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Investigation for the mechanism of quinolone antibacterial agent ofloxacin-induced chondrotoxicity in juvenile rats

（キノロン系抗菌薬オフロキサシンの幼若ラットにおける関節毒性発症機序に関する研究）

Koichi Goto

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Abbreviations

$AUC_{0\text{-}24h}$	area under the concentration-time curve up to 24 hours post-dose
C_{max}	maximum concentration
Dusp1	dual specificity phosphatase 1
Fos	FBJ murine osteosarcoma viral oncogene homolog
h	hour
Has2	hyaluronan synthase 2
Il6ra	interleukin 6 receptor, alpha
ISH	*in situ* hybridization
MC	methylcellulose
min	minutes
Mmp3	matrix metalloproteinase 3
Mt1a	metallothionein 1a
Nfkbia	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
OFLX	ofloxacin
Plaur	plasminogen activator, urokinase receptor
Ptgs2	prostaglandin-endoperoxide synthase 2
qPCR	quantitative real-time polymerase chain reaction
Rps27a	ribosomal protein S27a
qRT-PCR	quantitative real-time reverse transcription-polymerase chain reaction
s	second
Sstr1	somatostatin receptor 1
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a

General introduction

Quinolone antibacterial agents (Quinolones) have been developed so far modifying the chemical structure of nalidixic acid (NA) which was introduced into chemotherapy field as the first quinolone in 1962 (Lesher et al., 1962). The chemical structure of quinolones is shown in Figure 1. Quinolones exhibit their bactericidal activity primarily by inhibiting bacterial DNA gyrase. The early quinolones such as nalidixic acid, oxolinic acid (Kaminsky and Meetzer, 1967), and pipemidic acid (Shimizu et al., 1975) had a narrow spectrum of activity, low potency, high frequency of spontaneous bacterial resistance, low serum drug concentrations, and short half-lives, which restricted their use to urinary tract infection. However, the newly developed fluorinated quinolones, such as norfloxacin (Ito et al., 1980), ofloxacin (Sato et al., 1982), ciprofloxacin (Wise et al., 1983), levofloxacin (Hayakawa et al. 1986), and sparfloxacin (Nakamura et al., 1989), possess a broad antibacterial spectrum including both Gram-negative and Gram-positive bacteria, a low incidence of resistance, high oral bioavailability, extensive tissue penetration, low protein binding, and long elimination half-lives (Rosenstiel and Adam, 1994). Recently, garenoxacin that lacks a fluorine at the C-6 position has been reported to have a broad antibacterial activity against staphylococci, streptococci, pneumococci, and Enterococcus faecalis (Fung-Tomc et al., 2000). Furthermore, some new quinolones have been approved in the clinical fields (Muratani et al., 1992; Sato et al., 1992; Dalhoff et al., 1996).

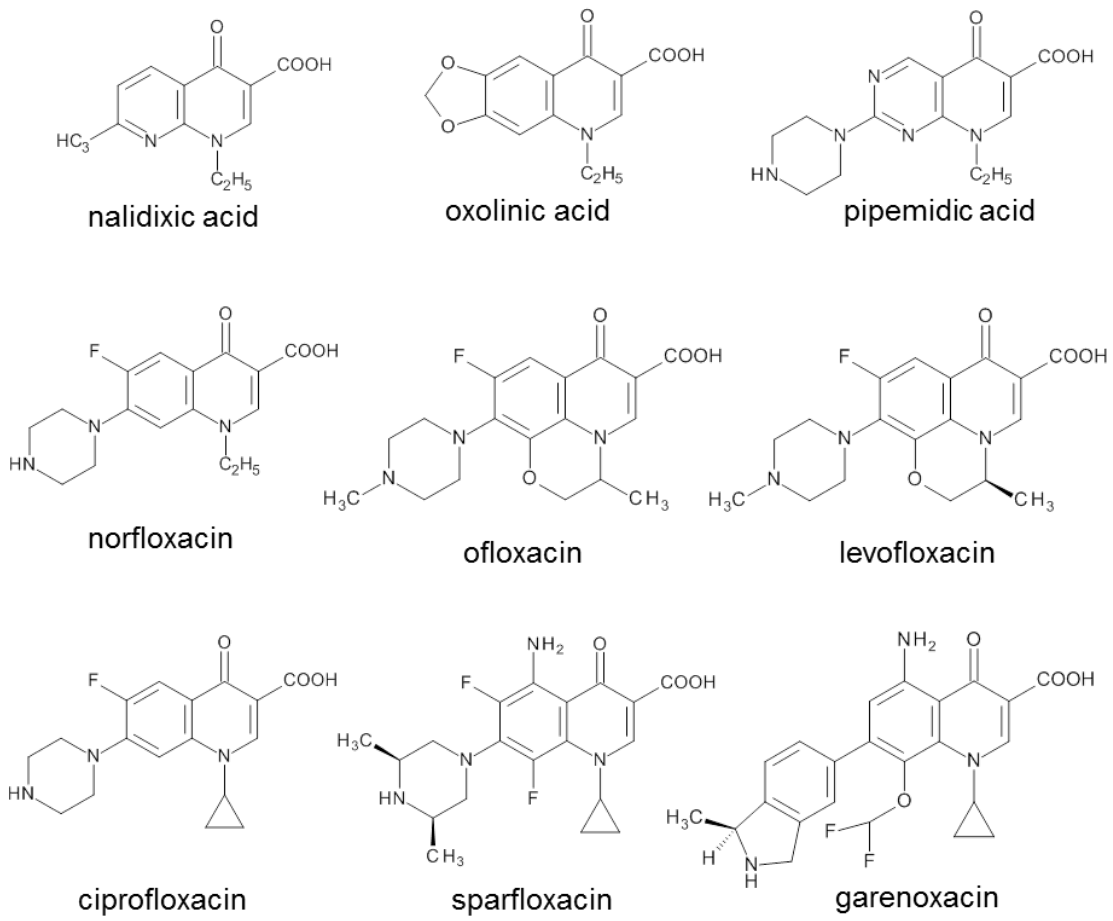


Figure 1 Chemical structures of quinolones.

Quinolones have been known to have several adverse effects such as central nervous system (CNS) effects, phototoxicity, chondrotoxicity (arthropathy), cardiotoxicity, and hypoglycemia (Andriole, 2005). In juvenile dogs treated with pipemidic acid, toxic effects on the immature joint cartilage were described for the first time in 1977 (Ingham et al., 1977). After that, some researchers have reported the quinolone-induced chondrotoxicity in juvenile animals such as mice (Linseman et al., 1995), rats (Kato and Onodera, 1988a), guinea pigs (Bendele et al., 1990), rabbits (Kato et al., 1995), dogs (Burkhardt et al., 1990), and non-human primates (Stahlman et al., 1990).

The quinolone-induced initial histopathological changes in the articular cartilage of juvenile animals have been demonstrated to occur in chondrocytes

(Burkhardt et al., 1992; Kato and Onodera, 1988a). Kato and Onodera (1988b) have reported that ofloxacin (OFLX) inhibits the uptake of ^{3}H-thymidine and $^{35}SO_4$ by the articular cartilage of juvenile rats 12 h after a single oral administration. Considering the inhibitory effect of the drug on topoisomerase II of mammalian cells, they speculated that the initial target of OFLX for the cartilage damage was the DNA synthesis of chondrocytes. Meanwhile, Bendele et al. (1990) has reported that the extracellular matrix was a site of the initiation of the lesions, although no changes in degrading enzymes such as collagenase and neutral protease were detected. Recent studies have shown that the synthesis of extracellular matrix components and the mitochondrial function of chondrocytes were susceptible to inhibitory effects of quinolones *in vitro* (Kato et al., 1995; Yabe et al., 2004). Moreover, it has been reported that chelation of magnesium ions with quinolones results in electrolyte imbalance, impairment of the integrin functions, and matrix degeneration in the cartilage (Shakibaei et al., 1996; Stahlmann et al., 1995). However, it has not yet been clarified whether the lesions seen in the cartilage exposed to quinolones were caused by direct toxic effects of quinolones on chondrocytes or were mediated by gene expression changes and chemical mediator productions in the cells. Thus, gene expression analysis for chondrocytes is considered to be useful to elucidate the mechanism of the chondrotoxicity.

Juvenile dogs have been commonly known to be the most sensitive species to the quinolone induced chondrotoxicity. However, to examine gene expression profile related to the chondrotoxicity in the articular cartilage, juvenile rats were used for the present study because information about genes and analytical tools such as microarray were poorly available in dogs. In this work, in order to prove some factors leading to the onset of quinolone-induced chondrotoxicity, OFLX was used as the chondrotoxic quinolone, and gene expression analysis was mainly conducted by using juvenile rats. This work contains 3 chapters: in Chapter 1, gene expression profile was investigated in the distal femoral articular cartilage of male juvenile rats treated with OFLX by GeneChip, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), and *in situ* hybridization (ISH). In Chapter 2, toxicokinetics and gene

expression were examined in the cartilage of male juvenile rats given non-chondrotoxic quinolones, and were compared with those in juvenile rats receiving OFLX. In Chapter 3, effects of body-weight loading onto the articular cartilage on the occurrence of the OFLX-induced chondrotoxicity was investigated in the articular cartilage of juvenile rats by using tail suspension method.

Some parts of this thesis have been published as follows:

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2. Goto, K., Yabe, K., Suzuki, T., Jindo, T., Sanbuissho, A., 2010. Chondrotoxicity and toxicokinetics of novel quinolone antibacterial agents DC-159a and DX-619 in juvenile rats. Toxicology 276, 122-127.

3. Goto, K., Imaoka, M., Goto, M., Kikuchi I., Suzuki, T., Jindo, T., Takasaki, W., 2013. Effect of body-weight loading onto the articular cartilage on the occurrence of quinolone-induced chondrotoxicity in juvenile rats. Toxicol. Lett. 216, 124-129.

Chapter 1.

Gene expression profiles in the articular cartilage of juvenile rats receiving the quinolone antibacterial agent ofloxacin

Introduction

Quinolone-induced chondrotoxicity has been reported to show cartilage lesions such as fissure and cavity formations in the articular cartilage of juvenile animals (Bendele et al., 1990; Burkhardt et al., 1992; Ingham et al., 1977; Kato and Onodera, 1988a; Kato et al., 1995; Linseman et al., 1995; Stahlman et al., 1990). The chondrotoxicity has been suggested to be caused by inhibitory effects of quinolones on the DNA synthesis of chondrocytes (Kato and Onodera, 1988b) or the synthesis of extracellular matrix components and the mitochondrial function of chondrocytes (Kato et al., 1995; Yabe et al., 2004). Moreover, chelation of magnesium ions with quinolones has been reported to induce electrolyte imbalance, impairment of the integrin functions, and matrix degeneration in the cartilage (Shakibaei et al., 1996; Stahlmann et al., 1995). Taken together, quinolones are considered to affect chondrocyte functions directly. However, it has not been elucidated how the effect of quinolones on chondrocytes can induce cartilage lesions. Therefore, in order to clarify molecular events occurring in chondrocytes exposed to quinolones and the mechanism of the cartilage lesions, OFLX at 900 mg/kg was orally administered once to male juvenile rats, and then gene expression profiles in the articular cartilage of the distal femur were analyzed at 2, 4, 8, and 24 h post-dose by using Affymetrix GeneChip Rat Genome 230 2.0 Array. Moreover, some of the genes whose functions were known well were subjected to qRT-PCR and ISH analyses.

Materials and methods

Test substance

OFLX was synthesized at former Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), and was suspended in 1% methylcellulose (MC) aqueous solution (Nacalai Tesque Inc., Kyoto, Japan).

Animals

Male juvenile Sprague-Dawley (SD) rats, 3 weeks of age, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were maintained in a room controlled with a temperature of 22-25°C and a relative humidity of 35-75%, ventilation of 15 times or more per hour, and lighting for 12 consecutive hours per day. The animals were allowed free access to a commercial laboratory diet (F-2, Funabashi Farm Co., Ltd., Chiba, Japan) and tap water ad libitum. All experimental procedures were performed in accordance with the "Law concerning the Protection and Control of Animals" and "Standards Relating to the Care and Management, etc. of Experimental Animals" in Japan.

Tissue preparation

For GeneChip and ISH analyses, OFLX suspension was administered by gavage at a dose level of 900 mg/kg once (10 mL/kg). For qRT-PCR analysis, OFLX at 100, 300, or 900 mg/kg was administered once to confirm the reliability of GeneChip data and to investigate a dose response of gene expression. Dose levels were selected because matrix rarefaction and cavity formation of the articular cartilage in the distal femur were observed at 6 and 24 h post-dose, respectively, in male juvenile SD rats, 3 weeks of age, treated with a single oral administration of OFLX at 900 mg/kg (in-house data) but not in rats receiving a single oral dosing of OFLX at 300 mg/kg (Stahlmann et al., 1995). Rats given 1% MC solution alone in the same way served as the vehicle control. The dose composition is shown in Table 1.

All animals were euthanized by exsanguination under ether anesthesia 2, 4, 8, and 24 h after administration. About 20 mg articular cartilages were carefully

removed from the bilateral distal femurs of all animals for gene expression analysis. For GeneChip analysis, the cartilage specimens of 2 or 3 animals in each group were pooled and submerged in RNAlater Stabilization Reagent (QIAGEN, Valencia, CA, USA) at 4°C until RNA isolation. For qRT-PCR analysis, the cartilage sample of each rat was stored in the reagent at 4°C. For ISH analysis, the left distal femur was removed from all animals and fixed in Tissue Fixative (Genostaff Inc., Tokyo, Japan).

RNA extraction for GeneChip and GeneChip analysis

The reagent was replaced by 692 μL Buffer RLT supplied in an RNeasy Fibrous Tissue Mini Kit (QIAGEN), and the cartilage samples were homogenized using a Mixer Mill MM300 (QIAGEN) at 27 Hz for 10 min. After adding 8 μl proteinase K (QIAGEN) to them, the lysate was incubated at 55°C for 20 min. The lysate was transferred onto a QIAshredder Spin Column (QIAGEN), and centrifuged at 18,000 × g for 2 min at room temperature. Afterward, 650 μl lysate was again spun down at 18,000 × g for 15 min at room temperature, and then an equal volume of 70% ethanol was added to the supernatant (approximately 500 μl). Subsequent steps for RNA isolation were in accordance with the manufacturer's instruction for the kit. After isolation of total RNA, each RNA sample extracted from 2 or 3 animals in the respective groups was merged into 1 sample (total 5-animal RNA) and concentrated by using an RNeasy MiniElute Cleanup Kit (QIAGEN) up to a concentration of 1 μg/μl or more of RNA (n = 3). RNA concentration was determined by the optical density at 260 nm. RNA quality was accessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples were stored at -80°C until the following steps for GeneChip analysis.

Gene expression was analyzed using GeneChip Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) according to the Affymetrix GeneChip protocol (3 arrays/each group, total 24 arrays). Arrays were scanned with a GeneChip Scanner 3000 (Affymetrix). The amount of a transcript mRNA (signal intensity) was calculated by use of a GeneChip Operating Software (GCOS) version 1.3 (Affymetrix). All signal intensities for each gene probe were determined with the "all probe sets

scaling option" that adjusts the trimmed mean signal of a probe to a target signal value (100).

GeneChip data analysis

The GCOS pre-processed data were additionally normalized and then analyzed with a GeneSpring software version 7.3 (Agilent Technologies). A "per gene" normalization was achieved by dividing a signal intensity of each gene probe in the respective sample by a mean signal intensity of each gene probe in the corresponding control group. To remove gene probes not expressed or always expressed at low levels, expression data were filtered to select gene probes detected as "present" for all samples. Analysis of variance (ANOVA) was performed using parametric tests and multiple testing corrections, and then gene probes showing statistical significance between the OFLX and control groups at each time point were selected by using Tukey post-hoc test. Subsequently, gene probes whose normalized signal intensities were between 0.66 and 1.5 across all samples at each time point were filtered out. Moreover, gene probes whose "Annotation grade" showed A, B, or C in the public data base, NetAffy Analytical Center (Affymetrix, http://www.affymetrix.com/analysis/index.affx) were chosen. For grouping selected gene probes, hierarchical clustering was conducted using Pearson's correlation coefficient and average-linkage methods. Information of molecular functions of the genes were obtained from NetAffy Analytical Center (Affymetrix) and The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://niaid.abcc.ncifcrf.gov/).

Analysis of gene expression by qRT-PCR

RNA isolation was performed in the same method as that for "*RNA extraction for GeneChip and GeneChip analysis*". After isolation of total RNA, a concentration of each RNA sample was determined by the optical density at 260 nm, and RNA quality was accessed with an Agilent 2100 bioanalyzer (Agilent Technologies). After that, 500 ng of each RNA sample was reverse-transcribed with a SuperScriptⅢ First-Strand synthesis system for qRT-PCR (Invitrogen Corp., Carlsbad, CA, USA) at 25°C for

10 min, 42°C for 50 min, 85°C for 5 min and 37°C for 20 min in accordance with the manufacturer's instructions. Dual specificity phosphatase 1 (Dusp1); nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia); interleukin 6 receptor, alpha (Il6ra); tumor necrosis factor receptor superfamily, member 12a (Tnfrsf12a); prostaglandin-endoperoxide synthase 2 (Ptgs2); FBJ murine osteosarcoma viral oncogene homolog (Fos); CCAAT/enhancer binding protein (C/EBP), beta (C/EBPb); metallothionein 1a (Mt1a); plasminogen activator, urokinase receptor (Plaur); complement component 3 (C3); matrix metalloproteinase 3 (Mmp3); somatostatin receptor 1 (Sstr1); and hyaluronan synthase 2 (Has2) genes were quantified by quantitative real-time PCR (qPCR) using primers described in Table 2.

An aliquot 1.5 μL each of the cDNA solution was used for the respective PCR. For qPCR, cDNA was amplified in duplicate by a Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) containing 300 nM of forward and reverse primers. The thermal cycling conditions for PCR were 50°C for 2 min, 95°C for 2 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s. The qPCR was conducted with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All data were normalized to ribosomal protein S27a (Rps27a) as an internal control under the aforementioned thermal cycling conditions (primers shown in Table 2). Each PCR product was proven as a target by a melting curve analysis and an analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Histopathological examination and ISH analysis

The DNA fragment of Tnfrsf12a (accession number, NM_181086; nucleotide position, 22-457), Ptgs2 (NM_017232, 256-570), Plaur (NM_134352, 182-612) or Mmp3 (NM_133523, 1482-1722) was subcloned into pGEM-T vector (Promega, Madison, WI, USA) and used for generation of sense or antisense RNA probe. Digoxigenin (DIG) labeled-RNA probes were prepared with DIG RNA labeling Mix (Roche, Mannheim, Germany). The left distal femur was decalcified with a decalcifying solution (Genostaff), embedded in paraffin, and then sectioned at 4 μm. The tissue sections were stained with Safranin-O for a microscopic examination. For

ISH, they were dewaxed with xylene, and rehydrated through an ethanol series and phosphate buffered saline (PBS). The specimens were fixed with 4% paraformaldehyde in PBS for 15 min and then washed with PBS. They were treated with 2 μg/ml Proteinase K in PBS at 37°C for 30 min, washed with PBS, refixed with 4% paraform-aldehyde in PBS, again washed with PBS, and placed in 0.2N HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1M triethanolamine-HCl, pH 8.0/0.25% acetic anhydride for 10 min. After washing with PBS, the specimens were dehydrated through a series of ethanols. Hybridization was performed with probes at concentrations of 300 ng/ml in Probe Diluent (Genostaff) at 60°C for 16 h. After that, the sections were washed with 5×HybriWash (Genostaff) at 50°C for 20 min and then in 50% formamide and 2×HybriWash at 50°C for 20 min, followed by RNase treatment in 50 μg/ml RNaseA in 10 mM Tris-HCl, pH 8.0, 1 M NaCl and 1 mM EDTA at 37°C for 30 min. Then they were washed twice with 2×HybriWash at 50°C for 20 min, twice with 0.2×HybriWash at 50°C for 20 min, and once with 0.1% Tween20 in Tris buffered saline (TBST). After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated in anti-DIG AP conjugate (Roche) diluted 1:1000 with TBST for 2 h at room temperature. The sections were washed twice with TBST and then incubated in 100 mM NaCl, 50 mM MgCl2, 0.1% Tween20 and 100 mM Tris-HCl, pH 9.5. Coloring reactions were made in NBT/BCIP solution (Sigma-Aldrich Inc., St. Louis, MO, USA) overnight and then the sections were washed with PBS. They were counterstained with Kernechtrot (Mutoh Pure Chemicals, Tokyo, Japan), dehydrated, and then mounted with Malinol (Mutoh Pure Chemicals).

Statistical analysis

For GeneChip data, mean normalized signal intensity of each gene probe in OFLX group is expressed as fold change in tables. For qPCR data, gene expression value of each gene was normalized to that of Rps27a, and then fold change value of each gene was calculated by dividing an individual normalized value of control or ofloxacin-treated group at each time point by the mean value of the corresponding

control group. The data are expressed as the mean ± standard deviation at each time point and analyzed statistically with the biostatistical package EXSAS ver. 7.10 (Arm Systecs Corp., Osaka, Japan) for the difference between the OFLX and control groups.

Results

Expressed genes in the articular cartilage of juvenile rats treated with OFLX

In the GeneChip analysis, the expression of 134 gene probes showed statistically significant differences in expression in the OFLX group as compared with that in the control group with at least 1.5-fold difference between the groups at each time point. Based on hierarchical clustering, these probes were classified into following 6 clusters (Figure 2A): cluster 1, 15 probes, included intracellular signaling cascade-, stress response- and transcription-related genes (Table 3); cluster 2, 22 probes, carboxylic acid metabolism-, cell death- and inflammatory response-related genes (Table 4); cluster 3, 22 probes, basic-leucine zipper transcription factor genes, and stress response-, glycoprotein- and cell communication-related genes (Table 5); cluster 4, 46 probes, glycoprotein-, stress response-, proteolysis-, cell death- and transcription-related genes (Table 6); cluster 5, 4 probes, not categorized (Table 7); cluster 6, 25 probes, carboxylic acid metabolism-, glycoprotein- and transcription-related genes (Table 8). In particular, cluster 1 included Dusp1, Nfkbia, and Il6ra; cluster 2 did Tnfrsf12a, Ptgs2, and Fos; cluster 3 did C/EBPb and C/EBP, delta (C/EBPd), Mt1a, and Plaur; cluster 4 did C3 and Mmp3; cluster 6 did Sstr1 and Has2. Furthermore, time-course changes in the expression levels in each cluster were as follows: cluster 1, up-regulated at 2 h post-dose; cluster 2, up-regulated at 4 and 8 h; cluster 3, up-regulated at 8 and 24 h; cluster 4, up-regulated at 24 h; cluster 5, down-regulated at 8 h; cluster 6, down-regulated at 24 h (Figure 2B). These results suggest that intracellular signaling cascade- (up-regulated at 2 h post-dose), cell death- (up-regulated at 4 and 8 h post-dose), inflammatory response- (up-regulated at 4 and 8 h post-dose), stress response- (up-regulated at 2, 8 and 24 h post-dose), proteolysis- (up-regulated at 24 h post-dose) and glycoprotein-related genes (down-regulated at 24 h post-dose) mainly changed in turn in the articular cartilage of juvenile rats treated with OFLX. However, the involvement of the cluster 5 genes in the occurrence of cartilage lesions was unclear because little information of the cluster 5 genes was available in chondrocytes.

Gene expression of selected 13 genes in qRT-PCR analysis

Of the aforementioned genes, 13 genes known well regarding their functions and identified as related to intracellular signaling cascade, cell death, inflammatory response, stress response, proteolysis and glycoprotein were selected for qRT-PCR analysis to verify the GeneChip data and to confirm a dose response of each gene expression. These genes were as follows: Dusp1, Nfkbia and Il6ra in the cluster 1; Tnfrsf12a, Ptgs2 and Fos in the cluster 2; C/EBPb, Mt1a and Plaur in the cluster 3; C3 and Mmp3 in the cluster 4; Sstl1 and Has2 in the cluster 6. In the qRT-PCR analysis, significant increases in Il6ra, Tnfrsf12a, Ptgs2, Fos, C/EBPb, Mt1a Plaur, C3, and Mmp3 were observed (Figure 3), and significant decreases in Sstr1 and Has2 were noted (Figure 4). A tendency to increase in Dusp1 (300 and 900 mg/kg at 2 h post-dose), Ptgs2 (300 mg/kg at 4 and 24 h), Fos (900 mg/kg at 8 and 24 h), Plaur (100 mg/kg at 4 h and 300 mg/kg at 8 h) was seen; a tendency to decrease in Has2 (300 mg/kg at 2 h). But, no obvious change in Nfkbia was observed. The expression patterns of these genes at 900 mg/kg were nearly equal to those of the GeneChip data, confirming the reliability of the data. In addition, dose-dependent increases in Dusp1 (2 h post-dose), Tnfrsf12a (2, 8 and 24 h), Ptgs2 (4, 8 and 24 h), Fos (2 and 4 h), Mt1a (24 h), Plaur (4 and 8 h), and Mmp3 (24 h), and decreases in Sstr1 (24 h) and Has2 (2 h) were seen.

Histopathological examination and ISH analysis

In the histopathological examination, a slightly decreased Safranin-O staining was observed in the cartilage matrix of the middle zone at 4 h post-dose, and this expanded to the cartilage surface at 8 and 24 h post-dose. Furthermore, rarefaction and cavity formation were observed in the middle zone of the cartilage from 8 h post-dose (Figure 5). No histopathological changes in the cartilage were noted at 2 h post-dose (data not shown). In the ISH analysis, hybridization signals for antisense probe of Tnfrsf12a, Ptgs2, Plaur, or Mmp3 were positively stained in a small number of chondrocytes around the region of slightly decreased Safranin-O staining from 4 h post-dose, and the number of the positively stained cells increased around the cartilage

lesions with time dependency (Figure 6). On the contrary, no signals for each sense probe were observed in the cartilage at 24 h post-dose (Figure 6). No chondrocytes stained with each antisense probe were noted in the cartilage of control animals (data not shown).

Discussion

Gene expression changes related to OFLX chondrotoxicity were investigated in the articular cartilage of male juvenile rats by Affymetrix GeneChip Rat Genome 230 2.0 Array, qRT-PCR, and ISH analyses.

In GeneChip data, 134 gene probes that were selected by statistical analyses and fold-change filtering were classified into 6 clusters by hierarchical clustering. Among the 6 clusters, gene probes in the cluster 1 were up-regulated at 2 h post-dose, indicating that OFLX first mainly affects expressions of intracellular signaling cascade- and stress response-related genes in the articular cartilage of juvenile rats (Table 3). Of these genes, Dusp1, Nfkbia, and Il6ra genes were up-regulated. In qRT-PCR analysis, dose-dependent increases in Dusp1 were observed at 2 h post-dose (Figure 3). Dusp1 protein has been known to inactivate c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways (Theodosiou and Ashworth, 2002). Recently, OFLX has been reported to inactivate ERK/MAPK signaling pathway in microencapsulated juvenile rabbit chondrocytes (Sheng et al., 2007). Although it is unclear whether increased Dusp1 results from activation of MAPK pathways or causes inactivation, the present results suggest that OFLX affects intracellular signaling cascades in vivo, such as MAPK signaling pathways in chondrocytes in the early stage of OFLX chondrotoxicity.

The cluster 2 included gene probes showing changes in the early to middle stage of chondrotoxicity with up-regulation at 4 and 8 h post-dose of OFLX (Table 4). They predominantly consisted of cell death- and inflammatory response-related genes. Of these genes, Tnfrsf12a, Ptgs2, and Fos genes were increased. In qRT-PCR analysis, Tnfrsf12a, Ptgs2, and Fos were up-regulated with dose dependency (Figure 3), and Tnfrsf12a and Ptgs2 were shown in chondrocytes around the cartilage lesions in ISH analysis (Figure 6). Tnfrsf12a protein termed as fibroblast growth factor-inducible 14 (Fn14) has been identified to be a TNF-like weak inducer of apoptosis (TWEAK) receptor (Wiley et al., 2001). TWEAK has been reported to induce cell death (Schneider et al., 1999) and some pro-inflammatory chemokines (Harada et al., 2002). However, TWEAK-induced cell death has been demonstrated to be generally relatively

weak, characterized by a narrow spectra of sensitive cell lines, requiring longer incubation periods (Burkly et al., 2007), suggesting that TWEAK-induced cell death is not associated with chondrotoxicity *in vivo*. In contrast, Perper et al. (2006) have revealed that TWEAK/Fn14 pathway may contribute to joint tissue degeneration by inducing productions of not only proinflammatory cytokines and chemokines but also matrix metalloproteinases in human primary chondrocytes. Based on these information, TWEAK/Fn14 pathway was considered to be directly involved in the onset of cartilage lesion by inducing cytokines, chemokines and/or matrix metalloproteinases productions. Ptgs2 protein (also termed cyclooxygenase-2) has been recognized to catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins (Smith et al., 1996). In chondrocytes of human articular cartilage with osteoarthritis, Ptgs2 and prostaglandin E2 have been found elevated, and considered to play a key role of Ptgs2 in the diseased cartilage (Hardy et al., 2002), implying that Ptgs2 is implicated in the induction of chondrotoxicity. Fos (synonym of c-fos) is one of the representative nuclear oncogenes, and is known as an immediately early response gene induced by pro-inflammatory cytokines (Angel and Karin, 1991). Fos protein has been documented to represent the transcription factor activator protein-1 (AP-1) with c-Jun or its related proteins (JunB or JunD), and may act as an intracellular messenger that converts short term signals generated by extracellular stimuli into long term changes in cell phenotype by regulating the expression of downstream genes that possess an AP-1 binding site (Angel and Karin, 1991). The AP-1 binding sequences have been found in promoters of many genes, including Ptgs2 mentioned above, Mt1a and Plaur in the cluster 3 and Mmp3 in the cluster 4 genes as mentioned below (Guo et al., 2001; Haq et al., 2003; Schewe et al., 2005; Tsuji et al., 2000). In the present study, Fos was up-regulated from 2 h post-dose in qRT-PCR, suggesting that Fos was involved in inductions of some key genes related to chondrotoxicity. Histopathological examination revealed that a decreased Safranin-O staining from 4 h post-dose onward in the middle zone of the articular cartilage (Figure 5). Taken together, it is conceivable that cytokines and/or chemokines produced by increases in cell death- and inflammatory

response-related genes, such as Tnfrsf12a, Ptgs2, and Fos are strongly involved in the onset of cartilage lesions in the early to middle stage of OFLX chondrotoxicity.

The cluster 3 included middle to late response genes, which were up-regulated at 8 and 24 h post-dose of OFLX (Table 5). They mainly consisted of basic leucine zipper transcription factor genes and stress response-related genes. Among these genes, C/EBPb and d, Mt1a, and Plaur were up-regulated. In qRT-PCR analysis, Mt1a and Plaur were elevated in a dose dependent manner (Figure 3). Further, Plaur-specific signals were detected in chondrocytes around the cartilage lesions in ISH analysis (Figure 6). Metallothioneins are a family of heavy metal binding and single-chain proteins with 4 classes, designated as metallothionein-1 through -4 (Haq et al., 2003). Mt1a is a part of metallothionein-1 gene (confirmed by the website of NCBI). Metallothionein-1 gene is highly inducible in mammalian cells by not only many heavy metals, but also oxidative stress, acute-phase proteins such as IL-6 and steroid hormones (Haq et al., 2003). Furthermore, in the rheumatic cartilage, metallothioneins in chondrocytes have been reported to enhance the activity of metalloproteinases (Zafarullah et al., 1993). Plaur protein (also known as urokinase-type plasminogen activator receptor, u-PAR) has been reported to interact with the membrane receptor of the urokinase-type plasminogen activator (u-PA) that activates the proenzyme plasminogen to the broad-spectrum serine proteinase plasmin, which is able to degrade extracellular matrix directly and indirectly through activation of secreted pro-matrix metalloproteinases (Hashimoto et al., 1998). Therefore, our results point out the possibility that increases in stress response-related genes, such as Mt1a and Plaur play an important part in the occurrence of the cartilage lesions directly or through induction of metalloproteinases in the middle to late stage of OFLX chondrotoxicity.

The cluster 4 included late response genes, which were up-regulated at 24 h post-dose (Table 6). They mostly consisted of stress response- and proteolysis-related genes. Among these genes, C3 and Mmp3 genes were elevated. In qRT-PCR analysis, Mmp3 were increased in a dose-dependent manner (Figure 3), and Mmp3-positive signals were observed in chondrocytes around the cartilage lesions in

ISH analysis (Figure 6). Mmp3 is one of the metalloproteinase genes. Mmp3 protein (MMP3) has been shown to degrade the proteoglycan core proteins, laminin, fibronectin, elastin, gelatin, and non-helical collagen (Lijnen, 2002). Therefore, MMP3 is known to play a pivotal role in the cartilage destruction in some pathological conditions. It has been demonstrated that collagenase or neutral protease is not involved in the pathogenesis of cartilage lesions in guinea pigs treated with nalidixic acid (Bendele et al., 1990). However, MMP3 has not been reported to be related to induction of the quinolone chondrotoxicity. Based on these information, it is suggested that up-regulated proteolysis-related genes, such as Mmp3 strongly contribute to the onset or exacerbation of cartilage lesions in the late stage of OFLX chondrotoxicity.

The cluster 6 included down-regulated at 24 h post-dose (Table 8). They are mainly glycoprotein-related genes. Of these genes, Sstr1 and Has2 genes were down-regulated. In qRT-PCR analysis, Sstr1 and Has2 were decreased in a dose-dependent manner (Figure 4). Sstr1 protein (SSTR1) is one of the 5 receptor subtypes of neuropeptide, somatostatin (Hoyer et al., 1995). Somatostatin has been reported to inhibit monocyte/macrophase functions (Krantic, 2000), B lymphocyte immunoglobulin production, T lymphocyte proliferation, and cytokine production (Kolasinski et al., 1992). Furthermore, a previous study provided evidence that blocking the release of somatostatin aggravates chronic arthritis in Freund's complete adjuvant-induced arthritis model rats (Helyes et al., 2004). Hyaluronan synthase gene has 3 mammalian genes referred to as Has1, Has2, and Has3, and all hyaluronan synthases are capable to synthesize hyaluronan directly (Itano et al., 1999). Among these Has genes, Has2 is the major expressed genes in chondrocytes and the cartilage (Recklies et al., 2001). It has been reported that human chondrocytes treated with transforming growth factor-β1 and IL-1β inducing pro-inflammatory conditions down-regulated Has2 expression (Recklies et al., 2001). In addition, it has been demonstrated that decreased Has2 expression in human chondrocytes by Has2 antisense oligonucleotide displayed the reduction of hyaluronan, cell-associated matrix and capacity to retain newly synthesized proteoglycan in chondrocytes (Nishida et al., 1999). In the present study, Has2 gene was down-regulated at 2 h post-dose, suggesting that

cartilage matrix synthesis was inhibited in the cartilage shortly after administration of OFLX in juvenile rats. In contrast, Has2 gene was up-regulated 4 and 8 h after dosing of 300 mg/kg though the expression returned to normal or slight down-regulated level after administration of 900 mg/kg. It has been reported that a single oral administration of OFLX at 300 mg/kg does not cause cartilage lesions in the articular cartilage of juvenile rats (Stahlmann et al., 1995). This result points out the possibility that hyaluronan synthesis potential rapidly recovers in the cartilage exposed to 300 mg/kg of OFLX, which is associated with no cartilage lesions in juvenile rats treated with OFLX at 300 mg/kg. Thus, these results may imply that down-regulated glycoprotein-related genes, such as Sstr1 and Has2 induced by OFLX treatment result in inhibitions of anti-inflammatory potential and proteoglycan synthesis of the cartilage.

Recently, OFLX has been demonstrated to induce apoptosis in microencapsulated juvenile rabbit chondrocytes in vitro (Sheng et al., 2007). However, as shown in our results, OFLX has a potential to induce some genes related to cytokines, chemokines and/or proteases productions in chondrocytes but not genes showing activated apoptosis pathway. Yabe et al. (2004) have reported that necrotic chondrocytes not apoptotic chondrocytes are ultrastructurally observed in the middle zone of the articular cartilage in juvenile dogs receiving a single or 2-day oral administration of OFLX at 20 mg/kg/day. Therefore, cartilage lesions induced by OFLX were considered to be due to some chemical mediators rather than apoptosis of chondrocytes *in vivo*.

In conclusion, our results suggest that up-regulated intracellular signaling cascade-, cell death-, inflammatory response-, stress response-, and proteolysis-related genes and down-regulated glycoprotein-related genes are involved in the induction of OFLX-induced chondrotoxicity in juvenile rats. In particular, cytokines, chemokines and/or proteases produced by up-regulation of Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, and Mmp3 genes are considered to play an important role in the onset or exacerbation of cartilage lesions.

Summary

Quinolone antibacterial agents are extensively utilized in antimicrobial chemotherapy. However, they have been reported to induce arthropathy in juvenile animals, and the mechanism has not been clarified. In the present study, to investigate the molecular details of the chondrotoxicity of the quinolone OFLX, it was orally administered by gavage at a dose level of 900 mg/kg once to male juvenile SD rats, 3 weeks of age. Then gene expression profiles in the articular cartilage of the distal femur were analyzed at 2, 4, 8, and 24 h post-dose. In the GeneChip analysis, the expression of 134 gene probes in the OFLX-treated group showed statistically significant differences with at least 1.5-fold difference from the control. Among them, intracellular signaling cascade- and stress response-related genes changed at 2 h post-dose; cell death- and inflammatory response-related genes at 4 and 8 h post-dose; basic-leucine zipper transcription factor and stress response-related genes at 8 and 24 h post-dose; stress response-, proteolysis- and glycoprotein-related genes at 24 h post-dose. In qRT-PCR analysis, up-regulated Dusp1 (intracellular signaling cascade-related gene), Tnfrsf12a (cell death-related gene), Ptgs2 and Fos (inflammatory response-related genes), Mt1a and Plaur (stress response-related genes), and Mmp3 (proteolysis-related gene), and down-regulated Sstr1 and Has2 (glycoprotein-related genes) were observed with dose dependency in the articular cartilage of juvenile rats treated with OFLX at 100, 300, and 900 mg/kg. The expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 was also noted in chondrocytes around the cartilage lesions by ISH. In conclusion, our results suggest that cytokines, chemokines and/or proteases produced by up-regulation of cell death-, inflammatory response-, stress response- and proteolysis-related genes play an important role in the onset of OFLX-induced chondrotoxicity in juvenile rats.

Table 1 Dose composition.

For GeneChip or ISH analysis

Compound	Time point of euthanasia (h)	Dose (mg/kg)	n (GeneChip)	n (ISH)
Vehicle	2	0	15	3
OFLX	2	900	15	3
Vehicle	4	0	15	3
OFLX	4	900	15	3
Vehicle	8	0	15	3
OFLX	8	900	15	3
Vehicle	24	0	15	3
OFLX	24	900	15	3

For qRT-PCR analysis

Compound	Time point of euthanasia (h)	Dose (mg/kg)	n
Vehicle	2	0	5
OFLX	2	100	5
OFLX	2	300	5
OFLX	2	900	5
Vehicle	4	0	5
OFLX	4	100	5
OFLX	4	300	5
OFLX	4	900	5
Vehicle	8	0	5
OFLX	8	100	5
OFLX	8	300	5
OFLX	8	900	5
Vehicle	24	0	5
OFLX	24	100	5
OFLX	24	300	5
OFLX	24	900	5

Table 2 Primers for quantitative real-time PCR.

Gene	Accession No.	Forward primer (5’ →3’)	Reverse primer (5’ →3’)
Dusp1	NM_053769	CCCGATGACATACACGTTTGAGAG	ACAATGACATTTGTGAGGGCAGAC
Nfkbia	XM_343065	CCCAAGTACCCGGATACAGCAG	CACAGTCATCGTAGGGCAACTCA
Il6ra	NM_017020	CAGTGTCGGAAGCAGGTCCA	TGCCAACTGACTTTGAGCCAAC
Tnfrsf12a	NM_181086	CCTGCCCACTTCAGGATGCTA	CCTCACTGGATCAGTGCCACA
Ptgs2	NM_017232	GGAGCATCCTGAGTGGGATGA	AAGCAGGTCTGGGTCGAACTTG
Fos	NM_022197	TGGAGCCGGTCAAGAACATT	GCCTAGATGATGCCGGAAAC
C/EBPb	NM_024125	ACCGGGTTTCGGGACTTGA	CCCGCAGGAACATCTTTAAGTGA
Plaur	NM_134352	ACCACCGAATGGCTTCCAA	CACATCTAAGCCTGTAGCCTCCAA
Mt1a	NM_138826	TGTGCCCAGGGCTGTGT	GCAGCACTGTTCGTCACTTCA
C3	NM_016994	CGCTCAGTGCAGTTGATGGAA	GCTGGCAGCTGTACGGCATA
Mmp3	NM_133523	GTGCTCATCCTACCCATTGCAT	TTTGACAACAGGGCTACTGTCCTT
Sstr1	NM_012719	AGAGATTGGAGTCGCTGCTGAAG	CCACGGTAGTCAGTAAATGCAGACA
Has2	NM_013153	GTGACTGCACCAGTTCCGCTAA	CATGTCTAATGGGACTGCACACAAG
Rps27a	NM_031113	GAGGTTGAACCCTCGGACACTA	TTCCAACTGCTTACCAGCAAAGA

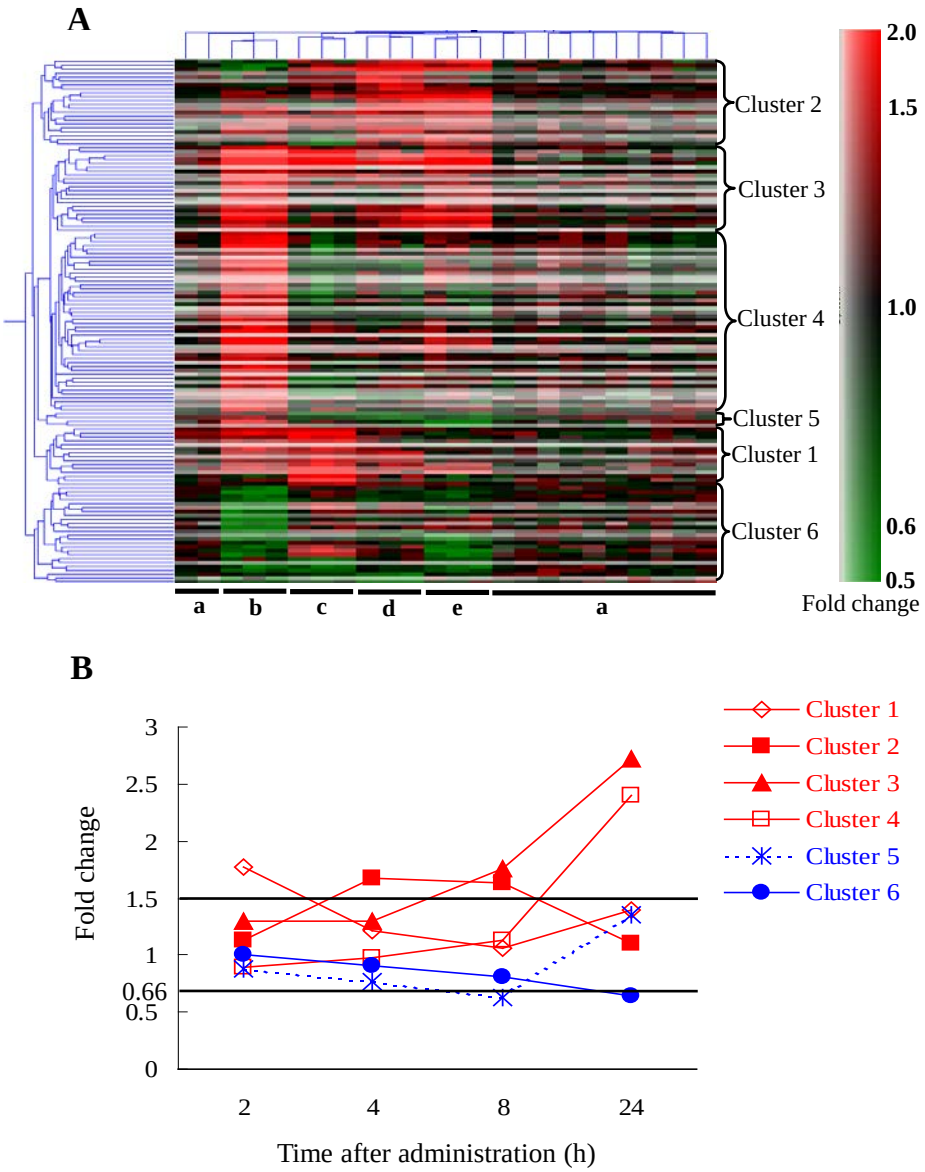


Figure 2 Gene expression patterns of gene probes separated by hierarchical clustering.

A: cluster tree of gene probes by unsupervised hierarchical clustering analysis. Heat map represents expression profile of 134 gene probes. Gene coloring was based on fold change, as indicated at the right side of the tree. a: Control; b: OFLX at 24 h post-dose; c: OFLX at 2 h post-dose; d: OFLX at 4 h post-dose; e: OFLX at 8 h post-dose. B: gene expression pattern of each cluster separated by hierarchical clustering. Each cluster indicates the cluster of gene probes separated by hierarchical clustering. Each spot represents the mean fold change in the respective clusters at each time point. The time point that the fold change value of each spot shows above 1.5 or below 0.66 is defined as “up-regulation” or “down-regulation”, respectively. Red color shows up-regulated cluster and blue color indicates down-regulated cluster.

Table 3 Gene probes in cluster 1 up-regulated at 2 h post-dose.

Gene name	Affymetrix probe ID	Fold change			
		2 [a]	4	8	24
Intracellular signaling cascade					
Dual specificity phosphatase 1 (Dusp1)	1368146_at	2.04 [b]	1.27	0.87	0.79
Dual specificity phosphatase 1 (Dusp1)	1368147_at	1.95	1.04	0.80	1.09
Epidermal growth factor (Egf)	1368325_at	1.65	1.20	0.70	1.85
Ras homolog gene family, member B (Rhob)	1369958_at	1.54	1.20	1.09	0.99
Ret proto-oncogene (Ret)	1370989_at	1.73	1.28	1.38	1.44
RAS-like family 11 member B (Rasl11b)	1383322_at	2.07	1.33	1.44	1.58
Ret proto-oncogene (Ret)	1388208_a_at	1.54	1.47	0.98	1.44
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia)	1389538_at	1.81	1.07	0.89	1.30
Stress response					
Serum/glucocorticoid regulated kinase (Sgk)	1367802_at	1.55	1.13	1.05	1.46
DNA-damage-inducible transcript 4 (Ddit4)	1368025_at	2.52	1.05	0.89	1.87
Interleukin 6 receptor, alpha (Il6ra)	1386987_at	1.95	1.25	1.76	1.40
Transcription					
Period homolog 2 (Per2)	1368303_at	1.42	1.55	0.97	1.40
Basic helix-loop-helix domain containing, class B2 (Bhlhb2)	1369415_at	1.61	1.12	1.04	1.66
Hairy and enhancer of split 1 (Hes1)	1387036_at	1.60	1.31	1.00	1.23
Miscellaneous					
Transducer of ERBB2, 2 (Tob2)	1375677_at	1.70	0.97	1.00	1.32

[a]: Time after administration (h). [b]: Mean value (n = 3).

Table 4 Gene probes in cluster 2 up-regulated at 4 and 8 h post-dose.

Gene name	Affymetrix probe ID	Fold change			
		2 [a]	4	8	24
Carboxylic acid metabolism					
Fructose-1,6-bisphosphatase 2 (Fbp2)	1368622_at	1.15 [b]	1.65	1.43	0.58
Phosphoribosyl pyrophosphate amidotransferase (Ppat)	1369785_at	1.16	1.74	1.35	1.53
Phosphoserine aminotransferase 1 (Psat1)	1372665_at	1.13	1.60	1.07	0.84
Solute carrier family 7 (cationic amino acid transporter, y+system), member 5 (Slc7a5)	1387280_a_at	1.58	1.62	1.62	1.24
Asparagine synthetase (Asns)	1387925_at	0.99	1.60	1.27	1.03
Cell death					
Heat shock 70kD protein 1A (Hspa1a)	1368247_at	0.79	1.79	1.89	1.22
Heat shock 70kD protein 1B (mapped) (Hspa1b_mapped)	1370912_at	0.75	1.56	1.70	1.06
Tumor necrosis factor receptor superfamily, member 12a (Tnfrsf12a)	1371785_at	1.31	2.15	2.60	1.26
Annexin A1 (Anxa1)	1394451_at	0.97	1.54	1.73	0.99
Inflammatory response					
Prostaglandin-endoperoxide synthase 2 (Ptgs2)	1368527_at	1.07	2.50	2.87	1.65
FBJ murine osteosarcoma viral oncogene homolog (Fos)	1375043_at	2.13	3.04	3.06	0.65
Miscellaneous					
Transgelin (Tagln)	1367570_at	1.08	1.45	2.00	0.79
Tetraspanin 2 (Tspan2)	1368105_at	1.14	1.38	1.53	1.20
Vesicle transport through interaction with t-SNAREs homolog 1A (Vti1a)	1368889_at	0.90	1.10	1.77	1.05
Phospholipase C, epsilon 1 (Plce1)	1368980_at	1.01	1.66	0.83	0.99
Eukaryotic elongation factor-2 kinase (Eef2K)	1369638_at	1.25	1.69	1.09	0.85
PDZ and LIM domain 7 (Pdlim7)	1370347_at	0.92	1.60	1.14	1.44
ElaC homolog 2 (Elac2)	1374107_at	1.14	1.38	1.53	1.20
SDA1 domain containing 1 (Sdad1)	1382921_at	1.29	1.26	1.53	1.45
Integrin alpha 5 (Itga5_mapped)	1385649_at	1.25	1.46	1.53	1.42
Eukaryotic elongation factor-2 kinase (Eef2K)	1387658_at	1.18	1.99	0.99	0.99
Immediate early response 5 (Ier5)	1389355_at	0.64	1.13	1.34	0.96

[a]: Time after administration (h). [b]: Mean value (n = 3).

Table 5 Gene probes in cluster 3 up-regulated at 8 and 24 h post-dose.

Gene name	Affymetrix probe ID	Fold change 2 [a]	4	8	24
Basic-leucine zipper transcription factor					
CCAAT/enhancer binding protein (C/EBP), delta (C/EBPd)	1368813_at	1.89 [b]	1.82	2.23	3.80
CCAAT/enhancer binding protein (C/EBP), beta (C/EBPb)	1387087_at	2.30	1.37	1.69	4.70
CCAAT/enhancer binding protein (C/EBP), delta (C/EBPd)	1387343_at	2.05	1.83	2.23	3.71
Jun-B oncogene (Junb)	1387788_at	1.15	1.61	2.61	3.15
Stress response					
Inhibin beta-A (Inhba)	1369012_at	1.17	0.93	1.85	2.27
Coagulation factor III (F3)	1369182_at	1.96	1.21	2.54	3.75
Superoxide dismutase 2, mitochondrial (Sod2)	1370173_at	0.97	1.39	1.35	2.50
Metallothionein 1a (Mt1a)	1371237_a_at	2.39	2.15	2.80	10.11
Plasminogen activator, urokinase receptor (Plaur)	1387269_s_at	0.60	1.17	2.69	2.08
Heat shock 70kDa protein 2 (Hspa2)	1394200_at	0.98	0.93	1.53	1.65
Glycoprotein					
Heparin-binding EGF-like growth factor (Hbegf)	1368983_at	0.96	1.28	1.50	1.80
Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 (Slc1a1)	1370367_at	0.90	0.79	1.19	1.73
Beta galactoside alpha 2,6 sialyltransferase 1 (St6gal1)	1370907_at	1.13	1.43	1.74	1.52
Urotensin 2 receptor (Uts2r)	1387304_at	1.48	1.28	1.26	2.93
Solute carrier family 29 (nucleoside transporters), member 1 (Slc29a1)	1398295_at	1.01	1.28	1.51	1.53
Cell communication					
Glutamate-ammonia ligase (Glul)	1367632_at	1.19	1.05	1.23	1.95
Ral guanine nucleotide dissociation stimulator (Ralgds)	1367825_at	1.17	0.95	1.42	1.87
Down syndrome critical region homolog 1 (Dscr1)	1388686_at	0.95	1.35	1.37	1.65
Interleukin 13 receptor, alpha 1 (Il13ra1)	1388711_at	0.99	0.99	1.33	1.66
Miscellaneous					
Cysteine and glycine-rich protein 2 (Csrp2)	1370282_at	1.13	1.29	1.23	1.75
Myeloid cell leukemia sequence 1 (Mcl1)	1372520_at	1.17	1.08	1.31	1.61
Immediate early response 3 (Ier3)	1388587_at	0.98	1.51	2.04	2.09

[a]: Time after administration (h). [b]: Mean value (n = 3).

Table 6 Gene probes in cluster 4 up-regulated at 24 h post-dose.

Gene name	Affymetrix probe ID	Fold change 2 [a]	4	8	24
Glycoprotein					
Glial cell line derived neurotrophic factor family receptor alpha 1 (Gfra1)	1367954_at	0.98 [b]	1.00	0.88	1.61
WNT1 inducible signaling pathway protein 2 (Wisp2)	1369484_at	0.98	1.14	1.27	3.45
Bone morphogenetic protein 3 (Bmp3)	1369773_at	0.61	0.91	0.74	2.54
Growth arrest specific 6 (Gas6)	1383047_at	0.94	1.17	1.17	1.99
Chitinase 3-like 1 (Chi3l1)	1392171_at	1.20	1.19	1.20	3.67
Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 (Slc1a1)	1387932_at	1.20	0.70	1.17	1.85
Stress response					
Complement component 3 (C3)	1368000_at	0.77	0.91	0.91	4.06
Serine (or cysteine) peptidase inhibitor, clade A, member 3N (Serpina3n)	1368224_at	1.13	0.99	1.27	7.27
CD14 antigen (Cd14)	1368490_at	1.01	0.90	1.11	1.60
Complement component 5, receptor 1 (C5r1)	1368742_at	0.80	0.74	1.00	2.15
Superoxide dismutase 2, mitochondrial (Sod2)	1370172_at	0.96	1.03	1.46	3.02
UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1 (mapped) (B4galt1_mapped)	1371073_at	0.95	0.90	0.88	1.59
Serine (or cysteine) peptidase inhibitor, clade G, member 1 (Serping1)	1372254_at	1.02	1.04	1.05	1.80
Heat shock 27kD protein family, member 7 (Hspb7)	1372649_at	0.88	1.12	0.98	2.11
Myeloid differentiation primary response gene 88 (Myd88)	1374468_at	0.81	0.91	0.99	1.54
Lipopolysaccharide binding protein (Lbp)	1387868_at	0.69	0.94	1.04	3.58
Proteolysis					
Tissue inhibitor of metalloproteinase 1 (Timp1)	1367712_at	0.87	1.01	1.06	1.66
Plasminogen activator, tissue (Plat)	1367800_at	1.11	0.96	1.07	1.63
Matrix metalloproteinase 3 (Mmp3)	1368657_at	0.97	1.10	1.47	4.03
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (Ace)	1370028_at	0.86	1.13	1.01	2.46
Proprotein convertase subtilisin/kexin type 5 (Pcsk5)	1393467_at	1.21	0.96	1.11	1.92
Cell death					
Tumor necrosis factor receptor superfamily, member 1a (Tnfrsf1a)	1367715_at	0.99 [b]	0.85	1.03	1.74
STEAP family member 3 (Steap3)	1370374_at	1.01	1.08	1.03	1.60
Angiopoietin-like 4 (Angptl4)	1388924_at	0.87	0.97	1.79	3.99

[a]: Time after administration (h). [b]: Mean value (n = 3). (*continued on next page*)

Table 6 (*continued)*

Gene name	Affymetrix probe ID	Fold change 2 [a]	4	8	24
Transcription					
Nuclear factor, erythroid derived 2, like 2 (Nfe2l2)	1367826_at	0.91 [b]	1.23	1.03	1.59
ClpB caseinolytic peptidase B homolog (Clpb)	1367997_at	0.96	0.98	1.13	1.80
Runt related transcription factor 1 (Runx1)	1368914_at	0.72	1.10	1.36	2.13
High mobility group AT-hook 1 (Hmga1)	1388309_at	0.66	1.03	1.45	3.02
Miscellaneous					
Flotillin 2 (Flot2)	1367746_a_at	0.80	0.97	0.89	1.60
Interferon-related developmental regulator 1 (Ifrd1)	1367795_at	0.67	1.10	1.09	1.55
5-oxoprolinase (ATP-hydrolysing) (Oplah)	1368091_at	0.89	0.78	0.90	2.43
Glutamate oxaloacetate transaminase 1 (Got1)	1368272_at	0.93	0.85	0.90	1.48
Bladder cancer associated protein homolog (Blcap)	1369032_at	0.67	0.97	1.01	1.52
Lamin B receptor (Lbr)	1370136_at	0.84	0.94	0.90	1.52
Homer homolog 3 (Homer3)	1370212_at	0.59	0.87	1.26	2.17
Laminin, alpha 3 (Lama3)	1370538_at	0.81	0.85	1.23	2.43
Alpha 1,3-galactosyltransferase 2 (A3galt2)	1370561_at	0.79	0.81	1.42	2.09
Interleukin 13 receptor, alpha 1 (Il13ra1)	1370728_at	1.07	0.92	1.15	2.05
Calcium regulated heat stable protein 1（Carhsp1)	1371536_at	0.88	0.88	1.14	1.63
Secreted and transmembrane 1 (Sectm1)	1376976_at	1.11	1.45	2.34	7.43
Activity and neurotransmitter-induced early gene protein 4 (Ania4)	1387276_at	0.95	0.86	1.02	1.78
Interferon induced transmembrane protein 3 (Ifitm3)	1387995_a_at	0.93	1.01	1.08	1.94
Calcium regulated heat stable protein 1（Carhsp1)	1388659_at	0.78	0.77	1.05	2.23
Phosphatidylinositol glycan, class Q (Pigq)	1388793_at	0.80	1.00	1.01	1.53
Prepro-Neuropeptide W polypeptide	1391059_at	0.62	0.97	1.08	2.37
PX domain containing serine/threonine kinase (Pxk)	1395279_at	0.79	0.94	0.96	1.52

[a]: Time after administration (h). [b]: Mean value (n = 3).

Table 7 Gene probes in cluster 5 down-regulated at 8 h post-dose.

Gene name	Affymetrix probe ID	Fold change			
		2 [a]	4	8	24
Brain protein 16 (Brp16)	1376519_at	0.66 [b]	0.70	0.74	1.05
D site albumin promoter binding protein (Dbp)	1387874_at	1.01	0.81	0.49	1.80
Dehydrogenase/reductase (SDR family) member 1 (Dhrs1)	1389407_at	0.80	0.65	0.75	1.26
Calcium activated nucleotidase 1 (Cant1)	1396446_at	1.02	0.90	0.50	1.31

[a]: Time after administration (h). [b]: Mean value (n = 3).

Table 8 Gene probes in cluster 6 down-regulated at 24 h post-dose.

Gene name	Affymetrix probe ID	Fold change 2 [a]	4	8	24
Carboxylic acid metabolism					
Dopa decarboxylase (Ddc)	1368064_a_at	1.00 [b]	0.94	0.90	0.62
Aldolase C (Aldoc)	1386998_at	1.09	0.87	0.66	0.78
ELOVL family member 6, elongation of long chain fatty acids (Elovl6)	1394401_at	0.84	0.61	0.92	0.78
Glycoprotein					
Gremlin 1 homolog, cysteine knot superfamily (Grem1)	1369113_at	1.07	0.85	0.73	0.37
Somatostatin receptor 1 (Sstrl)	1369770_at	0.89	0.74	0.51	0.62
Alpha-1-inhibitor III murinoglobulin 1 homolog (Mug1)	1370027_a_at	1.07	0.84	0.79	0.65
Prolactin receptor (Prlr)	1370384_a_at	0.61	0.46	0.97	0.68
Spondin 2, extracellular matrix protein (Spon2)	1370847_at	1.11	1.07	1.23	0.49
Hyaluronan synthase 2 (Has2)	1387548_at	0.64	0.89	0.70	0.66
Transcription					
Myeloid/lymphoid or mixed-lineage leukemia,translocated to, 3 (Mllt3)	1368279_at	1.33	1.06	1.03	0.64
Mesenchyme homeobox 2 (Meox2)	1368422_at	0.62	0.98	0.71	0.93
Jun oncogene (Jun)	1369788_s_at	1.30	1.13	0.68	0.63
Aryl hydrocarbon receptor nuclear translocator-like (Arntl)	1370510_a_at	1.16	1.06	1.15	0.64
Inhibitor of DNA binding 4 (Id4)	1375120_at	0.87	0.89	0.59	0.61
Nuclear receptor subfamily 4, group A, member 1 (Nr4a1)	1386935_at	1.60	1.07	0.32	0.75
S100 calcium binding protein A1 (S100a1)	1388456_at	1.14	0.79	1.02	0.64
Jun oncogene (Jun)	1389528_s_at	1.45	1.04	0.58	0.61
Miscellaneous					
Purkinje cell protein 4 (Pcp4)	1368145_at	0.94	0.83	1.18	0.47
7-dehydrocholesterol reductase (Dhcr7)	1368189_at	0.82	0.61	0.89	0.92
Testis specific X-linked gene (Tsx)	1368736_at	1.41	1.06	0.68	0.41
Adenosine deaminase, RNA-specific, B1 (Adarb1)	1368933_at	1.12	1.15	0.82	0.55
Asparaginase like 1 (Asrgl1)	1374871_at	0.82	0.80	0.81	0.60
Pyruvate dehydrogenase phosphatase isoenzyme 2 (Pdp2)	1380045_at	0.65	0.99	0.79	0.70
Protein phosphatase 1, regulatory (inhibitor) subunit 3B (Ppp1r3b)	1384262_at	0.61	0.88	0.60	0.62
RAB3A, member RAS oncogene family (Rab3a)	1391434_at	1.05	0.96	0.99	0.59

[a]: Time after administration (h). [b]: Mean value (n = 3).

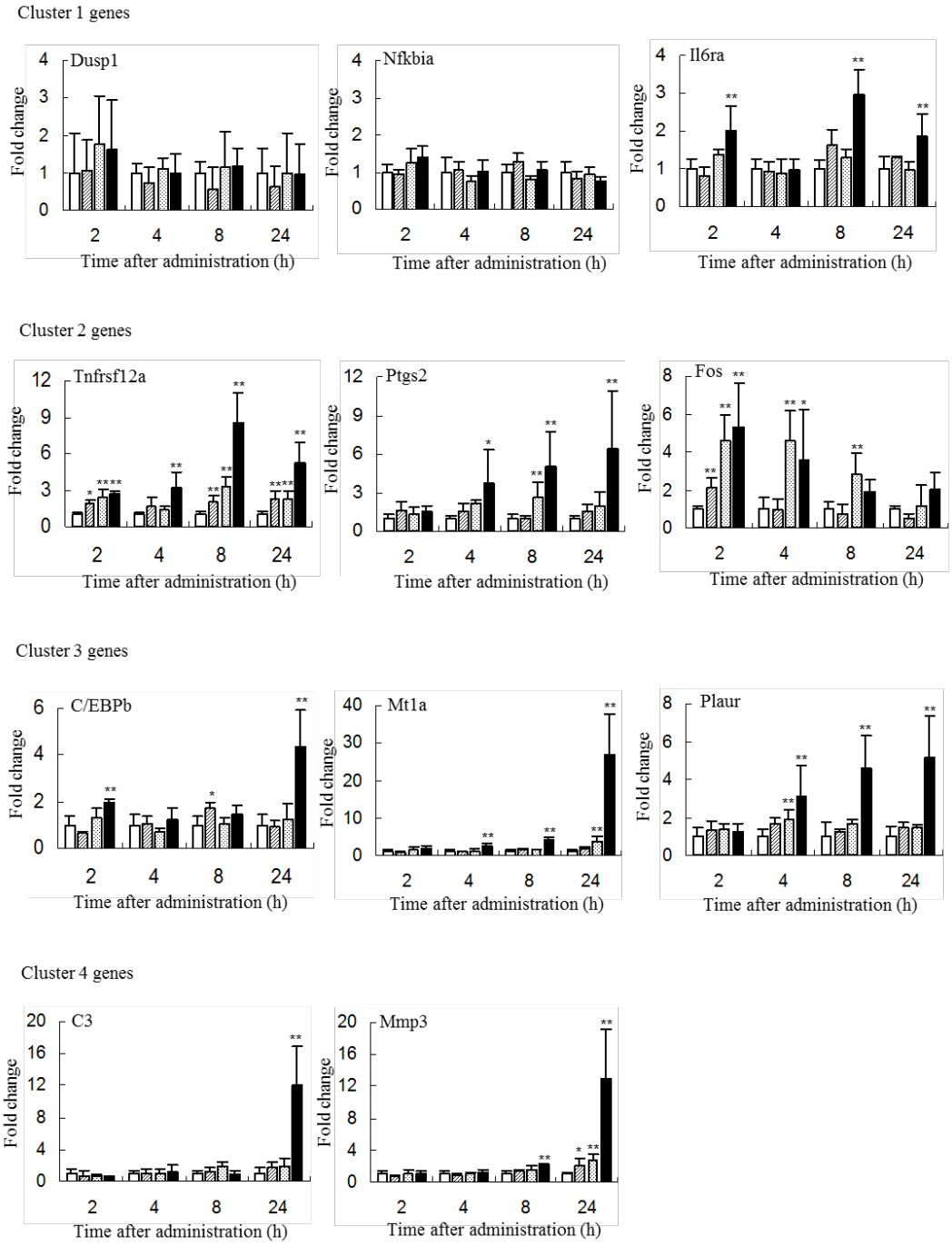


Figure 3 Time-course changes in up-regulated genes in qRT-PCR analysis. OFLX was orally administered to male juvenile rats at 100, 300, and 900 mg/kg, and qRT-PCR was conducted in the distal femoral articular cartilage. Data of qRT-PCR analysis show the mean ± standard deviation of 5 animals. $^{*}p<0.05$ and $^{**}p<0.01$: significantly different from the corresponding control group by Dunnett's multiple comparison test. (□): vehicle control; (▨): OFLX 100 mg/kg; (⁙): OFLX 300 mg/kg; (■): OFLX 900 mg/kg.

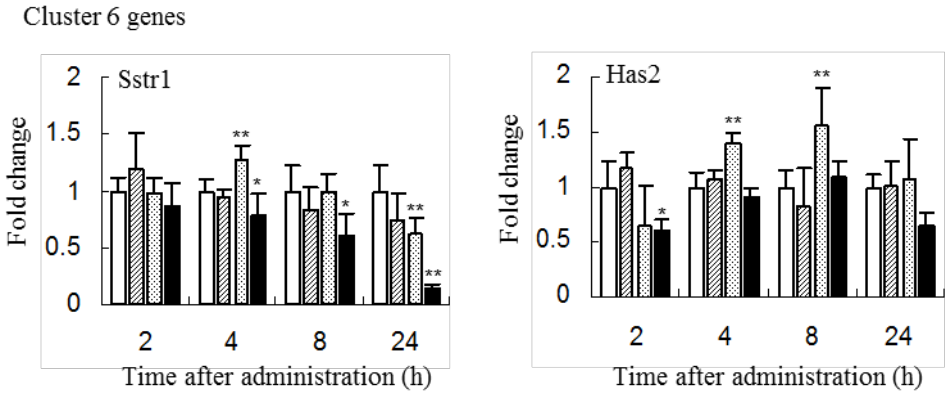


Figure 4 Time-course changes in down-regulated genes in qRT-PCR analysis. OFLX was orally administered to male juvenile rats at 100, 300, and 900 mg/kg, and qRT-PCR was conducted in the distal femoral articular cartilage. Data of qRT-PCR analysis show the mean ± standard deviation of 5 animals. $^{*}p<0.05$ and $^{**}p<0.01$: significantly different from the corresponding control group by Dunnett's multiple comparison test. (□): vehicle control; (▨): OFLX 100 mg/kg; (⁙): OFLX 300 mg/kg; (■): OFLX 900 mg/kg.

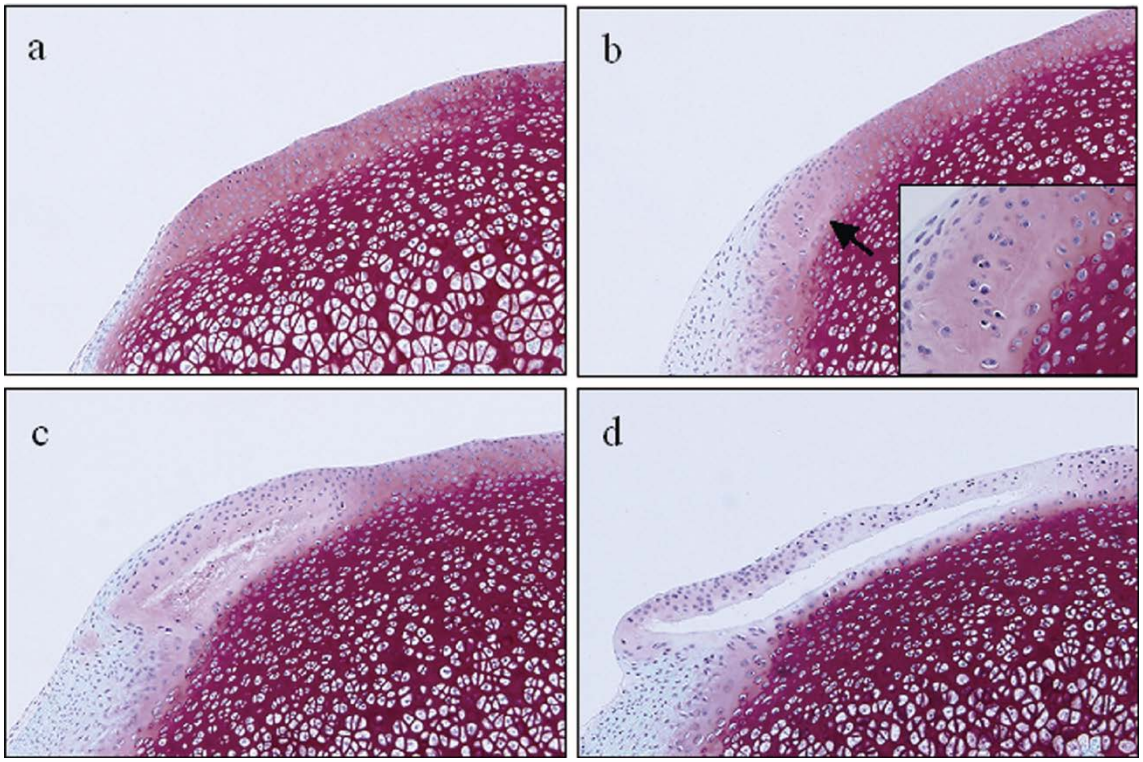


Figure 5 Histopathological examination in the distal femoral articular cartilage of juvenile rats treated with ofloxacin. OFLX was orally administered to male juvenile rats at 900 mg/kg, and Safranin-O staining was conducted in the distal femoral articular cartilage. Original magnification, ×100. a: control at 24 h post-dose. b: 4 h post-dose. A slight decrease in Safranin-O staining (arrow) was observed. Inset shows a high magnification of the region of the decreased staining (×200). c: 8 h post-dose. d: 24 h post-dose. From 8 h post-dose, rarefaction and cavity were observed in the middle zone of the articular cartilage, and a decrease in Safranin-O staining of the cartilage matrix was expanded to the cartilage surface.

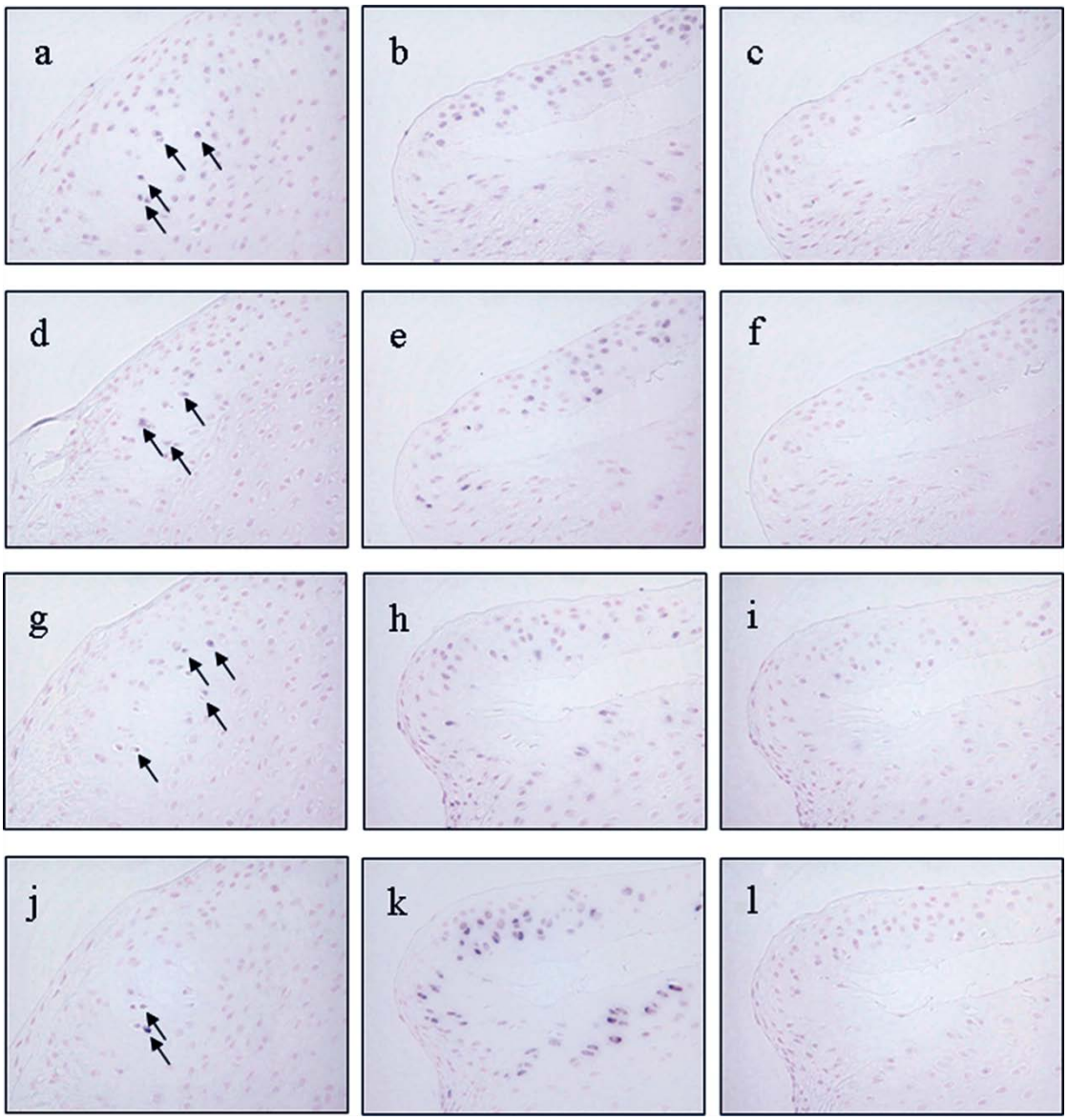


Figure 6 ***in situ*** **hybridization analysis in the distal femoral articular cartilage of juvenile rats treated with ofloxacin.** OFLX was orally administered to male juvenile rats at 900 mg/kg, and *in situ* hybridization for Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were conducted in the distal femoral articular cartilage. a-c:gene expression of Tnfrsf12a; d-f: Ptgs2; g-i: Plaur; j-l: Mmp3. a, d, g and j: 4 h-post dose; b, e, h and k: 24 h post-dose; c, f, i and l: sense probe at 24 h post-dose. Each gene of hybridization signals was positively stained in chondrocytes around the region of a slight decrease in Safranin-O staining from 4 h post-dose (arrow), and the number of the positively stained cells increased around the lesions with time dependency. Original magnification, ×200.

Chapter 2.

Chondrotoxicity and toxicokinetics of novel quinolone antibacterial agents DC-159a and DX-619 in juvenile rats

Introduction

In Chapter 1, intracellular signaling cascade-, cell death-, stress response-, inflammatory response-, and proteolysis-related genes were up-regulated, and glycoprotein-related genes were down-regulated in the articular cartilage of juvenile rats given a single oral administration of OFLX in GeneChip analyses. In addition, Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, and Mmp3 genes increased; and Sstr1 and Has2 genes decreased in the distal femoral articular cartilage of juvenile rats following a single oral administration of OFLX in qRT-PCR analysis. Furthermore, the expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes was also noted in chondrocytes around the cartilage lesions by *in situ* hybridization . These results suggest that these changes were involved in the onset or exacerbation of cartilage lesions caused by OFLX.

DC-159a is a new 8-methyoxy fluoroquinolone (Figure 7) that possesses a broad-spectrum of antibacterial activity with extended activity against gram-positive pathogens, especially streptococci and staphylococci from patients with community-acquired infections (Hoshino et at., 2008). DX-619 is a novel des-fluoro (6) quinolone (Figure 7) that has potent antibacterial activity, especially against Gram-positive organisms including methicillin-resistant Staphylococcus aureus (MRSA), methicillin-resistant coagulase-negative staphylococci (MRCNS), and vancomycin-resistant enterococci (VRE, Fujikawa et al., 2005).

In the present study, to compare the chondrotoxicity among DC-159a, DX-619, and OFLX, each quinolone was orally administered to male juvenile rats (3 weeks of age) for 7 days. Then histopathological examination was conducted in the articular cartilage of distal humerus and femur. In addition, concentrations of DC-159a or DX-619 in serum and the distal femoral articular cartilage were measured in male juvenile rats following a single oral administration of each quinolone, and compared with those of OFLX. Furthermore, DC-159a or DX-619 was orally administered once to male juvenile rats, and then Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 genes were quantified in the distal femoral articular cartilage by

qRT-PCR. The expression of these genes was compared with that in juvenile rats treated with OFLX, which was reported in Chapter 1.

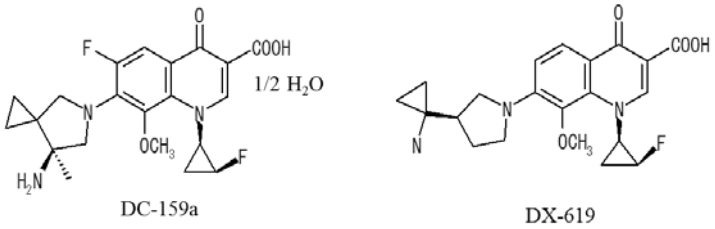


Figure 7 Chemical structure of DC-159a and DX-619.

Materials and methods

Test substance

DC-159a, DX-619, and OFLX were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan), and were suspended in 1% MC aqueous solution (Nacalai Tesque Inc.). DC-159a was used as the anhydrous free-base equivalent.

Animals

Male juvenile Sprague-Dawley rats, 3 weeks of age, were purchased from Japan SLC, Inc. They were maintained in a room controlled with a temperature of 22-25°C and a relative humidity of 35-75%, ventilation of 15 times or more per hour, and lighting for 12 consecutive hours per day. The animals were allowed free access to a commercial laboratory diet (F-2, Funabashi Farm Co., Ltd.) and tap water ad libitum. All experimental procedures were performed in accordance with the "Law Concerning the Protection and Control of Animals" and "Standards Relating to the Care and Management, etc., of Experimental Animals" in Japan.

Quinolone treatment

To conduct histopathological examination, DC-159a, DX-619, or OFLX suspension was administered by gavage at a dose level of 300 or 900 mg/kg/day for 7 days (10 mL/kg, n = 5). To measure quinolone concentrations in the distal femoral articular cartilages, DC-159a, DX-619, or OFLX suspension was orally dosed once at a dose level of 100, 300 or 900 mg/kg (n = 3). To quantify the gene expression in the distal femoral articular cartilages, DC-159a or DX-619 suspension was orally administered once at a dose level of 900 mg/kg (n = 5). Rats receiving 1% MC solution alone in the same way served as the vehicle control.

Histopathological examination

All animals were euthanized by exsanguination under ether anesthesia on the next day of the last dosing, and the bilateral distal humeri and femurs were removed,

fixed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and decalcified with 10% EDTA-4Na solution. These tissues were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin and examined light-microscopically.

Measurement of drug concentration

Blood was collected from the carotid artery of all animals under ether anesthesia 1, 2, 4, and 24 h after administration of each quinolone. After being euthanized by exsanguination under ether anesthesia, about 30 mg articular cartilages were removed from the bilateral distal femurs of all animals. About 0.5 mL of serum obtained by centrifugation at 2,150 × g for 15 min at 4ºC and the cartilage sample were stored frozen at -80ºC until analysis. Cartilage specimens were melted in 2 mL of 1N NaOH per g of cartilage at 100°C for 30 min, and an equal volume to 1N HCl was added for neutralization. Concentrations of DC-159a, DX-619, and OFLX were measured by an agar-well bioassay method with Bacillus subtilis ATCC6633 (Lot No. 020417).

RNA isolation from the articular cartilage

All animals were euthanized by exsanguination under ether anesthesia 2, 4, 8, and 24 h after administration. About 20 mg articular cartilages of each rat were carefully removed from the bilateral distal femurs of all animals and submerged in RNAlater Stabilization Reagent (QIAGEN) at 4°C until RNA isolation. RNA isolation was conducted in the same way as that for "*RNA extraction for GeneChip and GeneChip analysis*" in Chapter 1. After isolation of total RNA, a concentration of each RNA sample was determined by the optical density at 260 nm, and RNA quality was accessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were stored at -80°C until the following steps for qRT-PCR analysis.

Analysis of gene expression by qRT-PCR

Reverse-transcription reaction and qPCR were conducted in the same method as those for "*Analysis of gene expression by qRT-PCR*" in Chapter 1. Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 genes were quantified by qPCR using primers described in Table 2 (see Chapter 1). The qPCR was conducted with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All data were normalized to Rps27a as an internal control under the aforementioned thermal cycling conditions (primers shown in Table 2). Each PCR product was proven as a target by a melting curve analysis and an analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies).

Statistical analysis

For quinolone concentration data, the maximum serum or cartilage concentration (serum or cartilage C_{max}) and the area under the serum or cartilage concentration-time curve (serum or cartilage AUC) were calculated by Microsoft Excel 2000 (Microsoft Corporation, Redman, WA, USA), and then expressed as the group mean ± standard deviation. When drug concentrations were below the limit of quantification (BLQ), BLQ was regarded as "0" for the calculation. For qPCR data, gene expression value of each gene was normalized to that of Rps27a, and then the fold change value of each gene was calculated by dividing the individual normalized value of control or each quinolone-treated group at each time point by the mean value of the corresponding control group. The data are expressed as the mean ± standard deviation at each time point, and analyzed statistically. F-test was performed, and then Student's *t*-test (a P value was 0.05 or more by F-test) or Aspin-Welch's *t*-test (a P value was less than 0.05 by F-test) was conducted with the biostatistical package EXSAS ver. 7.10 (Arm Systex Corp., Osaka, Japan) for the difference between each quinolone and control groups.

Results

Histopathological examination in the articular cartilage

No changes were observed in any of the cartilages of rats given DC-159a or DX-619 at up to 900 mg/kg/day for 7 days (Table 9). On the contrary, cavity formation and chondrocyte cluster were observed in the distal humerus of 5/5 animals treated with OFLX at 300 or 900 mg/kg and in the distal femur of 2/5 and 5/5 animals receiving OFLX at 300 and 900 mg/kg, respectively (Table 9, Figure 8).

Toxicokinetic parameters of DC-159a, DX-619, or OFLX in serum

The C_{max} and $AUC_{0\text{-}24\ h}$ values of DC-159a, DX-619, or OFLX increased in accordance with the dose increment (Table 10, Figure 9). Serum C_{max} values of DC-159a were higher than those of DX-619 at all doses, and were nearly equal to those of OFLX at up to 300 mg/kg. At 900 mg/kg, the C_{max} value of DC-159a was about 2 times lower than that of OFLX. Serum $AUC_{0\text{-}24\ h}$ values of DC-159a were higher than those of DX-619 and OFLX at all doses.

Toxicokinetic parameters of DC-159a, DX-619, or OFLX in the articular cartilage

The C_{max} and $AUC_{0\text{-}24\ h}$ values of DC-159a, DX-619, or OFLX increased with increasing dose levels (Table 11, Figure 10). Cartilage C_{max} values of DC-159a were similar to those of DX-619 at 300 and 900 mg/kg, and were about 2 times lower than those of OFLX at all doses. Cartilage $AUC_{0\text{-}24\ h}$ values of DC-159a were higher than those of DX-619 at all doses, and were nearly equal to those of OFLX at up to 300 mg/kg. At 900 mg/kg, $AUC_{0\text{-}24\ h}$ values of DC-159a were lower than those of OFLX.

Gene expression in the articular cartilage in qRT-PCR analysis

In DC-159a, significant increases in Tnfrsf12a (8 and 24 h after dosing) and Mt1a (2, 4, and 24 h), and significant decreases in Tnfrsf12a (4 h), Ptgs2 (2 h), Fos (24 h), and Sstr1 (4 and 24 h) were observed, and a tendency to increase in Dusp1 (2 h)

was noted (Figure 11). In DX-619, significant increases in Fos (4 h) and Mt1a (24 h), and significant decreases in Ptgs2 (8 h), Fos (24 h), Plaur (4 h), Sstr1 (8 h), and Has2 (2 and 8 h) were seen, and tendencies to increase in Dusp1 (2 h), Fos (2 h), and Mt1a (2 and 4 h) were observed (Figure 11). However, Mmp3 gene did not change in the cartilage of any animal treated with DC-159a or DX-619.

Discussion

Chondrotoxicity of quinolone antibacterial agents DC-159a, DX-619, and OFLX was investigated in male juvenile rats treated orally with each quinolone at 300 or 900 mg/kg/day for 7 days. In addition, concentrations in serum and the articular cartilage were measured in male juvenile rats receiving a single oral administration of each quinolone at 100, 300, or 900 mg/kg. Furthermore, the gene expression was quantified in the articular cartilage of rats receiving a single oral dosing of DC-159a or DX-619 at 900 mg/kg by qRT-PCR.

In histopathological examination, no changes in the distal humeral and femoral articular cartilages were observed in animals receiving DC-159a or DX-619. On the contrary, cartilage lesions were noted in rats treated with OFLX. These results suggest that DC-159a and DX-619 have no or low chondrotoxic potential in juvenile rats.

In measurement of drug concentrations, serum C_{max} and $AUC_{0\text{-}24\ h}$ values of DC-159a were higher than those of DX-619. In contrast, cartilage C_{max} values of DC-159a were almost similar to those of DX-619. These results indicate that DC-159a could distribute similarly to DX-619 into the cartilage but could show longer exposure time in the cartilage than DX-619. On the contrary, OFLX showed a high capability to distribute into the cartilage, and cartilage C_{max} value of OFLX was higher than that of DC-159a or DX-619 at each dose. In this work, cartilage lesions were observed at 300 mg/kg or more of OFLX, but not at 900 mg/kg of DC-159a or DX-619 following 7-day oral administration. Given that the cartilage $AUC_{0\text{-}24\ h}$ value of OFLX at 300 mg/kg was nearly equal to that of DC-159a at 300 mg/kg or lower than those of DC-159a and DX-619 at 900 mg/kg, high penetration of quinolones into the cartilage may be important to evoke chondrotoxicity in juvenile rats. This result suggests that 75.63 ± 16.06 μg/g or more of OFLX may be able to induce cartilage lesions in juvenile rats. However, cartilage C_{max} value of OFLX at 300 mg/kg was little higher than or almost comparable to that of DC-159a or DX-619 at 900 mg/kg. Norfloxacin has been reported to induce arthropathy in the knee joint cartilage of male

juvenile rats treated subcutaneously with 100 mg/kg/day for 7 consecutive days, and cartilage concentration of norfloxacin was 20.6 ± 4.6 μg/g at 0.5 h post-dose (Machida et al., 1990). Sparfloxacin has been identified to cause arthropathy in the knee joint cartilage of male juvenile rats given a single oral dosing at 1,800 mg/kg, and cartilage concentration of sparfloxacin was 114.8 ± 80 μg/g at 1.5 h post-dose (Stahlmann et at., 1998). Garenoxacin has demonstrated that no changes were observed in the cartilage of the knee joint in male juvenile rats receiving 5-day oral administration at 600 mg/kg/day, and cartilage concentration of garenoxacin was 37.4 ± 7.7 μg/g 2 h after the first dosing (Kastner et al., 2004). Based on these information, the cause of no cartilage lesion in juvenile rats treated with DC-159a or DX-619 was considered to be due to not only lower penetrating potential of each quinolone into the cartilage than that of OFLX, but also other unknown factors.

Regarding an antibacterial activity, DC-159a and DX-619 have reported to possess a more potent activity against various pathogens than levofloxacin which is the optical active material of OFLX (Fujikawa et al., 2005; Hoshino et al., 2008). Thus, the antibacterial activity was not considered to be associated with the induction of quinolone-induced chondrotoxicity.

It has been reported that cartilage lesions, increases in Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, and Mmp3 genes, and decreases in Sstr1 and Has2 genes were observed in the distal femoral articular cartilage of rats given OFLX, implying that these genes are thought to be related to the onset of chondrotoxicity in juvenile rats (see Chapter 1). Therefore, these genes in the distal femoral articular cartilage exposed to DC-159a or DX-619 were quantified by qRT-PCR. In qRT-PCR analyses, significant increases or a tendency to increase in Dusp1, Tnfrsf12a, and Mt1a, and significant decreases in Tnfrsf12a, Ptgs2, Fos, and Sstr1 were observed in rats treated with DC-159a (Figure 11). Furthermore, significant increases or tendencies to increase in Dusp1, Fos, and Mt1a, and significant decreases in Ptgs2, Fos, Plaur, Sstr1, and Has2 were noted in rats receiving DX-619 (Figure 11). However, changes in Tnfrsf12a, Ptgs2, and Plaur were very slight as compared with those in juvenile rats

treated with 300 mg/kg or more of OFLX (see Chapter 1), although Dusp1, Fos, Mt1a, Sstr1, and Has2 genes were up- or down-regulated. In addition, no change in Mmp3 gene that increased in the cartilage of juvenile rats receiving 300 mg/kg or more of OFLX (see Chapter 1) was noted in any animal treated with DC-159a or DX-619. Tnfrsf12a protein, termed as fibroblast growth factor-inducible 14 (Fn14), has been found to be a TNF-like weak inducer of apoptosis (TWEAK) receptor (Wiley et al., 2001). TWEAK/Fn14 has been shown that it may be involved in the degeneration of the joint tissue in human primary chondrocytes (Perper et al., 2006). Ptgs2 has been known to catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins (Smith et al., 1996), and has been reported to play a key role in the diseased cartilage (Hardy et al., 2002). Plaur protein, known as urokinase-type plasminogen activator receptor u-PAR, has been found to interact with the membrane receptor of the urokinase-type plasminogen activator (u-PA) that activates the proenzyme plasminogen to the broad-spectrum serine proteinase plasmin, and it degrades extracellular matrix (Hashimoto et al., 1998). Mmp3 protein has been known to degrade the proteoglycan core proteins, laminin, fibronectin, elastin, gelatin, and non-helical collagen (Lijnen, 2002), and is considered to play a pivotal role in the cartilage destruction in some pathological conditions. Furthermore, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were seen in chondrocytes around the cartilage lesions induced by a single oral administration of OFLX in juvenile rats by ISH, suggesting that these genes were involved in the induction of chondrotoxicity (see Chapter 1). Taken together, our results raise the possibility that no obvious increases in Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes are associated with non-chondrotoxic potentials of DC-159a and DX-619 in juvenile rats.

In conclusion, it is suggested that the non-chondrotoxic potentials of DC-159a and DX-619 result from the low penetration of these compounds into the articular cartilage and low or no inductions of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes in the cartilage in juvenile rats.

Summary

The author have demonstrated that Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 genes change in the articular cartilage of juvenile rats with a single oral administration of OFLX, suggesting that these genes are involved in the induction of OFLX-induced chondrotoxicity. In the present study, to compare the chondrotoxic potential between new synthesized quinolones DC-159a and DX-619, and OFLX, they were orally administered by gavage at a dose level of 300 or 900 mg/kg/day to male juvenile SD rats, 3 weeks of age, for 7 consecutive days. Then the distal humerus and femur were subjected to microscopic examination. Moreover, concentrations of these quinolones in the femoral articular cartilage were measured in male juvenile rats following a single oral administration at 100, 300, or 900 mg/kg. Furthermore, gene expression of Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 was investigated in the articular cartilage of the distal femur in male juvenile rats treated with 900 mg/kg of DC-159a or DX-619 by qRT-PCR analysis. In a microscopic examination, no changes in the articular cartilage were observed in any animal administered DC-159a or DX-619. On the contrary, cavity formation and chondrocyte cluster in the cartilage of distal humerus and femur were noted in animals receiving OFLX at 300 mg/kg/day or more. In toxicokinetic analysis, the cartilage C_{max} value of DC-159a or DX-619 at 900 mg/kg was little lower than or almost comparable to that of OFLX at 300 mg/kg. Furthermore, the cartilage $AUC_{0\text{-}24\ h}$ value of DC-159a or DX-619 at 900 mg/kg was higher than that of OFLX at 300 mg/kg. In qRT-PCR analysis, up-regulated Dusp1, Fos, and Mt1a, and down-regulated Sstr1 and Has2 genes were seen in the femoral articular cartilage of rats given DX-619 or DC-159a at 900 mg/kg. However, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes, which were up-regulated in the distal femoral articular cartilage exposed to OFLX, did not increase or slightly changed. In conclusion, the penetration of DC-159a or DX-619 into the cartilage was low compared with that of OFLX, and no obvious changes in Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were observed in the articular cartilage of juvenile rats treated with

DC-159a or DX-619, which was likely to be responsible for non-chondrotoxic potentials of DC-159a and DX-619.

Table 9 Microscopic findings in male juvenile rats receiving 7-day oral administration of DC-159a, DX-619, or OFLX.

	Compound	Vehicle	DC-159a		DX-619		OFLX	
	Dose (mg/kg/day)	0	300	900	300	900	300	900
	n	5	5	5	5	5	5	5
Position	Findings							
Distal	Cavity	0 [a]	0	0	0	0	5	5
humerus	Chondrocyte cluster	0	0	0	0	0	5	5
Distal	Cavity	0	0	0	0	0	2	5
femur	Chondrocyte cluster	0	0	0	0	0	2	5

Each quinolone was orally administered to male juvenile rats for 7 days, and all animals were sacrificed 24 h after completion of treatment.

[a]: Number of animals showing the finding.

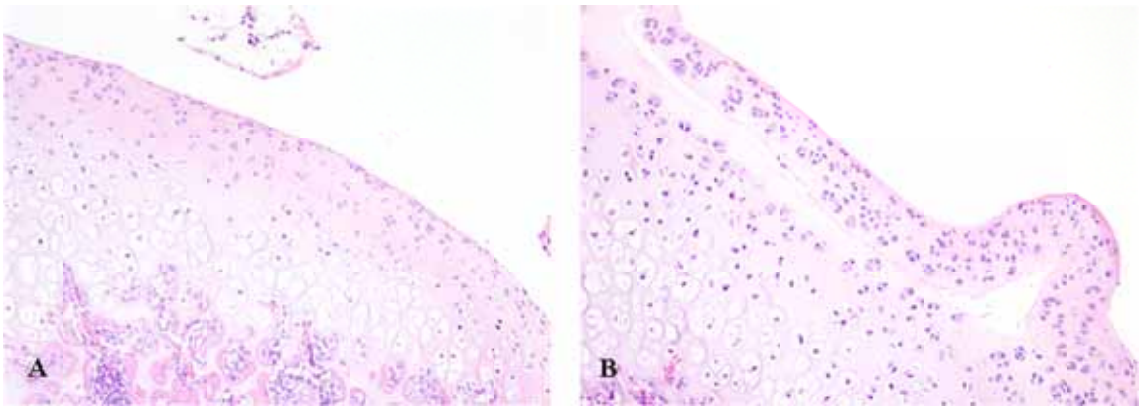


Figure 8 Histopathological examination in the distal femoral articular cartilage of juvenile rats treated with OFLX. OFLX was orally administered to male juvenile rats at 900 mg/kg for 7 days, and hematoxylin and eosin staining was conducted in the distal femoral articular cartilage. Original magnification, × 200.

(A) Articular cartilage in juvenile rats treated with 1% MC solution alone. (B) Articular cartilage in juvenile rats given 900 mg/kg/day of ofloxacin. Cavity was observed in the middle zone of the articular cartilage, and chondrocyte clusters were noted around the cavity.

Table 10　Toxicokinetic parameters of DC-159a, DX-619, and OFLX in serum of male juvenile rats receiving a single oral administration at 100, 300, or 900 mg/kg.

Dose	Parameters					
	C_{max} (μg/mL)			$AUC_{0\text{-}24\ h}$ (μg·h/mL)		
(mg/kg)	DC-159a	DX-619	OFLX	DC-159a	DX-619	OFLX
100	6.11 ± 0.29 [a]	3.44 ± 1.08	6.67 ± 1.82	31.51 ± 1.24	10.53 ± 1.58	21.72 ± 1.74
300	18.23 ± 4.14	10.75 ± 0.60	15.85 ± 4.29	135.79 ± 32.99	40.54 ± 9.53	72.55 ± 6.89
900	27.00 ± 1.52	19.49 ± 4.17	52.53 ± 17.34	294.78 ± 53.27	125.65 ± 33.22	239.08 ± 38.17

Each quinolone was orally administered to male juvenile rats for 7 days, and all animals were sacrificed 24 h after completion of treatment.

[a]: Number of animals showing the finding.

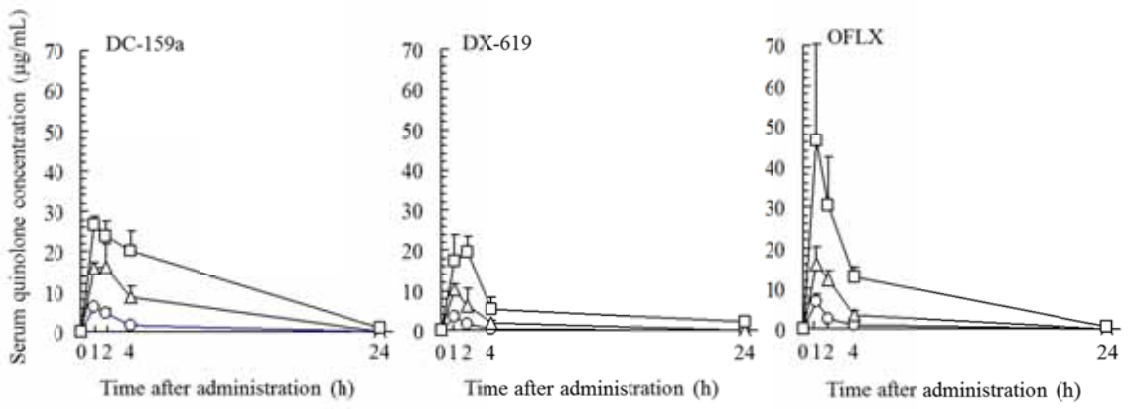


Figure 9 Serum quinolone concentrations in male juvenile rats given a single oral administration of DC-159a, DX-619 or OFLX. Serum quinolone concentrations were measured by an agar-well bioassay method. Data are represented as the mean ± standard deviation of 3 animals. (—○—): 100 mg/kg; (—△—): 300 mg/kg; (—□—): 900 mg/kg.

Table 11 Toxicokinetic parameters of DC-159a, DX-619, and OFLX in the femoral articular cartilage of male juvenile rats receiving a single oral administration at 100, 300, or 900 mg/kg

Dose	Parameters					
	C_{max} (μg/g)			$AUC_{0\text{-}24\ h}$ (μg·h/g)		
(mg/kg)	DC-159a	DX-619	OFLX	DC-159a	DX-619	OFLX
100	15.43 ± 2.92 [a]	7.77 ± 1.80	23.81 ± 2.30	83.47 ± 12.88	29.60 ± 2.19	81.27 [b]
300	38.50 ± 4.18	30.27 ± 4.10	75.63 ± 16.06	334.81 ± 27.40	135.75 ± 17.95	361.58 ± 138.88
900	54.80 ± 7.32	60.01 ± 14.23	119.89 ± 21.86	639.90 ± 111.84	434.44 ± 119.92	1012.13 ± 146.15

Each quinolone was orally administered once to male juvenile rats, and animals were sacrificed 1, 2, 4, and 24 h after dosing.

[a]: Mean ± standard deviation (n = 3). [b]: n = 2.

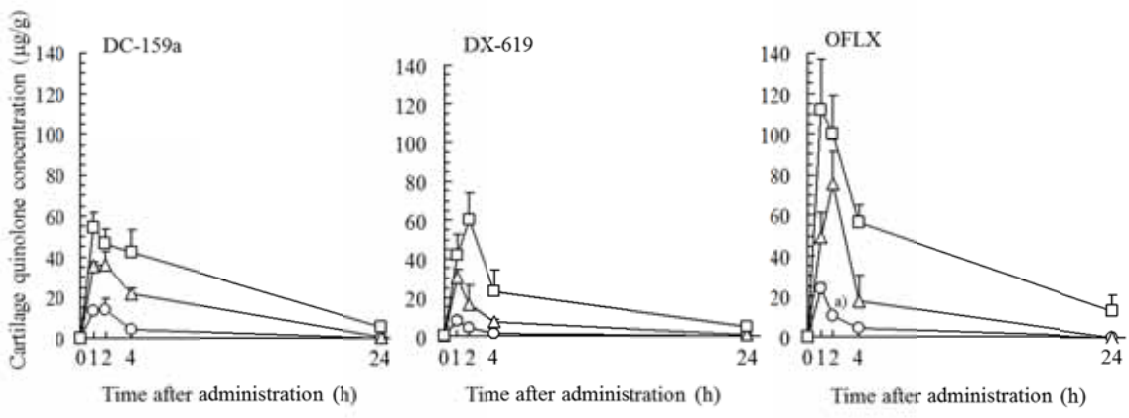


Figure 10 Cartilage quinolone concentrations in male juvenile rats receiving a single oral administration of DC-159a, DX-619, or OFLX. Cartilage quinolone concentrations were measured by an agar-well bioassay method. Data are represented as the mean ± standard deviation of 3 animals. (—○—): 100 mg/kg; (—△—): 300 mg/kg; (—□—): 900 mg/kg. a): n = 2.

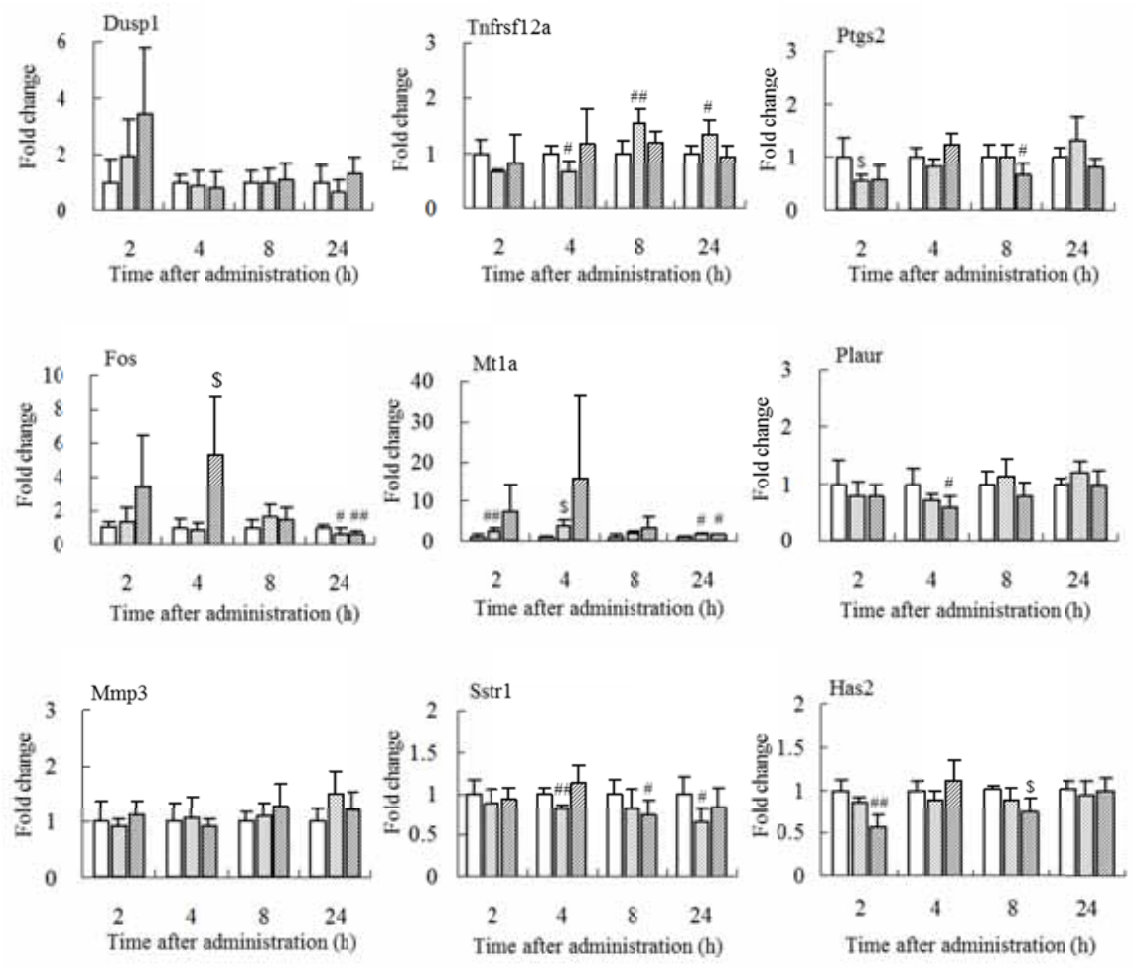


Figure 11　Time-course changes in genes in qRT-PCR analysis.

DC-159a or DX-619 was orally administered to male juvenile rats at 900 mg/kg, and qRT-PCR was conducted in the distal femoral articular cartilage. Data of qRT-PCR analysis show the mean ± standard deviation of 5 animals. $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$: significantly different from the corresponding control group by Student's *t*-test. $^{\$}$ $p<0.05$: significantly different from the corresponding control group by Aspin-Welch's *t*-test. (□): vehicle control; (▨): DC-159a 900 mg/kg; (▨): DX-619 900 mg/kg.

Chapter 3.

Effect of body-weight loading onto the articular cartilage on the occurrence of quinolone-induced chondrotoxicity in juvenile rats

Introduction

In Chapter 1, OFLX increased gene expression of Tnfrsf12a at 2 h post-dose or later; Ptgs2 and Plaur at 4 h post-dose or later; Mmp3 at 8 h post-dose or later; and induced histological changes (rarefaction and cavity formation) in the distal femoral articular cartilage at 8 h post-dose or later in male juvenile rats given a single oral administration at 300 or 900 mg/kg. On the contrary, DC-159a and DX-619, new synthesized quinolones, did not up-regulate expression of these genes and cause any histological change in male juvenile rats receiving a single or 7-day oral administration at 900 mg/kg (see Chapter 2). Based on these information, it is suggested that an increase in these gene expression is involved in the OFLX-induced chondrotoxicity in juvenile rats.

Tatsumi et al. (1978) have demonstrated that 3-day oral dosing of pipemidic acid, an old quinolone, at 1,000 mg/kg/day does not cause any blisters in the joints protected from mechanical pressure, implying that body-weight loading onto the articular cartilage is associated with the occurrence of chondrotoxicity. In the present study, the effects of body-weight loading on histological changes in the cartilage and gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 were investigated in male juvenile rats treated orally with OFLX once at 900 mg/kg under a hindlimb unloading condition by a tail suspension method.

Material and methods

Test substance

OFLX were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), and were suspended in 1% MC (Nacalai Tesque Inc.) aqueous solution to achieve a concentration of 90 mg/mL.

Animals

Male juvenile SD rats, 3 weeks of age, were purchased from Japan SLC, Inc. They were maintained in a room controlled with a temperature of 22 - 25°C and a relative humidity of 35 - 75%, ventilation of 15 times or more per hour, and lighting for 12 consecutive hours per day. The animals were allowed free access to a commercial laboratory diet (F-2, Funabashi Farm Co., Ltd.) and tap water *ad libitum*. All experimental procedures were performed in accordance with the "Law Concerning the Protection and Control of Animals" and "Standards Relating to the Care and Management, etc., of Experimental Animals" in Japan. The study was also approved by the Animal Use Committee at Daiichi Sankyo Co., Ltd.

Quinolone treatment

OFLX suspension was orally administered once at 900 mg/kg (10 mL/kg). Dose level of 900 mg/kg was selected because rarefaction and cavity formation were observed in the distal femoral articular cartilage of male juvenile SD rats given a single oral administration of OFLX 900 mg/kg at 8 h post-dose (see Chapter 1) but not in rats treated orally with OFLX at 300 mg/kg once (Stahlmann et al., 1995). Rats receiving 1% MC solution alone in the same way served as the vehicle control.

Tail suspension method

For the tail suspension group, a clip for tail suspension (Yamashita-Giken, Tokushima, Japan) with slight modification was attached to the base to middle of the tail just after administration of 1% MC aqueous solution or OFLX. After that, the

clip was connected to the stainless rod attached to the cage cover. Animals were kept in the cage individually. During the tail suspension, animals were able to move by forelimb in the cage, access the diet, and take water freely. In 2- or 4-h tail suspension group, the clip was disconnected from the tail 2 or 4 h after the start of the suspension, and then rats were kept in normal cages for the remaining time (6 h or 4 h, respectively). In the 8-h tail suspension group, the clip was detached from the tail, and then animals were sacrificed for collecting the articular cartilage for histological examination or gene expression analysis. In the 0-h tail suspension group, animals were given the test substance, and then were kept in normal cages for 8 h.

Histopathological examination

All animals were euthanized by exsanguination under isoflurane anesthesia 8 h after dosing, and the bilateral distal femurs were removed, fixed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd.) and decalcified with 10% EDTA-4Na solution. These tissues were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin, and examined by light-microscopy.

RNA isolation from the articular cartilage

All animals were euthanized by exsanguination under isoflurane anesthesia 8 h after administration. About 20 mg articular cartilages of each rat were carefully removed from the bilateral distal femurs of all animals and submerged in RNAlater Stabilization Reagent (QIAGEN) at 4°C until RNA isolation. RNA isolation was conducted in the same way as that for "*RNA extraction for GeneChip and GeneChip analysis*" in Chapter 1. After isolation of total RNA, a concentration of each RNA sample was determined by the optical density at 260 nm, and RNA quality was accessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples were stored at -80°C until the following steps for qRT-PCR analysis.

Analysis of gene expression by qRT-PCR

Five hundred nanograms of each RNA sample were reverse-transcribed with a SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies Corporation, Carlsbad, CA, USA) at 25ºC for 10 min, 42ºC for 50 min, 85ºC for 5 min, and 37ºC for 20 min in accordance with the manufacturer's instructions. Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were quantified by qPCR using primers described in Table 2 in Chapter 1. An aliquot (2 μL each) of the cDNA solution was used for the respective PCR. For qPCR, cDNA was amplified in duplicate by Platinum SYBR Green qPCR SuperMix-UDG with ROX (Life Technologies) containing 200 nM of forward and reverse primers. The thermal cycling conditions for PCR were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The qPCR was conducted with an 7900HT Fast Real-Time PCR System (Life Technologies). All data were normalized to ribosomal protein S27a (Rps27a) as an internal control under the aforementioned thermal cycling conditions (primer also shown in Table 2). Each PCR product was proven as a target by the melting curve analysis.

Statistical analysis

The gene expression value of each gene was normalized to that of Rps27a, and then the fold change value of each gene was calculated by dividing the individual normalized value of the control or OFLX-treated group by the mean value of the corresponding control group. The data was expressed as the mean ± standard deviation and analyzed statistically. F-test was performed, and then Student's *t*-test (a P value was 0.05 or more by F-test) or Aspin-Welch's *t*-test (a P value was less than 0.05 by F-test) was conducted with the biostatistical package EXSUS ver. 7.7.1 (CAC EXICARE Corporation, Tokyo, Japan) for the difference between the corresponding control group and OFLX-treated group or OFLX-treated group without and with tail suspension.

Results

Histopathological examination in the articular cartilage

Firstly, the effects of body-weight loading onto the articular cartilage on the chondrotoxicity were evaluated histologically in rats given OFLX at 900 mg/kg by the tail-suspension method. In the histological examination of the distal femoral articular cartilage, pyknosis of chondrocyte and fissure formation were observed in rats treated with OFLX at 900 mg/kg at 8 h post-dose under a normal condition (Table 12, Figure 12). In contrast, no change in the cartilage was noted in rats receiving OFLX at 900 mg/kg by the 8-h tail suspension (Table 12). Following that, histological changes in the articular cartilage by the 2-h or 4-h tail suspension were compared with those in rats treated with OFLX at 900 mg/kg under a normal condition. In the histological examination, pyknosis of chondrocyte and rarefaction of the cartilage matrix were observed in the normal condition group and also the 2-h and 4-h tail suspension groups. However, a tendency to decrease in the incidence of the cartilage lesions was seen with a tail-suspension time dependency (Table 12).

Gene expression in the articular cartilage in qRT-PCR analysis

The effect of body-weight loading onto the articular cartilage on gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 in the cartilage of rats treated orally with OFLX were evaluated by the tail suspension for 0, 2, 4 or 8 h. Significant increases in expression of Plaur and Mmp3 and increasing tendency toward Tnfrsf12a and Ptgs2 were observed at 8 h post-dose in rats under a normal condition (Figure 13). In the 2-h tail-suspension group, a significant increase in expression of Tnfrsf12a, Ptgs2, and Plaur was seen in rats receiving OFLX at 8 h post-dose as well. In the 4-h tail-suspension group, only Tnfrsf12a was increased in rats given OFLX at 8 h post-dose. However, a tendency to decrease in the expression of Tnfrsf12a, Ptgs2, and Plaur genes was observed in rats by 4-h tail suspension as compared with that in rats under a normal condition (Figure 13). A significant decrease in expression of Mmp3 was seen from 2-h tail suspension. In addition, a significant decrease or

tendency to decrease in the expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes was observed by 8-h tail suspension (Figure 13).

Discussion

The effects of body-weight loading on histological changes in the distal femoral articular cartilage and gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 were investigated in male juvenile SD rats treated orally with OFLX at 900 mg/kg by using of the tail-suspension method.

In the histological examination, changes seen in the cartilage of rats given OFLX under a normal condition were not observed in any animal receiving OFLX followed by the 8-h tail suspension (Table 12). In addition, the incidence of the cartilage lesions were decreased in rats with the 2-h and 4-h tail suspension as compared with that in rats kept in a normal condition (Table 12). The cartilage lesions induced by OFLX have been known to be observed in the caudal portion of the medial femoral condyle that is thought to be exposed to continuous body-weight loading (Kato and Onodera, 1986; Kato and Onodera, 1988). Our results clearly indicate that body-weight loading onto the cartilage is necessary to induce OFLX-induced chondrotoxicity in juvenile rats. In gene expression analysis, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were up-regulated in the cartilage of rats given OFLX under a normal condition. However, the expression of these genes showed a tendency to decrease with tail-suspension time dependency, and was reduced under the condition of 8-h tail suspension as compared with that under a normal condition (Figure 13). These results suggest that body-weight loading onto the cartilage is essential to up-regulate these genes, and apparent increases in these genes are necessary to induce cartilage lesions in the cartilage exposed to OFLX by some mechanisms. Mechanical loading to the articular cartilage due to weight-bearing has been considered important to maintain cartilage functions. However, it has been reported that constant or static compression leads to increased IL-1β (Murata et al., 2003), cyclooxygenase-2 (COX-2, synonym of Ptgs2), reactive oxygen species (Zhu et al., 2010), and IL-6 (Wang et al., 2010). IL-1β has been revealed to enhance fibroblast growth factor-inducible 14 (Fn14, synonym of Tnfrsf12a) expression in human gingival fibroblasts (Hosokawa et al., 2006). Fn14 has been identified as a

receptor of tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) with a physiological affinity (Wiley et al., 2001). It has been reported that TWEAK induces the production of MMPs by chondrocytes and specifically blocks chondrogenesis in the articular cartilage, suggesting that the TWEAK/Fn14 pathway may directly target chondrocytes (Perper et al., 2006). It has been demonstrated that IL-1β also increases gene expression of COX-2 in human chondrocytes (Geng et al., 1995). In addition, reactive oxygen species (ROS) have also been reported to induce COX-2 and produce prostaglandin E2 (PGE2, synthesis from COX-2) in human chondrocytes (Cillero-Pastor et al., 2008). Increased prostaglandin E2 in the articular cartilage has been known as an important component in the pathogenesis of arthritis (Harris, 1990; Hardy et al., 2002). As for COX-2, the author has demonstrated that Ptgs2 (COX-2) expression was increased in the cartilage of juvenile rats from 4 h post-dose of OFLX at 900 mg/kg (see Chapter 1). Therefore, up-regulated COX-2 expression is considered to be caused by IL-1β, ROS or other factors in the occurrence of chondrotoxicity. Expression of Plaur was revealed to be mediated by IL-1β in human chondrocyte (Schwab et al., 2004). PGE2 has also been shown to induce u-PAR mRNA expression in gastric fibroblasts (Iwamoto et al., 2003). u-PAR has been identified as a specific receptor of u-PA that can convert plasminogen into the less-specific plasmin, which has various substrates, including fibrin, cytokines, and pro-matrix metalloproteinases (Irigoyen et al., 1999; Busso and Hamilton, 2002). IL-1β has also been found to increase gene expression of Mmp3 in cultured human chondrocytes (Chubinskaya et al., 1999). Gosset et al. (2010) has reported that not only IL-1β but PGE2 play a key role in the induction of Mmp3 gene and protein in mouse chondrocytes because the Mmp3 expression is significantly reduced even with IL-1β using chondrocytes from microsomal PGE synthase-1 (rate-limiting step of PGE2 synthesis) knockout mice. Furthermore, IL-6 has been shown to induce matrix metalloproteinases (including Mmp3) in human chondrocytes (Aida et al., 2012). In the present study, Mmp3 expression did not increase under any tail-suspension condition (Figure 13). It has been shown that TWEAK makes murine disc cells

induce Mmp3 gene and protein (Wako et al., 2007). Moreover, u-PA has been reported to be associated with increased matrix metalloproteinases as described above. Thus, decreased expression of Tnfrsf12a, Ptgs2, and Plaur is likely to be involved in the reduction of Mmp3 expression. Based on these information, our results raise the possibility that OFLX could change in response to body-weight loading onto the cartilage from a normal to catabolic reaction, and then released IL-1β, ROS and/or IL-6 by the body-weight loading could induce expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3, which may cause histological changes in the cartilage of juvenile rats.

It has been reported that α5β1 integrin may have a function as a mechanoreceptor, which binds fibronectin and interacts with intracellular signaling molecules and the actin cytoskeleton (Millward-Sadler and Salter, 2004). Moreover, after recognition and transduction of the mechanical signal by the α5β1 integrin, IL-4 has been found to be released from normal human chondrocytes (Millward-Sadler et al., 1999). In this report, IL-4 has been described to be chondroprotective by inhibiting cartilage degradation and promoting matrix synthesis in normal articular cartilage. In addition, GRGDSP oligopeptides, which can reduce the effective binding and signaling through the α5β1 integrin, and IL-4 antibodies have been reported to block increased gene expression of aggrecan and decreased Mmp3 in human chondrocytes (Millward-Sadler et al., 2000). Furthermore, Förster et al. (1996) has revealed that magnesium deficiency due to the chelating activity of quinolones could impair chondrocyte-matrix interaction which is mediated by integrin receptors of the beta 1-subfamily. Taken together, it seems that the α5β1 integrin impairment by chelating magnesium could reduce chondroprotective activity in chondrocytes by treatment of OFLX, which is essential to induce cartilage lesions accompanied by increased expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 in juvenile rats.

In conclusion, our results indicate that body-weight loading onto the cartilage is necessary to induce cartilage lesions and expression of Tnfrsf12a, Ptgs2, Plaur, and

Mmp3 in the articular cartilage of juvenile rats, which may result from the impaired response to weight bearing due to OFLX treatment in chondrocytes.

Summary

The effect of body-weight loading onto the articular cartilage on the occurrence of chondrotoxicity was investigated in male juvenile SD rats given OFLX orally once at 900 mg/kg. Just after dosing of OFLX, hindlimb unloading was performed for 0, 2, 4, or 8 h by a tail-suspension method. Animals were sacrificed at 8 h post-dose, and then the distal femoral articular cartilage was subjected to a histological examination and an investigation for gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 by qRT-PCR analysis. As a result, cartilage lesions and up-regulations of these 4 genes that were seen in rats without the tail suspension were not observed in rats with the 8-h tail suspension, and a tendency to decrease in the incidence of the cartilage lesions and the gene expression was noted in a tail-suspension time dependent manner. Our results clearly indicate that body-weight loading onto the cartilage is necessary to induce cartilage lesions and gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 in juvenile rats treated with OFLX.

Table 12 Microscopic findings in male juvenile rats given a single oral administration of ofloxacin at 900 mg/kg, followed by a 2-, 4-, or 8-h tail suspension

Compound		Vehicle	Ofloxacin	Vehicle	Ofloxacin	Vehicle	Ofloxacin	Vehicle	Ofloxacin
Dose level (mg/kg)		0	900	0	900	0	900	0	900
Time of tail suspension (h)		0		2		4		8	
n		5[a], 5[b]	5[a], 5[b]	5[b]	5[b]	5[b]	5[b]	5[a]	5[a]
Organ	Findings								
distal femur (Number of animals)	pyknosis, chondrocyte	0	3[a], 3[b]	0	2	0	2	0	0
	fissure formation	0	1[a]	0	0	0	0	0	0
	rarefaction, cartilage matrix	0	1[b]	0	1	0	1	0	0
distal femur (Incidence of the lesion*)	pyknosis, chondrocyte	0/10[a], 0/10[b]	3/10[a], 4/10[b]	0/10	3/10	0/10	2/10	0/10	0/10
	fissure formation	0/10[a], 0/10[b]	1/10[a]	0/10	0/10	0/10	0/10	0/10	0/10
	rarefaction, cartilage matrix	0/10[a], 0/10[b]	1/10[b]	0/10	1/10	0/10	1/10	0/10	0/10

Histological grade of all findings were slight.

*: Number of femurs with the change/number of femurs examined.

a: Data of the first study, b: data of the second study.

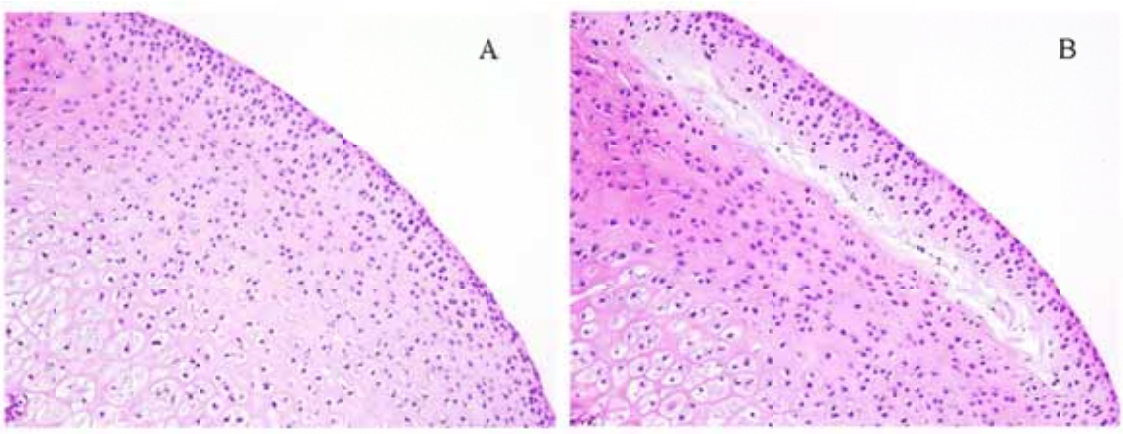


Figure 12　Histopathological examination in the distal femoral articular cartilage of juvenile rats treated with OFLX.

One % MC or OFLX at 900 mg/kg was orally administered once to male juvenile rats under a normal condition, and then the articular cartilage was collected at 8 h post-dose. Hematoxylin and eosin staining was conducted in the distal femoral articular cartilage. Original magnification, × 200. (A) Articular cartilage in juvenile rats treated with 1% MC alone. (B) Articular cartilage in juvenile rats given 900 mg/kg of OFLX. Pyknosis of chondrocytes and fissure formation were observed in the middle zone of the articular cartilage.

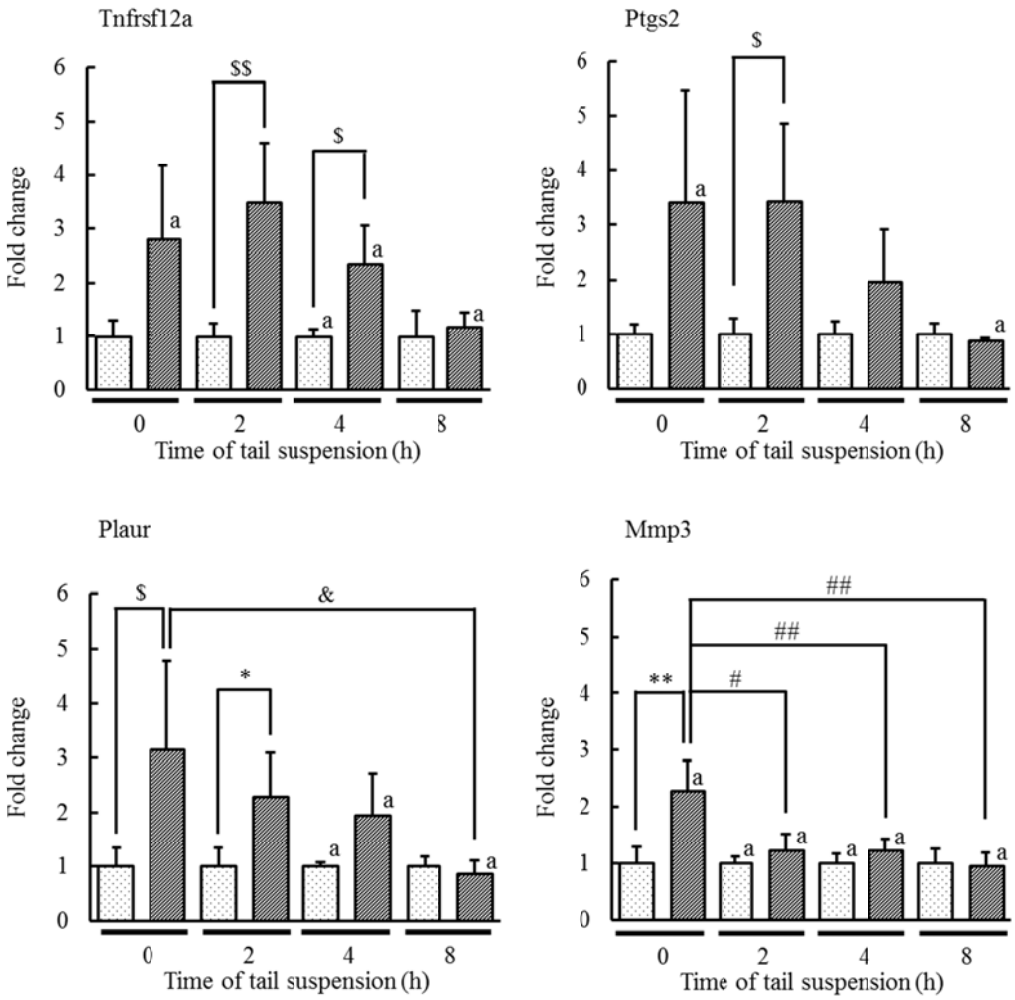


Figure 13 Gene expression in the articular cartilage of male juvenile rats given a single oral administration of OFLX at 900 mg/kg, followed by the treatment of tail suspension. Just after the dosing of OFLX, tail suspension was conducted for 0, 2, 4, or 8 h. qRT-PCR analysis was performed in the articular cartilage of the distal femoral articular cartilage at 8 h post-dose. The animals given 1% MC alone in the same way served as the vehicle control. Individual fold change value of each gene in qRT-PCR analysis was calculated by dividing the individual normalized value of control or OFLX-treated group at each time point by the mean value of the corresponding control group. Data of qRT-PCR analysis show the mean ± standard deviation of 4 or 5 animals. a: n = 4.

* p<0.05 and ** p<0.01: Significantly different from the corresponding control group by Student's t-test. $^{\#}$ p<0.05 and $^{\#\#}$ p<0.01: significantly different from the OFLX-treated group without tail suspension by student's t-test. $^{\$}$ p<0.05 and $^{\$\$}$ p<0.01: significantly different from the corresponding control group by Aspin-Welch's t-test. $^{\&}$ p<0.05: significantly different from the OFLX-treated group without tail suspension by Aspin-Welch's t-test. (□): vehicle control; (▨): OFLX 900 mg/kg.

Conclusion

Quinolones have been widely used for patients because of their high antibacterial activity, broad spectra, and good pharmacokinetics. However, quinolones have been reported to induce arthropathy in juvenile animals. Due to their arthropathogenic effects, the prescription of quinolones has been contraindicated for children and adolescents. Some researchers have reported the mechanism of the chondrotoxicity in juvenile animals, but it has not yet been clarified whether quinolones directly injure cartilage matrix or some factors induce the cartilage lesions. Therefore, in order to prove some factors leading to the onset of the chondrotoxicity, the mechanism of chondrotoxicity was investigated in male juvenile rats using the quinolone OFLX as follows: Firstly, gene expression profile was assessed in the distal femoral articular cartilage of juvenile rats treated orally with OFLX by GeneChip, qRT-PCR, and ISH. Secondly, expression of genes which were changed in the articular cartilage of juvenile rats receiving OFLX was evaluated in juvenile rats given DC-159a or DX-619 that did not cause chondrotoxicity in juvenile rats. Finally, tail-suspended juvenile rats was studied to examine the effect of body-weight loading onto the articular cartilage on the occurrence of the chondrotoxicity.

In Chapter 1, OFLX was orally administered once to male juvenile rats at 900 mg/kg, and then gene expression in the distal femoral articular cartilage was assessed by GeneChip analysis. As a result, intracellular signaling cascade-, stress response-, cell death-, inflammatory response-, proteolysis-, and glycoprotein-related genes, and basic-leucine zipper transcription factor genes were changed in the articular cartilage. Among these genes, an increase in expression of Dusp1 (intracellular signaling cascade-related gene), Tnfrsf12a (cell death-related gene), Ptgs2 and Fos (inflammatory response-related genes), Mt1a and Plaur (stress response-related genes), and Mmp3 (proteolysis-related gene), and a decreases in expression of Sstr2 and Has2 (glycoprotein-related genes) were observed in qRT-PCR analysis with dose

dependency at 100, 300, and 900 mg/kg. The expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 was also noted in chondrocytes around the cartilage lesions by ISH. Taken together, our results suggest that cytokines, chemokines and/or proteases produced by up-regulation of cell death-, inflammatory response-, stress response-, and proteolysis-related genes play an important role in the onset of OFLX-induced chondrotoxicity in juvenile rats.

In Chapter 2, new synthesized quinolones DC-159a and DX-619 were confirmed they did not induce chondrotoxicity in juvenile rats treated orally with DC-159a or DX-619 for 7 days. Therefore, concentrations of these 2 quinolones in the distal femoral articular cartilage were compared with that of OFLX in male juvenile rats given a single oral administration at 100, 300, and 900 mg/kg. Furthermore, gene expression of Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 was assessed in the articular cartilage of the distal femur in male juvenile rats treated with 900 mg/kg of DC-159a or DX-619 by qRT-PCR analysis. In toxicokinetic analysis, the cartilage C_{max} of DC-159a or DX-619 at 900 mg/kg was little lower than or almost comparable to that of OFLX at 300 mg/kg that can induce cartilage lesions in juvenile rats following 7-day oral administration. Moreover, the cartilage $AUC_{0\text{-}24\ h}$ value of DC-159a or DX-619 at 900 mg/kg was higher than that of OFLX at 300 mg/kg. Thus, not only quinolone concentration in the cartilage but some unknown factors are likely to be associated with the occurrence of chondrotoxicity. In qRT-PCR analysis, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes, which were up-regulated in the distal femoral articular cartilage exposed to OFLX, did not increase or slightly increased in the cartilage receiving DC-159a or DX-619 at 900 mg/kg. In conclusion, not only the low penetration of DC-159a or DX-619 into the cartilage but also no obvious changes in Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes in the cartilage were considered to be responsible for non-chondrotoxic potential of DC-159a and DX-619.

Results in Chapter 2 raise the possibility that Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes play an important role for the onset of the ofloxacin-induced

chondrotoxicity in juvenile rats. Tatsumi et al. (1978) have demonstrated that 3-day oral dosing of pipemidic acid at 1,000 mg/kg/day does not cause any blisters in the joints protected from mechanical pressure. In Chapter 3, therefore, the effect of body-weight loading onto the articular cartilage on the occurrence of the OFLX-induced chondrotoxicity in male juvenile rats. Just after dosing of OFLX, hindlimb unloading was performed for 0, 2, 4, or 8 h for male juvenile rats by a tail-suspension method. Animals were sacrificed at 8 h post-dose, and histological examination and gene expression analysis of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 were conducted in the distal femoral articular cartilage. As a result, cartilage lesions and up-regulations of these 4 genes that were seen in rats without the tail suspension were not observed in rats with the 8-h tail suspension, and a tendency to decrease in the incidence of the cartilage lesions and the gene expression was noted in a tail-suspension time dependent manner. These results clearly indicate that body-weight loading onto the cartilage is necessary to induce cartilage lesions and gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 in juvenile rats treated with OFLX.

In conclusion, the possible mechanism of OFLX-induced chondrotoxicity in juvenile rats were shown in Figure 14. When OFLX exposes to the articular cartilage over 75.63 ± 16.06 μg/g with body-weight loading onto the cartilage, the response to the body-weight loading is considered to change from a normal to catabolic reaction, and then catabolic factors such as IL-1β, ROS, and/or IL-6 could be produced in the cartilage. α5β1 integrin impairment by chelating magnesium seems to contribute to this mechanism. After that, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes are up-regulated in the cartilage. Finally, TWEAK, prostaglandins, plasmin, and MMP3 proteins due to the increased expression of the corresponding genes could cause histological changes in the articular cartilage.

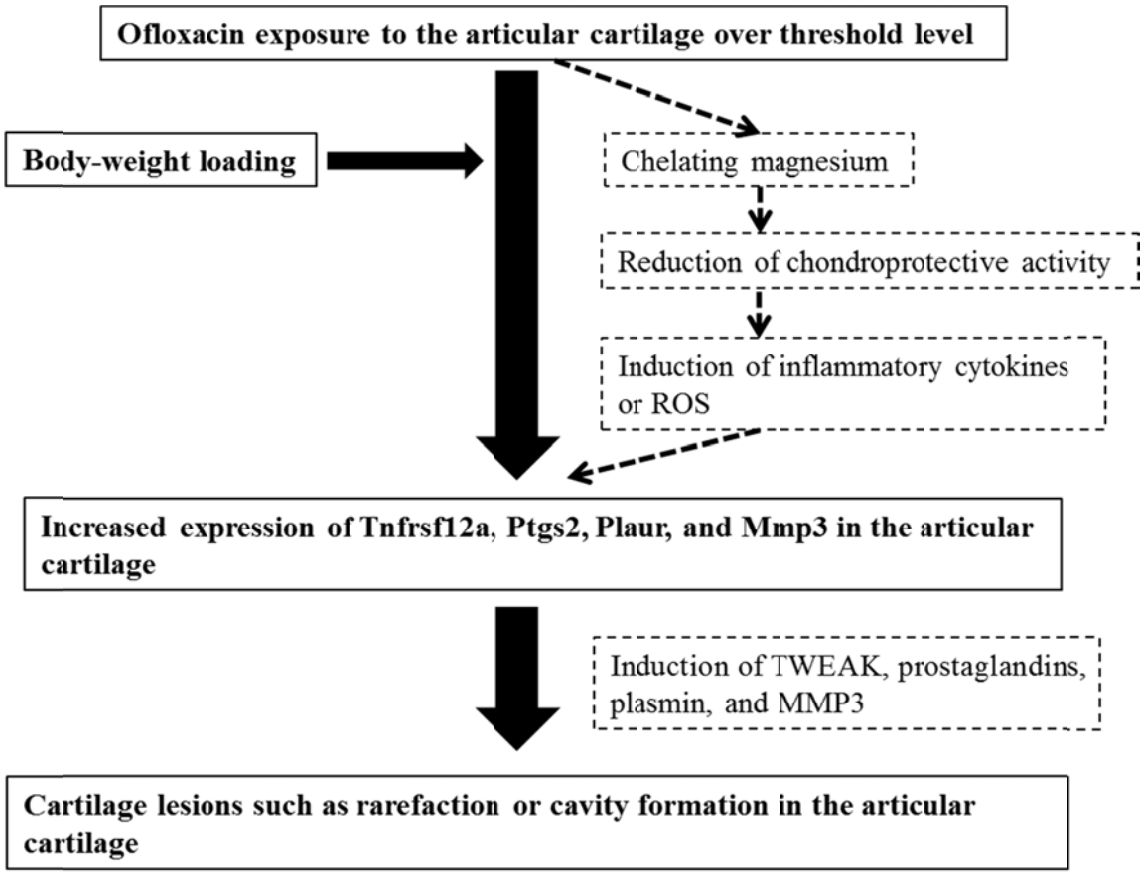


Figure 14 Possible mechanism underlying the onset of OFLX-induced chondrotoxicity in juvenile rats.

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References

Aida, Y., Honda, K., Tanigawa, S., Nakayama, G., Matsumura, H., Suzuki, N., Shimizu, O., Takeichi, O., Makimura, M., Maeno, M., 2012. IL-6 and soluble IL-6 receptor stimulate the production of MMPs and their inhibitors via JAK-STAT and ERK-MAPK signalling in human chondrocytes. Cell Biol. Int. 36, 367-376.

Andriole, V.T., 2005. The quinolones: past, present, and future. Clin. Infect. Dis. 41 Suppl 2, S113-119.

Angel, P., Karin, M., 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta 1072, 129-157.

Bendele, A.M., Hulman, J.F., Harvey, A.K., Hrubey, P.S., Chandrasekhar, S., 1990. Passive role of articular chondrocytes in quinolone-induced arthropathy in guinea pigs. Toxicol. Pathol. 18, 304-312.

Burkhardt, J.E., Hill, M.A., Carlton, W.W., Kesterson, J.W., 1990. Histologic and histochemical changes in articular cartilages of immature beagle dogs dosed with difloxacin, a fluoroquinolone. Vet. Pathol. 27, 162-170.

Burkhardt, J.E., Hill, M.A., Turek, J.J., Carlton, W.W., 1992. Ultrastructural changes in articular cartilages of immature beagle dogs dosed with difloxacin, a fluoroquinolone. Vet. Pathol. 29, 230-238.

Burkly, L.C., Michaelson, J.S., Hahm, K., Jakubowski, A., Zheng, T.S., 2007. TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease. Cytokine 40, 1-16.

Busso, N., Hamilton, J.A., 2002. Extravascular coagulation and the plasminogen activator/plasmin system in rheumatoid arthritis. Arthritis Rheum. 46, 2268-2279.

Chubinskaya, S., Kuettner, K.E., Cole, A.A., 1999. Expression of matrix metalloproteinases in normal and damaged articular cartilage from human knee and ankle joints. Lab. Invest. 79, 1669-1677.

Cillero-Pastor, B., Carames, B., Lires-Dean, M., Vaamonde-Garcia, C., Blanco, F.J., Lopez-Armada, M.J., 2008. Mitochondrial dysfunction activates cyclooxygenase 2 expression in cultured normal human chondrocytes. Arthritis Rheum. 58, 2409-2419.

Dalhoff, A., Petersen, U., Endermann, R., 1996. *In vitro* activity of BAY 12-8039, a new 8-methoxyquinolone. Chemotherapy 42, 410-425.

Förster, C., Kociok, K., Shakibaei, M., Merker, H.J., Vormann, J., Gunther, T., Stahlmann, R., 1996. Integrins on joint cartilage chondrocytes and alterations by ofloxacin or magnesium deficiency in immature rats. Arch. Toxicol. 70, 261-270.

Fujikawa, K., Chiba, M., Tanaka, M., Sato, K., 2005. *In vitro* antibacterial activity of DX-619, a novel des-fluoro(6) quinolone. Antimicrob. Agents Chemother. 49, 3040-3045.

Fung-Tomc, J.C., Minassian, B., Kolek, B., Huczko, E., Aleksunes, L., Stickle, T., Washo, T., Gradelski, E., Valera, L., Bonner, D.P., 2000. Antibacterial spectrum of a novel des-fluoro(6) quinolone, BMS-284756. Antimicrob. Agents Chemother. 44, 3351-3356.

Geng, Y., Blanco, F.J., Cornelisson, M., Lotz, M., 1995. Regulation of cyclooxygenase-2 expression in normal human articular chondrocytes. J. Immunol.155,

796-801.

Gosset, M., Pigenet, A., Salvat, C., Berenbaum, F., Jacques, C., Inhibition of matrix metalloproteinase-3 and -13 synthesis induced by IL-1beta in chondrocytes from mice lacking microsomal prostaglandin E synthase-1. J. Immunol. 185, 6244-6252.

Guo, Y.S., Hellmich, M.R., Wen, X.D., Townsend, C.M., Jr., 2001. Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. J. Biol. Chem. 276, 22941-22947.

Haq, F., Mahoney, M., Koropatnick, J., 2003. Signaling events for metallothionein induction. Mutat. Res. 533, 211-226.

Harada, N., Nakayama, M., Nakano, H., Fukuchi, Y., Yagita, H., Okumura, K., 2002. Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. Biochem. Biophys. Res. Commun. 299, 488-493.

Hardy, M.M., Seibert, K., Manning, P.T., Currie, M.G., Woerner, B.M., Edwards, D., Koki, A., Tripp, C.S., 2002. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum. 46, 1789-1803.

Harris, E.D., Jr., 1990. Rheumatoid arthritis. Pathophysiology and implications for therapy. N. Engl. J. Med. 322, 1277-1289.

Hashimoto, K., Nagao, Y., Kato, K., Mori, Y., Ito, A., 1998. Human urinary trypsin inhibitor inhibits the activation of pro-matrix metalloproteinases and proteoglycans release in rabbit articular cartilage. Life Sci. 63, 205-213.

Hayakawa, I., Atarashi, S., Yokohama, S., Imamura, M., Sakano, K., Furukawa, M., 1986. Synthesis and antibacterial activities of optically active ofloxacin. Antimicrob. Agents Chemother. 29, 163-164.

Helyes, Z., Szabo, A., Nemeth, J., Jakab, B., Pinter, E., Banvolgyi, A., Kereskai, L., Keri, G., Szolcsanyi, J., 2004. Antiinflammatory and analgesic effects of somatostatin released from capsaicin-sensitive sensory nerve terminals in a Freund's adjuvant-induced chronic arthritis model in the rat. Arthritis Rheum. 50, 1677-1685.

Hoshino, K., Inoue, K., Murakami, Y., Kurosaka, Y., Namba, K., Kashimoto, Y., Uoyama, S., Okumura, R., Higuchi, S., Otani, T., 2008. In vitro and in vivo antibacterial activities of DC-159a, a new fluoroquinolone. Antimicrob. Agents Chemother. 52, 65-76.

Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., 2006. Proinflammatory effects of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) on human gingival fibroblasts. Clin. Exp. Immunol. 146, 540-549.

Hoyer, D., Bell, G.I., Berelowitz, M., Epelbaum, J., Feniuk, W., Humphrey, P.P., O'Carroll, A.M., Patel, Y.C., Schonbrunn, A., Taylor, J.E., et al., 1995. Classification and nomenclature of somatostatin receptors. Trends Pharmacol. Sci. 16, 86-88.

Ingham, B., Brentnall, D.W., Dale, E.A., MacFandzean, J.A., 1977. Arthropathy induced by antibacterial fused N-Alkyl-4-pyridone-3-carboxilic acids Toxicol. Lett. 1, 21-26.

Irigoyen, J.P., Munoz-Canoves, P., Montero, L., Koziczak, M., Nagamine, Y., 1999. The plasminogen activator system: biology and regulation. Cell Mol. Life Sci. 56, 104-132.

Itano, N., Sawai, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shinomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spicer, A.P., McDonald, J.A., Kimata, K., 1999. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. J. Biol. Chem. 274, 25085-25092.

Ito, A., Hirai, K., Inoue, M., Koga, H., Suzue, S., Irikura, T., Mitsuhashi, S., 1980. *In vitro* antibacterial activity of AM-715, a new nalidixic acid analog. Antimicrob. Agents Chemother. 17, 103-108.

Iwamoto, J., Takahashi, K., Mizokami, Y., Otsubo, T., Miura, S., Narasaka, T., Takeyama, H., Omata, T., Shimokoube, K., Matsuoka, T., 2003. Expression of urokinase-type plasminogen activator and its receptor in gastric fibroblasts and effects of nonsteroidal antiinflammatory drugs and prostaglandin. Dig. Dis. Sci. 48, 2247-2256.

Kaminsky, D., Meltzer, R.I., 1968. Quinolone antibacterial agents. Oxolinic acid and related compounds. J. Med. Chem. 11, 160-163.

Kastner, M., Rahm, U., Baumann-Wilschke, I., Bello, A., Stahlmann, R., 2004. Concentrations of the des-F(6)-quinolone garenoxacin in plasma and joint cartilage of immature rats. Arch. Toxicol. 78, 61-67.

Kato, M., Onodera, T., 1986. Observation on the development of osteochondrosis in young rats. Lab. Anim. 20, 249-256.

Kato, M., Onodera, T., 1988. Effect of ofloxacin on the uptake of [3H]thymidine by articular cartilage cells in the rat. Toxicol. Lett. 44, 131-142.

Kato, M., Onodera, T., 1988. Morphological investigation of cavity formation in

articular cartilage induced by ofloxacin in rats. Fundam Appl Toxicol 11, 110-119.

Kato, M., Takada, S., Ogawara, S., Takayama, S., 1995. Effect of levofloxacin on glycosaminoglycan and DNA synthesis of cultured rabbit chondrocytes at concentrations inducing cartilage lesions *in vivo*. Antimicrob. Agents Chemother. 39, 1979-1983.

Kolasinski, S.L., Haines, K.A., Siegel, E.L., Cronstein, B.N., Abramson, S.B., 1992. Neuropeptides and inflammation. A somatostatin analog as a selective antagonist of neutrophil activation by substance P. Arthritis Rheum. 35, 369-375.

Krantic, S., 2000. Peptides as regulators of the immune system: emphasis on somatostatin. Peptides 21, 1941-1964.

Lesher, G.Y., Froelich, E.J., Gruett, M.D., Bailey, J.H., Brundage, R.P., 1962. 1,8-Naphthyridine Derivatives. A New Class of Chemotherapeutic Agents. J. Med. Pharm. Chem. 91, 1063-1065.

Lijnen, H.R., 2002. Matrix metalloproteinases and cellular fibrinolytic activity. Biochemistry (Mosc) 67, 92-98.

Linseman, D.A., Hampton, L.A., Branstetter, D.G., 1995. Quinolone-induced arthropathy in the neonatal mouse. Morphological analysis of articular lesions produced by pipemidic acid and ciprofloxacin. Fundam. Appl. Toxicol. 28, 59-64.

Machida, M., Kusajima, H., Aijima, H., Maeda, A., Ishida, R., Uchida, H., 1990. Toxicokinetic study of norfloxacin-induced arthropathy in juvenile animals. Toxicol. Appl. Pharmacol. 105, 403-412.

Millward-Sadler, S.J., Salter, D.M., 2004. Integrin-dependent signal cascades in

chondrocyte mechanotransduction. Ann. Biomed. Eng. 32, 435-446.

Millward-Sadler, S.J., Wright, M.O., Davies, L.W., Nuki, G., Salter, D.M., 2000. Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. Arthritis Rheum. 43, 2091-2099.

Millward-Sadler, S.J., Wright, M.O., Lee, H., Nishida, K., Caldwell, H., Nuki, G., Salter, D.M., 1999. Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. J. Cell. Biol. 145, 183-189.

Murata, M., Bonassar, L.J., Wright, M., Mankin, H.J., Towle, C.A., 2003. A role for the interleukin-1 receptor in the pathway linking static mechanical compression to decreased proteoglycan synthesis in surface articular cartilage. Arch. Biochem. Biophys. 413, 229-235.

Muratani, T., Inoue, M., Mitsuhashi, S., 1992. *In vitro* activity of T-3761, a new fluoroquinolone. Antimicrob. Agents Chemother. 36, 2293-2303.

Nakamura, S., Minami, A., Nakata, K., Kurobe, N., Kouno, K., Sakaguchi, Y., Kashimoto, S., Yoshida, H., Kojima, T., Ohue, T., et al., 1989. *In vitro* and *in vivo* antibacterial activities of AT-4140, a new broad-spectrum quinolone. Antimicrob. Agents Chemother. 33, 1167-1173.

Nishida, Y., Knudson, C.B., Nietfeld, J.J., Margulis, A., Knudson, W., 1999. Antisense inhibition of hyaluronan synthase-2 in human articular chondrocytes inhibits proteoglycan retention and matrix assembly. J. Biol. Chem. 274, 21893-21899.

Perper, S.J., Browning, B., Burkly, L.C., Weng, S., Gao, C., Giza, K., Su, L., Tarilonte,

L., Crowell, T., Rajman, L., Runkel, L., Scott, M., Atkins, G.J., Findlay, D.M., Zheng, T.S., Hess, H., 2006. TWEAK is a novel arthritogenic mediator. J. Immunol. 177, 2610-2620.

Recklies, A.D., White, C., Melching, L., Roughley, P.J., 2001. Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma cells. Biochem. J. 354, 17-24.

Sato, K., Hoshino, K., Tanaka, M., Hayakawa, I., Osada, Y., 1992. Antimicrobial activity of DU-6859, a new potent fluoroquinolone, against clinical isolates. Antimicrob. Agents Chemother. 36, 1491-1498.

Sato, K., Matsuura, Y., Inoue, M., Une, T., Osada, Y., Ogawa, H., Mitsuhashi, S., 1982. *In vitro* and *in vivo* activity of DL-8280, a new oxazine derivative. Antimicrob. Agents Chemother. 22, 548-553.

Schewe, D.M., Biller, T., Maurer, G., Asangani, I.A., Leupold, J.H., Lengyel, E.R., Post, S., Allgayer, H., 2005. Combination analysis of activator protein-1 family members, Sp1 and an activator protein-2alpha-related factor binding to different regions of the urokinase receptor gene in resected colorectal cancers. Clin. Cancer Res. 11, 8538-8548.

Schneider, P., Schwenzer, R., Haas, E., Muhlenbeck, F., Schubert, G., Scheurich, P., Tschopp, J., Wajant, H., 1999. TWEAK can induce cell death via endogenous TNF and TNF receptor 1. Eur. J. Immunol. 29, 1785-1792.

Schwab, W., Schulze-Tanzil, G., Mobasheri, A., Dressler, J., Kotzsch, M., Shakibaei, M., 2004. Interleukin-1beta-induced expression of the urokinase-type plasminogen activator receptor and its co-localization with MMPs in human articular chondrocytes.

Histol. Histopathol. 19, 105-112.

Shakibaei, M., Kociok, K., Forster, C., Vormann, J., Gunther, T., Stahlmann, R., Merker, H.J., 1996. Comparative evaluation of ultrastructural changes in articular cartilage of ofloxacin-treated and magnesium-deficient immature rats. Toxicol. Pathol. 24, 580-587.

Sheng, Z.G., Peng, S., Wang, C.Y., Li, H.B., Hajela, R.K., Wang, Y.E., Li, Q.Q., Liu, M.F., Dong, Y.S., Han, G., 2007. Apoptosis in microencapsulated juvenile rabbit chondrocytes induced by ofloxacin: role played by beta(1)-integrin receptor. J. Pharmacol. Exp. Ther. 322, 155-165.

Shimizu, M., Takase, Y., Nakamura, S., Katae, H., Minami, A., 1975. Pipemidic acid, a new antibacterial agent active against Pseudomonas aeruginosa: in vitro properties. Antimicrob. Agents Chemother. 8, 132-138.

Smith, W.L., Dewitt, D.L., 1996. Prostaglandin endoperoxide H synthases-1 and -2. Adv. Immunol. 62, 167-215.

Stahlmann, R., Forster, C., Shakibaei, M., Vormann, J., Gunther, T., Merker, H.J., 1995. Magnesium deficiency induces joint cartilage lesions in juvenile rats which are identical to quinolone-induced arthropathy. Antimicrob. Agents Chemother. 39, 2013-2018.

Stahlmann, R., Merker, H.J., Hinz, N., Chahoud, I., Webb, J., Heger, W., Neubert, D., 1990. Ofloxacin in juvenile non-human primates and rats. Arthropathia and drug plasma concentrations. Arch. Toxicol. 64, 193-204.

Stahlmann, R., Zippel, U., Forster, C., Schwabe, R., Shakibaei, M., Merker, H.J.,

Borner, K., 1998. Chondrotoxicity and toxicokinetics of sparfloxacin in juvenile rats. Antimicrob. Agents Chemother. 42, 1470-1475.

Tatsumi, H., Senda, H., Yatera, S., Takemoto, Y., Yamayoshi, M., Ohnishi, K., 1978. Toxicological studies on pipemidic acid. V. Effect on diarthrodial joints of experimental animals. J. Toxicol. Sci. 3, 357-367.

Theodosiou, A., Ashworth, A., 2002. MAP kinase phosphatases. Genome biology 3, REVIEWS3009.

Tsuji, M., Hirakawa, K., Kato, A., Fujii, K., 2000. The possible role of c-fos expression in rheumatoid cartilage destruction. J. Rheumatol. 27, 1606-1621.

von Rosenstiel, N., Adam, D., 1994. Quinolone antibacterials. An update of their pharmacology and therapeutic use. Drugs 47, 872-901.

Wako, M., Haro, H., Ando, T., Hatsushika, K., Ohba, T., Iwabuchi, S., Nakao, A., Hamada, Y., 2007. Novel function of TWEAK in inducing intervertebral disc degeneration. J. Orthop. Res. 25, 1438-1446.

Wang, P., Zhu, F., Lee, N.H., Konstantopoulos, K., 2010. Shear-induced interleukin-6 synthesis in chondrocytes: roles of E prostanoid (EP) 2 and EP3 in cAMP/protein kinase A- and PI3-K/Akt-dependent NF-kappaB activation. J. Biol. Chem. 285, 24793-24804.

Wiley, S.R., Cassiano, L., Lofton, T., Davis-Smith, T., Winkles, J.A., Lindner, V., Liu, H., Daniel, T.O., Smith, C.A., Fanslow, W.C., 2001. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. Immunity 15, 837-846.

Wise, R., Andrews, J.M., Edwards, L.J., 1983. In vitro activity of Bay 09867, a new quinoline derivative, compared with those of other antimicrobial agents. Antimicrob. Agents Chemother. 23, 559-564.

Yabe, K., Satoh, H., Ishii, Y., Jindo, T., Sugawara, T., Furuhama, K., Goryo, M., Okada, K., 2004. Early pathophysiologic feature of arthropathy in juvenile dogs induced by ofloxacin, a quinolone antimicrobial agent. Vet. Pathol. 41, 673-681.

Zafarullah, M., Su, S., Gedamu, L., 1993. Basal and inducible expression of metallothionein and heat shock protein 70 genes in bovine articular chondrocytes. Exp. Cell Res. 208, 371-377.

Zhu, F., Wang, P., Lee, N.H., Goldring, M.B., Konstantopoulos, K., 2010. Prolonged application of high fluid shear to chondrocytes recapitulates gene expression profiles associated with osteoarthritis. PLoS One 5, e15174.

Summary

Quinolone antibacterial agents (quinolones) have been widely used for patients because of their high antibacterial activity, broad spectra, and good pharmacokinetics. However, quinolones have been reported to induce arthropathy in juvenile animals. Due to their arthropathogenic effects, the prescription of quinolones has been contraindicated for children and adolescents. Some researchers have tried to elucidate the mechanism of the chondrotoxicity, but it has not yet been clarified whether quinolones directly injure cartilage matrix or some factors induce the cartilage lesions via molecular changes in chondrocytes. Therefore, in order to prove some factors leading to the onset of the chondrotoxicity, the mechanism of chondrotoxicity was investigated in male juvenile rats using the quinolone ofloxacin (OFLX) as follows: Firstly, gene expression profile was assessed in the distal femoral articular cartilage of juvenile rats treated orally with OFLX by GeneChip, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), and *in situ* hybridization (ISH). Secondly, expression of genes which were changed in the articular cartilage of juvenile rats receiving OFLX was evaluated in juvenile rats given DC-159a or DX-619 that did not cause chondrotoxicity in juvenile rats. Finally, tail-suspended juvenile rats was studied to examine the effect of body-weight loading onto the articular cartilage on the occurrence of the chondrotoxicity.

Firstly, OFLX was orally administered by gavage at a dose level of 900 mg/kg once to male juvenile Sprague-Dawley (SD) rats, 3 weeks of age. Then gene expression profiles in the articular cartilage of the distal femur were analyzed at 2, 4, 8, and 24 h post-dose. In the GeneChip analysis, the expression of 134 gene probes in the OFLX-treated group showed statistically significant differences with at least 1.5-fold difference from the control. Among them, intracellular signaling cascade- and stress response-related genes changed at 2 h post-dose; cell death- and inflammatory response-related genes at 4 and 8 h post-dose; basic-leucine zipper transcription factor and stress response-related genes at 8 and 24 h post-dose; stress

response-, proteolysis- and glycoprotein-related genes at 24 h post-dose. In qRT-PCR analysis, up-regulated gene expression of dual specificity phosphatase 1 (Dusp1, intracellular signaling cascade-related gene); tumor necrosis factor receptor superfamily, member 12a (Tnfrsf12a, cell death-related gene); prostaglandin-endoperoxide synthase 2 (Ptgs2, inflammatory response-related gene); FBJ murine osteosarcoma viral oncogene homolog (Fos, inflammatory response-related genes); metallothionein 1a (Mt1a, stress response-related gene); plasminogen activator, urokinase receptor (Plaur, stress response-related gene); and matrix metalloproteinase 3 (Mmp3, proteolysis-related gene), and down-regulated gene expresssion of somatostatin receptor 1 (Sstr1, glycoprotein-related gene) and hyaluronan synthase 2 (Has2, glycoprotein-related gene) were observed with dose dependency in the articular cartilage of juvenile rats treated with OFLX at 100, 300, or 900 mg/kg. The expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 was also noted in chondrocytes around the cartilage lesions by ISH. In conclusion, our results suggest that cytokines, chemokines and/or proteases produced by up-regulation of cell death-, inflammatory response-, stress response- and proteolysis-related genes play an important role in the onset of OFLX-induced chondrotoxicity in juvenile rats.

Secondly, in order to compare the chondrotoxic potential between new synthesized quinolones DC-159a and DX-619, and OFLX, they were orally administered by gavage at a dose level of 300 or 900 mg/kg/day to male juvenile SD rats, 3 weeks of age, for 7 consecutive days. Then the distal humerus and femur were subjected to microscopic examination. Moreover, concentrations of these quinolones in the femoral articular cartilage were measured in male juvenile rats following a single oral administration at 100, 300, or 900 mg/kg. Furthermore, gene expression of Dusp1, Tnfrsf12a, Ptgs2, Fos, Mt1a, Plaur, Mmp3, Sstr1, and Has2 was investigated in the articular cartilage of the distal femur in male juvenile rats treated with 900 mg/kg of DC-159a or DX-619 by qRT-PCR analysis. In a microscopic examination, no changes in the articular cartilage were observed in any animal administered DC-159a or DX-619. On the contrary, cavity formation and

chondrocyte cluster in the cartilage of distal humerus and femur were noted in animals receiving OFLX at 300 mg/kg/day or more. In toxicokinetic analysis, the maximum cartilage concentration (cartilage C_{max}) value of DC-159a or DX-619 at 900 mg/kg was little lower than or almost comparable to that of OFLX at 300 mg/kg. Furthermore, the area under the cartilage concentration-time curve up to 24 h post-dose (cartilage $AUC_{0\text{-}24\ h}$) value of DC-159a or DX-619 at 900 mg/kg was higher than that of OFLX at 300 mg/kg. In qRT-PCR analysis, up-regulated Dusp1, Fos, and Mt1a, and down-regulated Sstr1 and Has2 genes were seen in the femoral articular cartilage of rats given DX-619 or DC-159a at 900 mg/kg. However, Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes, which were up-regulated in the distal femoral articular cartilage exposed to OFLX, did not increase or slightly changed. In conclusion, the penetration of DC-159a or DX-619 into the cartilage was low compared with that of OFLX, and no obvious changes in Tnfrsf12a, Ptgs2, Plaur, and Mmp3 genes were observed in the articular cartilage of juvenile rats treated with DC-159a or DX-619, which was likely to be responsible for non-chondrotoxic potentials of DC-159a and DX-619.

Finally, the effect of body-weight loading onto the articular cartilage on the occurrence of chondrotoxicity was investigated in male juvenile SD rats given OFLX orally once at 900 mg/kg. Just after dosing of OFLX, hindlimb unloading was performed for 0, 2, 4, or 8 h by a tail-suspension method. Animals were sacrificed at 8 h post-dose, and then the distal femoral articular cartilage was subjected to a histological examination and an investigation for gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 by qRT-PCR analysis. As a result, cartilage lesions and up-regulations of these 4 genes that were seen in rats without the tail suspension were not observed in rats with the 8-h tail suspension, and a tendency to decrease in the incidence of the cartilage lesions and the gene expression was noted in a tail-suspension time dependent manner.

In conclusion, Tnfrsf12a, Plaur, Ptgs2, and Mmp3 genes which are considered related to the cartilage lesions increased in the articular cartilage of juvenile rats treated orally with OFLX. Further, our results clearly indicate that body-weight loading onto

the cartilage is necessary to induce gene expression of Tnfrsf12a, Ptgs2, Plaur, and Mmp3 and cartilage lesions in juvenile rats treated with OFLX.

Summary in Japanese

キノロン系抗菌薬（キノロン薬）は、幼若動物に関節毒性を引き起こすことから、多くのキノロン薬で小児への投与は禁忌である。これまでに関節毒性発症機序に関する報告はされているが、キノロン薬が直接軟骨基質に作用するのか、あるいは軟骨基質破壊に繋がる何らかの因子が関与するのかは未だ明らかではなかった。そこで本研究では、関節毒性発症に関与する因子を見出すため、1）キノロン薬 ofloxacin（OFLX）を投与した雄性幼若ラット（3 週齢）の関節軟骨における遺伝子発現変動を網羅的に解析するとともに、変動した遺伝子の局在を調べた。また、2）関節毒性を示さないキノロン薬を投与した幼若ラットの関節軟骨について軟骨内薬物濃度を測定するとともに、OFLX を投与した幼若ラット関節軟骨で見出された関節毒性と関連すると考えられた遺伝子の発現を調べた。さらに、3）尾懸垂処置を施した幼若ラットを用いて、軟骨に対する荷重負荷の病変形成及び関節毒性関連遺伝子の発現に及ぼす影響を調べることで関節毒性発現機作を考察した。

1）OFLX の 900 mg/kg を単回経口投与した幼若ラット大腿骨遠位関節軟骨について、投与 2、4、8、及び 24 時間後に GeneChip を用いた遺伝子発現解析を実施した。その結果、投与 2 時間後に細胞内シグナルカスケード関連及びストレス反応関連遺伝子の、投与 4 及び 8 時間後に細胞死関連及び炎症反応関連遺伝子の、投与 8 及び 24 時間後に塩基性ロイシンジッパー転写因子及びストレス反応関連遺伝子の、投与 24 時間後に蛋白溶解関連及び糖タンパク関連遺伝子の発現変動が認められた。次に、OFLX の 100、300、あるいは 900 mg/kg を単回経口投与した幼若ラットの大腿骨遠位関節軟骨について、quantitative real-time reverse transcription-polymerase chain reaction （qRT-PCR）により、GeneChip 解析で変動のあった遺伝子の発現を調べた。その結果、dual specificity phosphatase 1（Dusp1、細胞内シグナルカスケード関連遺伝子）、tumor necrosis factor receptor superfamily, member 12a （Tnfrsf12a、細胞死関連遺伝子）、prostaglandin-endoperoxide synthase 2（Ptgs2、炎症反応関連遺伝子）、FBJ murine osteosarcoma viral oncogene homolog（Fos、炎症反応関連遺伝子）、metallothionein

1a（Mt1a、ストレス反応関連遺伝子）、plasminogen activator, urokinase receptor（Plaur、ストレス反応関連遺伝子）、及び matrix metalloproteinase 3（Mmp3、蛋白溶解関連遺伝子）の用量依存的な発現の増加、並びに somatostatin receptor 1（Sstr1、糖タンパク関連遺伝子）及び hyaluronan synthase 2（Has2、糖タンパク関連遺伝子）の用量依存的な発現減少が認められた。さらに、Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子に対する *in situ* hybridization を実施した結果、これら遺伝子は、軟骨病変部位周辺で発現が増加することが確認された。以上、OFLX の関節毒性には、軟骨細胞における細胞死、炎症反応、ストレス反応、及び蛋白溶解関連遺伝子の発現増加により産生されると考えられるサイトカイン、ケモカイン、あるいはプロテアーゼが重要な役割を果たしていることが示唆された。

2）新規キノロン薬 DC-159a 及び DX-619 の関節毒性を OFLX と比較するため、DC-159a、DX-619、あるいは OFLX の 300 あるいは 900 mg/kg/day を幼若ラットに 7 日間反復経口投与し、上腕骨及び大腿骨遠位関節軟骨について組織学的検査を実施した。また、DC-159a、DX-619、あるいは OFLX の 100、300、あるいは 900 mg/kg を単回経口投与した幼若ラットの大腿骨遠位関節軟骨について、軟骨内薬物濃度を測定した。さらに、DC-159a あるいは DX-619 の 900 mg/kg を単回経口投与した幼若ラットの大腿骨遠位関節軟骨について、OFLX を投与した幼若ラット関節軟骨で変化が認められた Dusp1、Tnfrsf12a、Ptgs2、Fos、Mt1a、Plaur、Mmp3、Sstr1、及び Has2 遺伝子の発現を qRT-PCR で調べた。その結果、幼若ラットの上腕骨及び大腿骨遠位関節軟骨の組織学的検査では、OFLX の 300 mg/kg/day 以上で空洞形成及び軟骨細胞塊が認められたが、DC-159a 及び DX-619 では 900 mg/kg/day まで組織学的変化は認められなかった。大腿骨遠位関節軟骨内の薬物濃度測定では、DC-159a あるいは DX-619 の 900 mg/kg を単回経口投与した幼若ラットの軟骨内最高薬物濃度（軟骨内 C_{max}）は、7 日間反復投与により関節病変が認められた OFLX の 300 mg/kg を投与した幼若ラット軟骨内 C_{max} とほぼ同等あるいはやや低かった。また、DC-159a あるいは DX-619 の 900 mg/kg を単回経口投与した幼若ラットの軟骨内薬物濃度時間曲線下面積（軟骨内 $AUC_{0\text{-}24h}$）は、OFLX の 300 mg/kg を投与した幼若ラット

の軟骨内 $AUC_{0\text{-}24h}$ より高かった。さらに、DC-159a あるいは DX-619 を投与した幼若ラット大腿骨遠位関節軟骨では、Dusp1、Fos、及び Mt1a 遺伝子の発現増加並びに Sstr1 及び Has2 遺伝子の発現減少がみられたが、Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子には明らかな変化は認められなかった。以上のことから、OFLX と比較して DC-159a 及び DX-619 の関節軟骨内への分布が低いことに加えて、軟骨内 Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子に明らかな変化が無かったことが、DC-159a 及び DX-619 で関節毒性が認められない原因であると考えられた。

3）キノロン薬関節毒性発症と軟骨に対する荷重負荷の関係を調べるため、OFLX の 900 mg/kg を幼若ラットに単回経口投与し、投与後速やかに 2、4、及び 8 時間後まで尾懸垂処置を施した幼若ラットの大腿骨遠位関節軟骨について、投与 8 時間後に組織学的検査を実施するとともに、Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子の発現量を qRT-PCR で調べた。その結果、通常飼育条件下では、関節軟骨に軟骨細胞核濃縮並びに軟骨基質の疎鬆化及び亀裂形成が認められ、Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子の発現増加あるいは増加傾向が認められた。しかし、尾懸垂時間の延長に伴い通常飼育条件下で認められた関節軟骨の組織学的変化及び遺伝子発現は軽減する傾向が認められ、尾懸垂 8 時間処置ではこれらの変化は認められなかった。

以上のことから、OFLX を投与した幼若ラット関節軟骨では、関節病変形成に関与すると考えられる Tnfrsf12a、Ptgs2、Plaur、及び Mmp3 遺伝子の発現が増加することが示された。さらに、これら遺伝子の発現増加及び軟骨における組織学的変化には、軟骨への荷重負荷が重要な役割を果たしていることが明らかとなった。