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

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ABSTRACT

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 ofloxacin (OFLX), it was orally administered by gavage at a dose level of 900 mg/kg once to male juvenile Sprague–Dawley 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 a quantitative real-time reverse transcription-polymerase chain reaction analysis, up-regulated Dusp1 (intracellular signaling cascade-related gene), Tnfrsf12a (cell death-related gene), Ptgs2, Fos (inflammatory response-related genes), Mt1a, 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 *in situ* hybridization. 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 a important role in the onset of OFLX-induced chondrotoxicity in juvenile rats.

1. Introduction

Quinolone antibacterial agents (quinolones) have been widely used in clinical fields because of their high antibacterial activity, broad spectra, and good pharmacokinetics. However, quinolones have been reported to induce arthropathy 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 (Stahlmann et al., 1990). Due to their arthropathogenic effects, the prescription of quinolones has been contraindicated for children and adolescents.

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) have reported that the extracellular matrix is 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 are susceptible to inhibitory effect 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 been unclear whether the aforementioned events in the cartilage treated with quinolones were caused by direct toxic effects of quinolones on

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chondrocytes or were mediated by gene expression changes and chemical mediator productions in the cells.

In the present study, to investigate gene expression changes related to the chondrotoxicity, 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 quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and *in situ* hybridization (ISH) analyses. To the best of our knowledge, this is the first study to evaluate the molecular changes for the *in vivo* quinolone-induced chondrotoxicity.

2. Material and methods

2.1. Test substance

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

2.2. Animals

Male juvenile Sprague–Dawley 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.

2.3. 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 100, 300 and 900 mg/kg were administered once to confirm the reliability of GeneChip data and to investigate a dose response of gene expression. Dose levels were selected because matrix rarefraction and cavity formation of the articular cartilage in the distal femur was observed at 6 and 24 h post-dose, respectively, in male juvenile Sprague–Dawley 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).

2.4. 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) according to the Affymetrix GeneChip protocol (3 arrays/each group, total 24 arrays). Arrays were scanned with a GeneChip Scanner 3000 (Affymetrix).

Table 1
Dose composition

Compound	Timepoint of euthanasia (h)	Dose (mg/kg)	*n* (GeneChip)	*n* (ISH)
For GeneChip or ISH analysis				
Vehicle	2	0	15	3
Ofloxacin	2	900	15	3
Vehicle	4	0	15	3
Ofloxacin	4	900	15	3
Vehicle	8	0	15	3
Ofloxacin	8	900	15	3
Vehicle	24	0	15	3
Ofloxacin	24	900	15	3

Compound	Timepoint of euthanasia (h)	Dose (mg/kg)	*n*
For qRT-PCR analysis			
Vehicle	2	0	5
Ofloxacin	2	100	5
Ofloxacin	2	300	5
Ofloxacin	2	900	5
Vehicle	4	0	5
Ofloxacin	4	100	5
Ofloxacin	4	300	5
Ofloxacin	4	900	5
Vehicle	8	0	5
Ofloxacin	8	100	5
Ofloxacin	8	300	5
Ofloxacin	8	900	5
Vehicle	24	0	5
Ofloxacin	24	100	5
Ofloxacin	24	300	5
Ofloxacin	24	900	5

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).

2.5. 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 timepoint 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 timepoint 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/).

2.6. Analysis of gene expression by qRT-PCR

RNA isolation was performed in the same method as that for "2.4. 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 SuperScript III 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 osteosar-