

# Proteomic Analysis of Celecoxib on Chondrocytes from Patients with Osteoarthritis

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## Abstract

**Objective:** To study a comprehensive proteomic analysis of celecoxib in osteoarthritis (OA) chondrocytes. **Methods:** OA chondrocytes were stimulated with celecoxib, IL-1 $\beta$  and IL-1 $\beta$  together with celecoxib. Proteins were extracted from the cells and subjected to 2-dimensional differential image gel electrophoresis (2D-DIGE). Proteins of interest were identified by mass spectrometry. **Results:** Eighty-six protein spots showed significantly different intensities with each reagent or reagent combination. AAA+ protein, HSP47/Serpin, cAMP-dependent protein kinase type II-beta regulatory subunit, alpha-actin-4 and tubulin decreased with the addition of celecoxib, while apolipoprotein A-V, glutamate carboxipeptidase 2, mitochondrial stress-70 protein, sorting nexin-9 and GRP78 increased with the addition of celecoxib. GRP78 is a stress protein and may be chondroprotective. Celecoxib modulated IL-1 $\beta$  stimulated chondrocytes, and CD200R and moesin were identified as such resulting proteins. **Conclusion:** Protein profiles of OA chondrocytes changed after administration of celecoxib. Further investigation is needed to elucidate the function of each protein in OA chondrocytes.

## Keywords

Proteomic Analysis, Celecoxib, Chondrocytes, Osteoarthritis

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## 1. Introduction

Osteoarthritis (OA) is a common joint disorder affecting elderly populations. The prevalence of radiographic knee OA in adults was reported as 37.4% in the USA and 61.9% in Japan, and it increased with age [1] [2]. Indirect and nonmedical costs for OA have significant implications, given its high prevalence and an upcoming aging society [3].

One of the characteristic changes in OA is wear and tear of articular cartilage. Chondrocytes are the only cellular component in articular cartilage, and they maintain homeostasis of articular cartilage synthesizing matrix molecules such as collagen and proteoglycan. In OA, cartilage homeostasis is disrupted by the loss of balance between cartilage anabolism and catabolism [4]. Cytokines modulate the degradation of articular cartilage. Interleukin-1 (IL-1) plays a central role in the pathogenesis of OA [5]. IL-1 is present in OA chondrocytes [6], and it stimulates chondrocytes to produce chemical mediators such as prostaglandin E<sub>2</sub>, nitric oxide and metalloproteinases [7]-[9]. These mediators have catabolic effects on cartilage [10].

There are no evident treatments that cure OA or delay the disease progression as yet. Thus, the current goal of treatment is to modify symptoms of OA. Some guidelines recommend NSAIDs including selective COX-2 inhibitors [11] [12]. However, conventional NSAIDs have serious adverse effects and degrade articular cartilage [13]-[15]. In our *in vitro* experiments, both diclofenac and celecoxib induced apoptosis in chondrocytes in a dose-dependent manner, however the latter induced it to a lesser degree [16]. Recently, celecoxib is suggested to have potential disease-modifying properties in OA [17]. Thus, celecoxib might be preferable to use for the treatment of OA. The aim of this study is to identify potent candidate proteins that are targets of celecoxib in OA chondrocytes.

To investigate cellular phenotypes under different conditions, a method of comprehensive analysis of proteins has been developed [18] [19]. In the present study, chondrocytes from OA patients were prepared under four different conditions: 1) unstimulated (control), 2) treated with IL-1 $\beta$ , 3) treated with IL-1 $\beta$  and celecoxib, and 4) treated with celecoxib. To simulate *in vivo* conditions of OA, chondrocytes were stimulated with IL-1 $\beta$ , and celecoxib was added to simulate the clinical effects of celecoxib [16]. Moreover, stimulation by celecoxib alone was considered to reflect the effects of celecoxib on senescent chondrocytes.

## 2. Materials and Methods

### 2.1. Clinical Samples and Preparation of Chondrocytes

Human articular chondrocytes were obtained from 3 patients with OA (3 females, mean age, 73.3 years) who underwent knee joint arthroplasty. They fulfilled criteria of OA [20]. The patients were treated with neither NSAIDs nor celecoxib at least one month prior to the operation. Written informed consent was obtained from each patient and the study protocol was approved by the ethics committee of Nippon Medical School. The study was performed in compliance with the World Medical Association Declaration of Helsinki (1964).

After careful removal of synovial tissue, cartilage was minced, washed, and treated with collagenase. Isolated chondrocytes were then washed and grown *in vitro* in monolayer culture in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and antibiotics. The attached cells (P0) were expanded on type I collagen-coated culture dishes (P1 or P2), which were used in the experiments.

### 2.2. *In Vitro* Stimulation of Chondrocytes

Chondrocytes ( $1.0 \times 10^7$  cells/ $\phi$  100 mm type I collagen-coated dish) were cultured in the medium containing 5 ng/ml IL-1 $\beta$  (R & D, Minneapolis, MN, USA), 100  $\mu$ M celecoxib (a gift from Pfizer), or both for 24 hours. Chondrocytes for negative control were cultured without the reagents for 24 hours. Though the clinical relevant concentration of celecoxib is as low as 10  $\mu$ M [21], the higher concentration was used to enhance the cellular effects and to reveal the full spectrum of the pharmacological activities of celecoxib. Then the chondrocytes were harvested and cellular proteins were extracted into a lysis buffer (4% 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate/7 mol urea/2 mol thiourea/30 mmol Tris pH 8.0).

### 2.3. 2-Dimensional Differential Image Gel Electrophoresis (2D-DIGE) [19]

An equal amount of the 12 protein samples (IL-1 $\beta$ -treated, celecoxib-treated, both-treated, and control, n = 3)

were mixed and labeled with Cyanine dyes 3 (Cy3, Cy Dye DIGE Saturation dye; GE Healthcare, Buckinghamshire, UK) for internal standard. Similarly, each of the protein samples was labeled with Cyanine dyes 5 (Cy5). Then 2.5  $\mu\text{g}$  of the Cy3-labeled mixed sample (standard sample) and 2.5  $\mu\text{g}$  of the Cy5-labeled individual samples were mixed and applied onto an isoelectric focusing (IEF) gel (pH 3 - 11; GE Healthcare). After IEF, the proteins were further separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the gel images were obtained by an image analyzer (Typhoon). To compare protein spot intensity among the 4 groups, Cy5-fluorescent intensity of protein spots in each gel was normalized by Cy3-fluorescent intensity of identical spots by using the Progenesis program (Nonlinear Dynamics, Newcastle, UK), and the normalized Cy5-intensity was used for the comparison. In addition, 50 micrograms of proteins, labeled with Cy3, were used in 2DE for protein identification.

## 2.4. Protein Identification

For identification of proteins, 2-DE gel fragments with approximately 1 mm in diameter, which corresponded to protein spots of interest, were recovered. The protein in the gel fragment was digested with trypsin and the resulting peptides were recovered as described previously [19]. Masses of the digested peptides and their collision induced dissociation (CID) fragments were determined using a MALDI-TOF/TOF MS (Ultraflex, BurkerDaltonics, Germany). The MS/MS spectra obtained were used for searching of the National Center for Biotechnology Information protein database using the Mascot software program (Matrix Science, London, UK).

## 2.5. Statistical Analysis

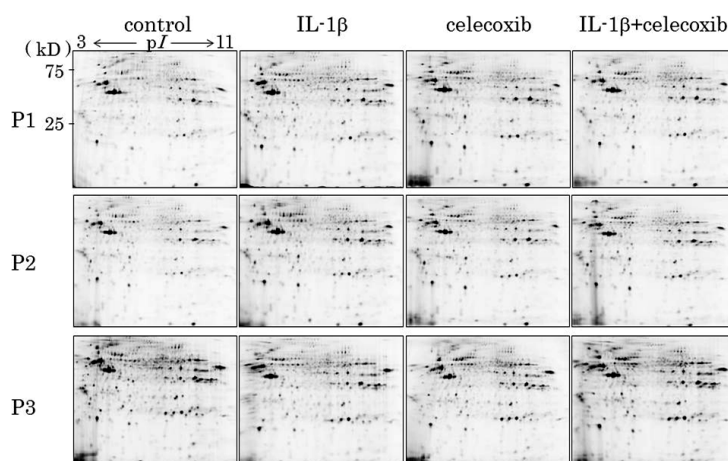
Statistical significance was calculated by Student's *t*-test.

## 3. Results

To investigate whether celecoxib affects protein profiles of chondrocytes, and also whether celecoxib modulates the effects of IL-1 $\beta$  on chondrocytes, we applied 2D-DIGE to chondrocyte cellular proteins. Then, we separated proteins extracted from unstimulated chondrocytes (control) and the stimulated chondrocytes (treated with IL-1 $\beta$ , celecoxib, IL-1 $\beta$  + celecoxib) by 2DE (**Figure 1**). As a result, more than 1100 protein spots were detected per gel image, of which 86 protein spots showed significantly different intensity among the 4 groups.

### 3.1. Effects of Celecoxib on Chondrocytes

In the 2D-GIGE comparison between the protein profiles of chondrocytes treated with celecoxib alone and those



**Figure 1.** Proteins extracted from the chondrocytes from 3 patients (P1, P2, P3) and stimulated chondrocytes (IL-1 $\beta$ , celecoxib and IL-1 $\beta$  + celecoxib) were applied to 2-dimensional electrophoresis (2-DE). More than 1100 protein spots were detected per gel image, of which 86 protein spots showed significantly different intensity among the 4 groups.

of control, 16 protein spots increased their intensity by celecoxib up to more than 1.5 folds and 12 protein spots down to less than 1/1.5 folds with statistical significance. Out of these protein spots, 4 spots increased their intensity up to more equal 2.0 folds and 3 spots down to less equal 2.0 folds, as shown in **Table 1**.

We then tried to identify these 28 protein spots ( $1.5 \leq$  or  $1/1.5 \geq$ ) by MALDI-TOF-TOF/MS and could identify 11 proteins out of them as summarized in **Table 2**. Each spot on the 2D gel was shown in **Figure 2**.

### 3.2. Effects of Celecoxib on IL-1 $\beta$ Treated Chondrocytes

Next, we tried to elucidate whether celecoxib cancels the effects of IL- $\beta$  on chondrocytes. We obtained 11 protein spots of which intensity was significantly increased by the IL-1 $\beta$  stimulation. In 4 out of the 11 protein spots, simultaneous addition of celecoxib decreased the intensity at the levels of control. Similarly, we obtained 4 protein spots of which intensity was significantly decreased by the IL-1 $\beta$  stimulation. In 3 out of the 4 protein spots, simultaneous addition of celecoxib increased to the levels of control. Thus, we obtained 7 protein spots in all, of which intensity was resumed by addition of celecoxib as shown in **Table 3** and **Figure 3**. We tried to identify the 7 proteins by mass spectrometry and could identify 2 proteins out of them, as listed in **Table 4**. **Figure 4** shows intensity of CD200R1 and moesin spots visualized by 3D images.

## 4. Discussion

Several comprehensive proteomic analyses of cancer cells treated with celecoxib have been studied in lung can-

**Table 1.** Difference of intensity and the number of protein spots.

Difference of intensity (celecoxib/control)	Number of spots
$3.0 \leq$	0
$2.0 \leq$	4
$1.5 \leq$	16
$1/1.5 \geq$	12
$1/2.0 \geq$	3
$1/3.0 \geq$	0

**Table 2.** Identification of chondrocyte proteins affected by celecoxib.

Spot No.	MW (Observed)	pI	Difference (celecoxib/control)	Protein
59	130	(5.2)	-1.37	Alpha-actinin-4 (ACTN4_HUMAN)
628	47	(5.8)	-2.39	ATPase family AAA domain-containing protein 3C (ATD3C_HUMAN)
453	55	(4.7)	-1.59	1) Tubulin beta chain (TBB5_HUMAN)
				2) Tubulin beta-2B chain (TBB2B_HUMAN)
630	42	(8.8)	-1.52	Serpin H1 (SERPH_HUMAN)
601	46	(5.0)	-2.08	cAMP-dependent protein kinase type II-beta regulatory subunit (KAP3_HUMAN)
615	47	(5.4)	2.11	Apolipoprotein A-V (APOA5_HUMAN)
306	78	(4.9)	2.55	78 kDa glucose-regulated protein (GRP78_HUMAN)
296	78	(5.0)	2.63	78 kDa glucose-regulated protein (GRP78_HUMAN)
362	80	(5.5)	1.5	Glutamate carboxypeptidase 2 (FOLH1_HUMAN)
290	63	(5.4)	1.57	Stress-70 protein, mitochondrial (GRP75_HUMAN)
324	63	(5.6)	1.9	Sorting nexin-9 (SNX9_HUMAN)

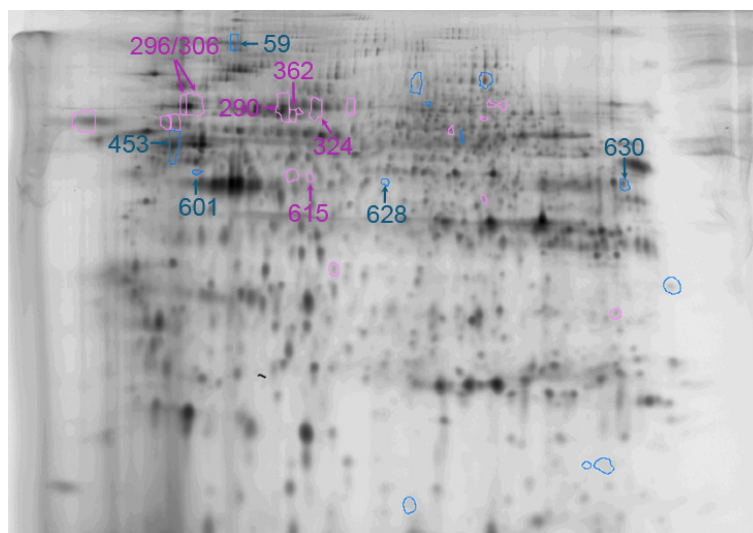
**Table 3.** Number of protein spots that were affected by IL-1 $\beta$  and celecoxib.

IL-1 $\beta$ /control ( $p < 0.05$ )		IL-1 $\beta$ + celecoxib/IL-1 $\beta$ ( $p < 0.05$ )	
Increase	11	Increase	1
		Decrease	4*
Decrease	4	Increase	3*
		Decrease	1
Total	15	Total	9

\*Intensity was resumed by addition of celecoxib.

**Table 4.** Proteins whose intensity was affected by IL-1 $\beta$  and resumed by addition of celecoxib.

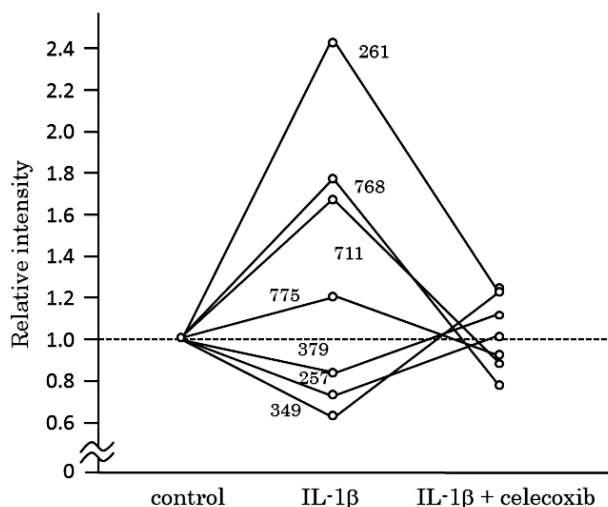
Spot No.	MW	pI	Relative difference			Protein
	(Observed)	Control	IL-1 $\beta$	IL-1 $\beta$ + celecoxib		
711	36	6	1	1.6	0.91	Cell surface glycoprotein CD200 receptor 1 (MO2R1_HUMAN)
349	63	6	1	0.69	1.2	Moesin (MOES_HUMAN)

**Figure 2.** Protein spots and their spot No. on 2D-gel.

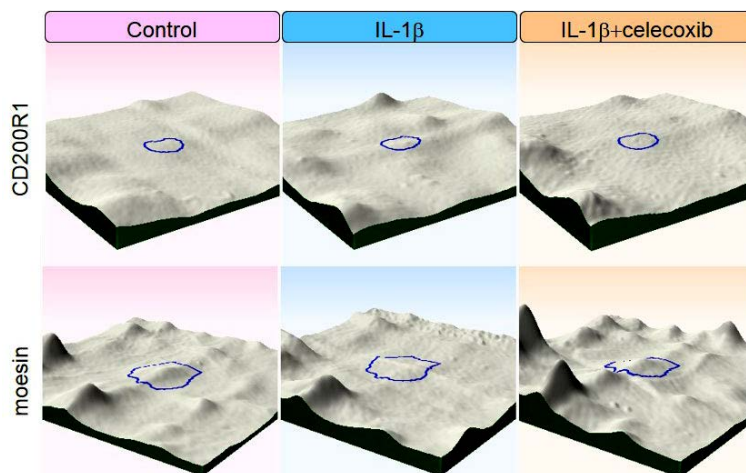
cer, oral squamous cell carcinoma and colorectal carcinoma [22]-[24]. Another study used cardiomyocytes to investigate the cardiovascular toxicity of NSAIDs including celecoxib [25]. The present study is believed to be the first one to investigate the effects of celecoxib on OA chondrocytes.

With the proteomic analysis, no less than 86 proteins were found to be affected by celecoxib and/or IL-1 $\beta$  in this study. Out of these 86 proteins, we could identify 5 decreased proteins and 6 enhanced proteins in chondrocytes treated with celecoxib. COX-2 was not implicated in this condition, as chondrocytes from OA as well as normal cartilage expressed extremely low levels of PGE<sub>2</sub> and COX-2 mRNA when incubated in an unstimulated condition [26]. Thus, the modification of these proteins was mediated by mechanisms unrelated to COX-2. Dimethyl-celecoxib, which is a close structural analogue of celecoxib, lacks the inhibitory function of COX-2, and has antitumor effects through activation of endoplasmic stress in tumor cells [27].

Two proteins out of the 5 decreased ones were cytoskeletons, actin and tubulin. The remaining proteins were ATPase family AAA domain-containing protein 3C, serpin H1 and cAMP-dependent protein kinase type II-beta regulatory subunit. The AAA+ (ATPases associated with various cellular activities) family is a large and func-



**Figure 3.** Protein spots of which intensity significantly changed by IL-1 $\beta$  and resumed by addition of celecoxib.



**Figure 4.** Intensity of CD200R1 and moesin spots were visualized by 3D images.

tionally diverse group of enzymes that are able to induce conformational changes in a wide range of substrate proteins [28]. Serpin H1 is one of the serine protease inhibitors and is commonly known as heat shock protein 47 (HSP47). HSP47/Serpin H1 is a molecular chaperon and maintains collagen biosynthesis. Antibodies against it are found in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematoses [29]. HSP47 is a stress protein that is induced under some stress in endoplasmic reticulum (ER). In early OA in a mouse model, HSP47 mRNA was up-regulated associated with IL-1 $\beta$ , IL-6 and TNF $\alpha$ , suggesting that celecoxib may suppress the stress in OA chondrocytes [30]. The significance of cAMP-dependent protein kinase and AAA+ family protein on osteoarthritis is unclear as far as we have found in the literature.

The 6 enhanced proteins included apolipoprotein A-V, 78 kDa glucose-regulated protein (GRP78), glutamate carboxy peptidase 2, mitochondrial stress-70 protein and sorting nexin-9. GRP78 was identified from 2 different spots. Apolipoprotein A-V was first found in 2001, and it maintains plasma levels of triglyceride [31]. Sorting nexin 9 is widely expressed and plays a role in endocytosis [32]. An alternative name for mitochondrial stress-70 protein is 75Kd glucose regulated protein (GRP75). It is located in mitochondria and causes cell proliferation [33]. No implication of chondrocytes in OA with these proteins has been reported as yet. On the other hand, GRP78 was physiologically induced by starvation [34], and interestingly, also by dimethyl celecoxib, the analog of celecoxib that lacks COX-2 activity [35], which suggested that GRP78 induction was COX-2 independent. Proteomic analysis of human chondrocytes has shown that GRP78 as well as GRP94 increased in late stage OA

[36], and advanced glycation end products (AGEs) induced GRP78 in chondrocytes [37]. GRP78 is an ER chaperon and is induced by stress to refold unfolded proteins, however, sustained ER stress leads to an inflammatory response and apoptosis [38]. Under stress, GRP78 is expressed and its specific overexpression was reported to reduce apoptosis in Chinese hamster ovary cells [39]. Thus, appropriate induction of GRP78 by celecoxib may have a chondroprotective effect, in part.

We identified 2 proteins whose IL-1 $\beta$ -induced expression was neutralized by celecoxib; CD200 receptor increased and moesin decreased with IL-1 $\beta$  stimulation, and both returned to baseline by addition of celecoxib. CD200 receptor (CD200R) is a ligand of CD200, also known as OX-2. Expression of CD200R is found in the macrophage lineage cells and delivers an inhibitory signaling [40]. Activation of CD200R by CD200-Fc ameliorates collagen-induced arthritis in mice [41]. Expression of CD200R in chondrocytes has not been studied yet, and further research is needed for the function of CD200R in chondrocytes of OA. Moesin together with Erzin and Radixin serves as a nexus between cell membrane and underlying cytoskeleton. It regulates signaling pathways by linking transmembrane receptors and downstream signaling components [42]. Though there is very little knowledge regarding the effects of moesin on chondrocytes, increased phosphorylation of moesin has been observed on stretched chondrocytes [43].

Some limitations of our study should be mentioned. First, the clinical serum concentration of celecoxib was not more than 1/10 of the concentration used in this study. As the aim of this study was to identify candidate proteins, we used higher concentration to detect as much potent proteins as possible. Validation of each protein should be necessary in the next step. Second, a number of patients who provided chondrocytes was only 3. As a larger number of patients are required for representing OA, validation of each protein will be necessary using an appropriate number of patients. Even though, it should be emphasized that differences of protein expression reached statistical significance in this study. Lastly, chondrocytes from healthy subjects were not evaluated as a control. Our previous study showed that responses of normal chondrocytes by IL-1 stimulation were different from those of OA [26] and the results might be different. While celecoxib is used for patients with symptom of OA, normal chondrocytes should also be evaluated as a scientific interest.

## 5. Conclusion

In conclusion, the protein profile of OA chondrocytes was found to change after the administration of celecoxib. AAA+ protein, HSP47/Serpin, cAMP-dependent protein kinase type II-beta regulatory subunit, alpha-actin-4 and tubulin decreased with administration of celecoxib, and apolipoprotein A-V, glutamate carboxipeptide 2, mitochondrial stress-70 protein, sorting nexin-9 and GRP78 increased with addition of celecoxib. Of these, GRP78 is a stress protein, and overexpression of it may have a chondroprotective effect by inhibiting apoptosis. In the present study, celecoxib modulated IL-1 $\beta$ -stimulated chondrocytes, and CD200R and moesin were identified as two such resulting proteins. Further investigation is needed to elucidate the function of each protein in OA.

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