto S, Lotz M. Cell death in cartilage. Osteoarthritis Cartilage. 2004;12:1–16.
14 Cawston TE, Ellis AJ, Humm G, Lean E, Ward D, Curry V. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture. Biochem Biophys Res Commun. 1995;215:377–385.
15 Bottomley KM, Borkakoti N, Bradshaw D, Brown PA, Broadhurst MJ, Budd JM, et al. Inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors. Biochem J. 1997;323:483–488.
16 Terauchi R, Takahashi KA, Arai Y, Ikeda T, Ohashi S, Imanishi J, et al. Hsp70 prevents nitric oxide-induced apoptosis in articular chondrocytes. Arthritis Rheum. 2003;48:1562–1568.
17 Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta. 1986;883:173–177.
18 Karran EH, Young TJ, Markwell RE, Harper GP. In vivo model of cartilage degradation – effects of a matrix metalloproteinase inhibitior. Ann Rheum Dis. 1995;54:662–669.
19 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
20 Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) transcription factors downregulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol. 2002;21:251–262.
21 Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, et al. c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J Clin Invest. 2001;108:73–81.
22 Presle N, Cipolletta C, Jouzeau JY, Abid A, Netter P, Terlain B. Cartilage protection by nitric oxide synthase inhibitors after intraarticular injection of interleukin-1β in rats. Arthritis Rheum. 1999;42:2094–2102.
23 Yasuhara R, Miyamoto Y, Akaike T, Akuta T, Nakamura M, Takami M, et al. Interleukin-1β induces death in chondrocyte-like ATDC5 cells through mitochondrial dysfunction and energy depletion in a reactive nitrogen and oxygen species-dependent manner. Biochem J. 2005;389:315–323.
24 Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci. 2006;11:529–543.
25 Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, et al. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest. 1996;97:761–768.
26 Neuhold LA, Killar L, Zhao W, Sung ML, Warner L, Kulik J, et al. Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. J Clin Invest. 2001;107:35–44.
27 Loeser RF, Erickson EA, Long DL. Motogen-activated protein kinases as therapeutic targets in osteoarthritis. Curr Opin Rheumatol. 2008;20:581–586.
28 Boileau C, Martel-Pelletier J, Brunet J, Tardif G, Schrier D, Flory C, et al. Oral treatment with PD-0200347, an $\alpha_2\delta$ ligand, reduces the development of experimental osteoarthritis by inhibiting metalloproteinases and inducible nitric oxide synthase gene expression and synthesis in cartilage chondrocytes. Arthritis Rheum. 2005;52:488–500.
29 Boileau C, Martel-Pelletier J, Brunet J, Schrier D, Flory C, Boily M, et al. PD-0200347, an $\alpha_2\delta$ ligand of the voltage gated calcium channel, inhibits in vivo activation of the Erk1/2

pathway in osteoarthritic chondrocytes: a PKCα dependent effect. Ann Rheum Dis. 2006;65:573–580.

30 Pelletier JP, Fernandes JC, Brunet J, Moldovan F, Schrier D, Flory C, et al. In vivo selective inhibition of mitogen-activated protein kinase kinase 1/2 in rabbit experimental osteoarthritis is associated with a reduction in the development of structural changes. Arthritis Rheum. 2003;48:1582–1593.

31 Gabay O, Gosset M, Levy A, Salvat C, Sanchez C, Pigenet A, et al. Stress-induced signaling pathways in hyalin chondrocytes: inhibition by Avocado-Soybean Unsaponifiables (ASU). Osteoarthritis Cartilage. 2008;16:373–384.

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

33 Loeser RF, Carlson CS, Carlo MD, Cole A. Detection of nitrotyrosine in aging and osteoarthritic cartilage. Arthritis Rheum. 2002;46:2349–2357.

34 Lee SW, Song YS, Shin SH, Kim KT, Park YC, Park BS, et al. Cilostazol protects rat chondrocytes against nitric oxide-induced apoptosis in vitro and prevents cartilage destruction in a rat model of osteoarthritis. Arthritis Rheum. 2008;58:790–800.

35 Yoon JB, Kim SJ, Hwang SG, Chang S, Kang SS, Chun JS. Non-steroidal anti-inflammatory drugs inhibit nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes independent of cyclooxygenase activity. J Biol Chem. 2003;278:15319–15325.

36 Kim SJ, Ju JW, Oh CD, Yoon YM, Song WK, Kim JH, et al. ERK-1/2 and p38 kinases oppositely regulate nitric oxide-induced apoptosis of chondrocytes in association with p53, caspase-3, and differentiation status. J Biol Chem. 2002;277:1332–1339.

37 Kim SJ, Kim HG, Oh CD, Hwang SG, Song WK, Yoo YJ, et al. p38 kinase-dependent and –independent inhibition of protein kinase C ζ and -α regulates nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes. J Biol Chem. 2002;277:30375–30381.

38 Lokey RS. Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia. Curr Opin Chem Biol. 2003;7:91–96.