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Increased Expression of the Histamine H_4 Receptor Subtype in Hypertrophic Differentiation of Chondrogenic ATDC5 Cells

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ABSTRACT

Histamine has been regarded as an inflammatory mediator of arthritic disorders. We have previously reported that the expression of histamine H_4 receptor (H_4R) mRNA in synovial tissues was significantly higher in patients with osteoarthritis (OA) compared to those with rheumatoid arthritis. Chondrocyte hypertrophy and endochondral ossification are essential processes in pathologic disorders such as osteophyte formation during OA progression. In the present study, we examined the expression of H_4R during differentiation into hypertrophic chondrocytes in the ATDC5 cells, a widely used in vitro model of chondrogenic differentiation. Quantitative reverse transcription polymerase chain reaction showed that the levels of histidine decarboxylase and H_4R mRNA on ATDC5 cells were increased in a time-dependent manner during the culture period. By contrast, the expressions of H_1R and H_2R were not increased from day 7 onwards. The mRNA expression of the hypertrophic chondrocyte marker type X collagen (COL X) was increased markedly from 14 to 21. Immunocytochemical analysis indicated that H_4R staining was strongly immunoreactive on the plasma membrane of ATDC5 cells. Flow cytometry showed increased expression of H_4R and COL X protein in ATDC5 chondrocytes. Furthermore, the majority of the COL X-positive cells expressed H_4R throughout the culture period. In summary, we showed for the first time that H_4R is expressed in ATDC5 chondrocytes. Moreover, we found that most hypertrophic chondrocytes express H_4R, suggesting that this receptor might be associated with the differentiation of chondrocytes into hypertrophic cells, which are abnormally observed in joint lesions in OA. J. Cell. Biochem. 113: 1054–1060, 2012.

KEY WORDS: HISTAMINE H_4 RECEPTOR (H_4R); ATDC5; OSTEOARTHRITIS (OA); TYPE X COLLAGEN; HYPERTROPHIC CHONDROCYTE

The histamine H_4 receptor (H_4R) is the most recently identified of the four histamine receptors belonging to the same G-protein-coupled receptor family. Several organs express H_4R, and immune tissues such as the spleen, thymus, bone marrow, and leukocytes show a wide range of H_4R expression [Oda et al., 2000]. Histamine has been regarded as an inflammatory mediator of arthritic disorders, and H_4R has been detected in synovial tissues from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) [Grzybowska-Kowalczyk et al., 2007, 2008]. The expression of H_4R mRNA in synovial tissues was found to be significantly higher in OA patients compared with RA patients [Yamaura et al., 2011]. Recently, histamine and histidine decarboxylase (HDC) as well as H_1R and H_2R were found in OA chondrocytes [Tetlow and Woolley, 2005]. Moreover, the presence of H_4R-like protein in tissue of OA cartilage has been reported [Grzybowska-Kowalczyk et al., 2008]. However, the expression and the function of H_4R in chondrocytes remain unclear.

Endochondral ossification is an essential process in pathologic disorders such as osteophyte formation during OA progression [Kronenberg, 2003; Kawaguchi, 2008]. After chondrocytes proliferate and differentiate into mature hypertrophic cells, the cells express vascular endothelial growth factor, stimulating blood vessels to invade the cartilage model [Goldring et al., 2006]. Groups of cells surrounding the cartilage model differentiate into osteoblasts, which calcify the surrounding matrix, leading to progressive replacement of degraded cartilage by bone. In proliferative chondrocytes, the extracellular matrix proteins are mainly type II collagen (COL II) and aggrecan, whereas type X collagen (COL X) and matrix metallo-

The authors declare that they have no conflicts of interest.

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proteinase (MMP) 13 are produced at high levels in hypertrophic chondrocytes [Ortega et al., 2004; Lefebvre and Smits, 2005].

The cell line ATDC5 has been shown to be a useful in vitro model for examining chondrogenic differentiation [Shukunami et al., 1997; Yamashita et al., 2009]. The proliferation and differentiation of chondroprogenitor ATDC5 cells in vitro can be mimicked in vivo by molecular analysis of early- and late-phase differentiation markers of chondrocytes. In this study, we showed for the first time that the H_4R subtype is expressed in ATDC5, and the majority of hypertrophic ATDC5 chondrocytes express H_4R, suggesting that this receptor is associated with the differentiation of chondrocytes into hypertrophic cells.

MATERIALS AND METHODS

CELL CULTURE

Mouse chondrogenic ATDC5 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Wako Pure Chemical Industries, Osaka, Japan) containing 5% fetal bovine serum (FBS; Sigma–Aldrich, St Louis, MO), 0.5% penicillin–streptomycin (Invitrogen, Carlsbad, CA), 10 μg/ml human transferrin (Sigma–Aldrich), and 3×10^{-8} M sodium selenite (Sigma–Aldrich) as described previously [Atsumi et al., 1990]. For induction of chondrogenic differentiation, 10 μg/ml bovine insulin (Sigma–Aldrich) was added to this medium. The cells were maintained at 37°C under a humidified atmosphere of 5% CO_2.The medium was replaced every other day throughout the differentiation period.

RNA ISOLATION AND QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR ANALYSIS)

ATDC5 cells were seeded onto 12-well plates at an initial density of 5×10^{-4} cells/well and cultured in insulin-supplemented medium for 7, 14, 21, and 28 days. At these time points, total RNA was isolated from ATDC5 cells using the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. Total RNA was reverse-transcribed into cDNA using PrimeScript® RT Master Mix (Takara Bio, Shiga, Japan).

Real-time RT-PCR was performed using SYBR® Premix Ex Taq (Takara Bio). Reactions were performed at 95°C for 30 s and then at 95°C for 3 s and 60°C for 30 s for 40 cycles using StepOne™ Real Time PCR System (Applied Biosystems, Carlsbad, CA). Primer sequences are shown in Table I. Expression of target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the mean value of day 7 set to 1.0.

ALIZARINE RED AND ALCIAN BLUE STAINING

ATDC5 cells were differentiated in 12-well plates and washed twice with phosphate-buffered saline (PBS). For Alizarin red staining, cells were fixed in 10% neutral buffered formalin solution (Wako) for 20 min and stained with 1% Alizarin red S (Wako) for 10 min. For Alcian blue staining, cells were fixed in ice-cold methanol (Wako) for 10 min and stained with 0.1% Alcian blue 8GX (Wako) in 0.1 M HCl overnight. Stained cells were washed with distilled water and photographed with a scanning camera.

TABLE I. Primers Used for Real-Time RT-PCR Amplification

Target genes	Primer sequence (5′–3′)
H_4R	5′ACGATCGATACCAGTCAGTTTCAA-3′
	5′-CCAGAATCATCGGGCCATTTAC-3′
H_1R	5′-CCAGAGCTTCGGGAAGATAA-3′
	5′-ACCACAGCATGAGCAAAGTG-3′
H_2R	5′-CGCGTTGCCATCTCTTTGGTCTTT-3′
	5′-TCGTTGACCTGCACTTTGCACTTG-3′
COL II	5′-CACACTGGTAAGTGGGGCAAGACCG-3′
	5′-GGATTGTGTTGTTTCAGGGTTCGGG-3′
COL X	5′-CTCCTACCACGTGCATGTGAA-3′
	5′-ACTCCCTGAAGCCTGATCCA-3′
Aggrecan	5′-CACGCTACACCCTGGACTTTG-3′
	5′-CCATCTCCTCAGCGAAGCAGT-3′
HDC	5′-ACTCCAAATGTGCAGCCTGGATACC-3′
	5′-GGCTAGATGCCCACGTGAATCCTAA-3′
GAPDH	5′-AACGACCCCTTCATTGAC-3′
	5′-TCCACGACATACTCAGCAC-3′

IMMUNOFLUORESCENCE STAINING

ATDC5 cells were seeded onto cover slides in 12-well plates and differentiated for 7 and 21 days. At each time point, cells were washed in PBS and fixed in 4% paraformaldehyde (Wako) for 15 min. Cells were then blocked in PBS containing 10% FBS and 10 mM NaN_3 (Wako) for 1 h, and incubated with goat anti-mouse histamine H_4 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2.5 h at room temperature. After washing with PBS containing 10% FBS and 10 mM NaN_3, cells were incubated with donkey anti-goat Cy2-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h in the dark. Cells were further washed and stained with propidium iodide (PI: Wako) for 30 min in the dark. To assess background staining, cells were processed identically with primary antibody excluded. Stained cells on cover glasses were mounted using Fluoromount™ mounting solution (Diagnostic BioSystems, Pleasanton, CA). Fluorescence images were obtained with an Olympus FV500 microscope (Olympus, Tokyo, Japan).

FLOW CYTOMETRY

ATDC5 cells were seeded in a 100-mm dish at an initial density of 6.5×10^5 cells, and differentiated for 7, 14, 21, and 28 days. Cultured ATDC5 cells were collected using 0.05% EDTA (DojinDo, Kumamoto, Japan) in PBS and fixed in 4% formalin (Wako) in PBS for 20 min. Following washing, cells were permeabilized by incubating in PBS containing 0.5% saponin (MP Biomedicals, Solon, OH), 2% FBS, and 0.1% NaN_3. Then cells were stained with goat anti-mouse histamine H_4 receptor antibody and rabbit anti-type X collagen antibody (Cosmo Bio, Tokyo, Japan) for 1 h. After washing, cells were incubated with donkey anti-goat Cy2-conjugated secondary antibody (Jackson Immunoresearch Laboratories) and donkey anti-rabbit PE-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h in the dark. Cells for the negative control were incubated with secondary antibody alone. After incubation, cells were assessed for H_4R and COL X positivity using flow cytometry (MoFlo®, Beckman Coulter, Brea, CA) and analytic software (SummitR v3.1, Beckman Coulter, Brea, CA).

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. For the statistical evaluation of the results of quantitative RT-PCR between day 7 in culture and other days, the Shirley–Williams test was performed. Dunnett's method was used for the results of flow cytometry. All statistical analyses were conducted using Stat Light 1997 (Yukms, Tokyo, Japan).

RESULTS

DIFFERENTIATION OF ATDC5 CHONDROCYTES IN CULTURE

First, to evaluate ATDC5 proliferation and differentiation in vitro, we analyzed the mRNA of typical chondrogenic differentiation markers and the proteoglycan production and calcium deposition (Fig. 1). The expression of mRNA levels of chondrogenic differentiation markers was measured by quantitative RT-PCR. COL II and aggrecan expression began to increase from day 7 in culture, indicating differentiation of mesenchymal cells into chondrocytes, and markedly increased on day 14, whereas aggrecan slightly decreased to day 28 (Fig. 1a,b). Expression of COL X mRNA levels increased from day 14, indicating late-phase differentiation of chondrocytes, and markedly increased from 14 to 21 and similar high levels at 21 and 28 days (Fig. 1c). Mineralization was determined by Alizarin red, and cartilage-specific proteoglycan synthesis, a major component of the proteoglycans, sulfated glycosaminoglycan, was verified by Alcian blue staining. The intensity of staining increased with culture period from day 14 to day 28, demonstrating that ATDC5 cells undergo differentiation and mineralization in culture (Fig. 1d).

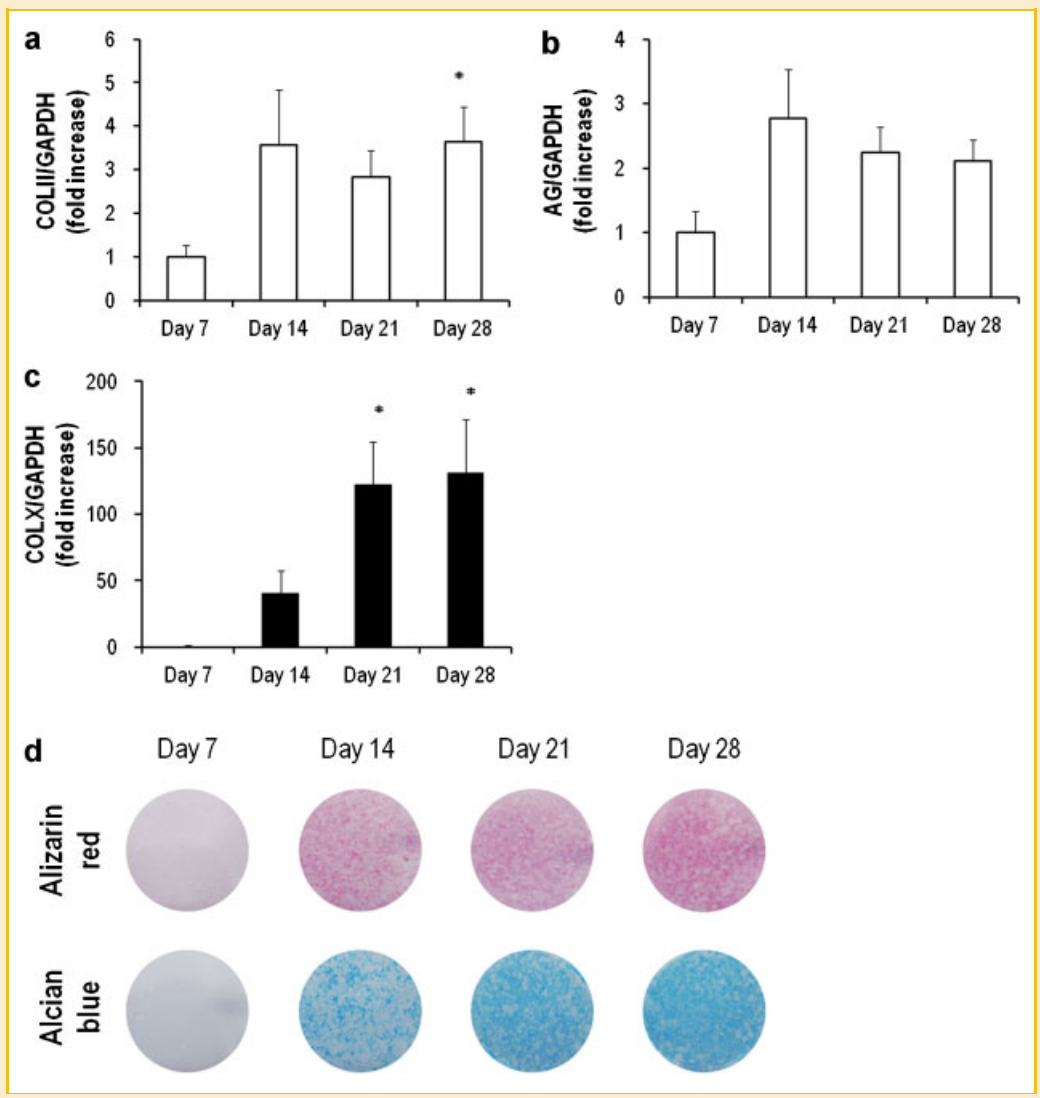

Fig. 1. ATDC5 cell differentiation in culture. To assess induction of cell proliferation and differentiation, cells were cultured in 12-well plates for 7, 14, 21, and 28 days. (a–c) Relative expressions of type II collagen (COL II), aggrecan (AG), and type X collagen (COL X) were determined by real-time RT-PCR. (d) After fixation, cells were stained with Alcian blue and Alizarin red. In all cases, the mRNA levels were normalized to the corresponding GAPDH mRNA levels and the mean value at day 7 was set to 1.0. Data are expressed as mean ± SEM (n = 4). $^{*}P < 0.05$, Shirley–Williams test compared with day 7.

EXPRESSION OF HISTAMINE RECEPTORS AND HDC mRNA IN ATDC5 CHONDROCYTES

HDC is the only enzyme that is able to catalyze the synthesis of histamine. We examined the expression of HDC mRNA during ATDC5 cell differentiation in culture by quantitative RT-PCR. The level of HDC mRNA was significantly increased in a time-dependent manner during the culture period (Fig. 2a). Furthermore, quantitative RT-PCR showed that the ATDC5 cells not only produce HDC but also express the three histamine receptor isoforms, H_1R, H_2R, and H_4R, throughout the differentiation phases. Our data show that, whereas the expression of H_1R and H_2R were not increased from day 7 onwards (Fig. 2b,c), the expression of H_4R on ATDC5 cells was increased in a time-dependent manner throughout the culture period (Fig. 2d).

IMMUNOHISTOCHEMICAL STAINING OF H_4R PROTEIN ON ATDC5 CHONDROCYTES

The expression of both H_1R and H_2R in human chondrocytes has been demonstrated by immunohistochemical studies [Tetlow and Woolley, 2005], but there have been no previous reports of expression of H_4R in chondrocytes. To confirm the expression of H_4R at the protein level, we carried out immunocytochemical analysis on cultured ATDC5 cells. Using H_4R staining, strong immunoreactivity was observed on the plasma membrane of ATDC5 cells (Fig. 3).

EXPRESSION OF H_4R AND COL X PROTEIN ON ATDC5 CHONDROCYTES

To examine whether the changes in gene expression measured at the level of mRNA correlated with changes in protein expression, H_4R and COL X protein were examined by flow cytometry. ATDC5 chondrocytes showed increased expression of H_4R and COL X protein as well as the result of mRNA quantitation in a time-dependent manner throughout the culture period (Fig. 4a–c). Moreover, we analyzed the percentage of H_4R-positive cells in COL X-positive hypertrophic chondrocytes; the majority of COL X-positive cells were H_4R positive on days 7, 21, and 28, at percentages of 72.6%, 72.3% and 74.4%, respectively (Fig. 4c).

DISCUSSION

Our results demonstrate, for the first time, the expression of H_4R in ATDC5 chondrocytes. The discovery of H_4R and its expression on numerous immune and inflammatory cells, such as mast cells, eosinophils, basophils, dendritic cells, and T cells, has prompted a re-evaluation of the actions of histamine in allergic inflammatory disorders [Thurmond et al., 2008]. Several ligands have been identified with affinity for this receptor, and modulation of the receptor may have effects on nociception, inflammation, pruritus, and autoimmune disorders [Tiligada et al., 2009]. The first potential selective H_4R antagonist, UR-63325, has completed a phase II clinical trial for the treatment of allergic asthma and rhinitis, and the pre-clinical investigation of UR-65318 as the first H_4R antagonist for the treatment of atopic dermatitis is at present in the advanced stages [Walter et al., 2011].

Histamine is involved in the pathogenesis of RA, characterized by synovial tissue inflammation and cartilage and bone degradation [Woolley and Tetlow, 1997]. Previously, we reported the expression of H_4R in two specific types of cell populations, fibroblast-like synoviocytes and macrophage-like cells, from RA synovial cell

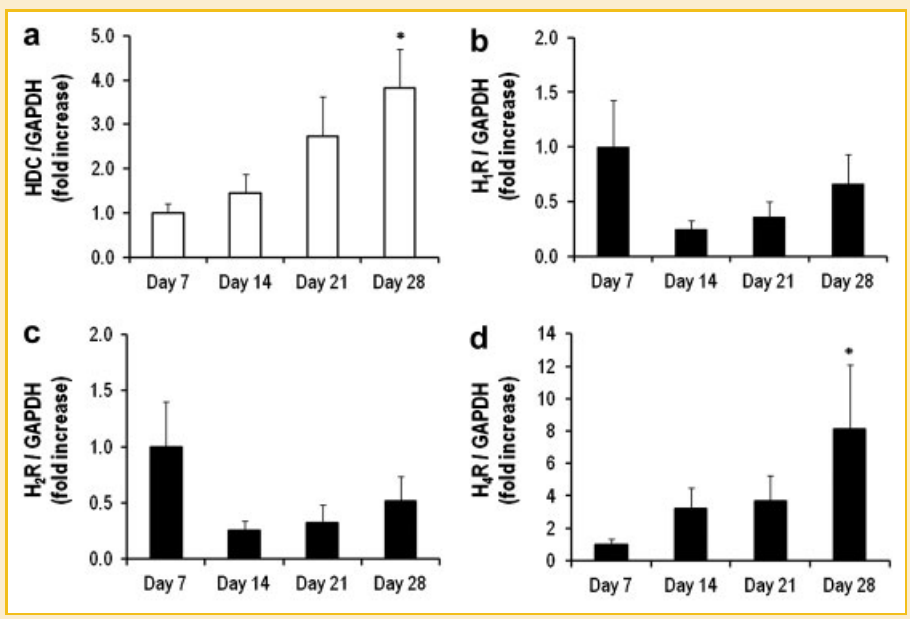

Fig. 2. Expression of histidine decarboxylase (HDC) and histamine receptors on ATDC5 cells. Cells were cultured in 12-well plates for 7, 14, 21, and 28 days. (a) Relative expression of HDC, (b) histamine H_1 receptor (H_1R), (c) H_2 receptor (H_2R), and (d) H_4 receptor (H_4R) were determined by real-time RT-PCR. In all cases, mRNA levels were normalized to the corresponding GAPDH mRNA levels and the mean value at day 7 was set to 1.0. Data are expressed as mean ± SEM ($n = 4$). $^*P < 0.05$, Shirley–Williams test compared with day 7.

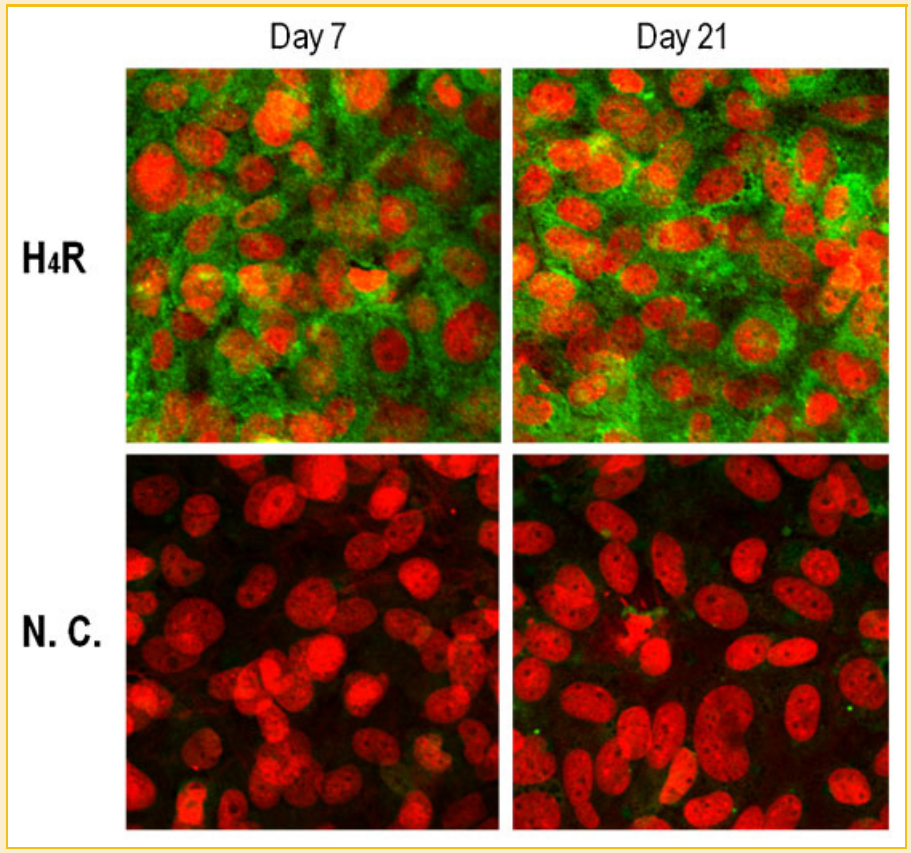

Fig. 3. Histamine H_4 receptor (H_4R) expression on ATDC5 cells on days 7 and 21. Cells were cultured in 12-well plates for 7 and 21 days and H_4R (green) and nuclei (red) were detected by immunofluorescence and confocal microscopic analysis. Negative controls (NC) were exposed to the secondary antibody and propidium iodide alone. Magnification, ×400.

cultures, which indicate the involvement of this receptor in disease progress [Ikawa et al., 2005; Ohki et al., 2007]. Recently, we demonstrated that the expression of H_4R in synovial tissue is significantly higher in patients with OA than in those with RA [Yamaura et al., 2011]. These results indicate the possibility that the H_4R is strongly involved in OA, a progressive disease of the joints characterized by degradation of articular cartilage. The degeneration of cartilage is caused by the activity of enzymes produced by the activated chondrocytes, which digests the matrix, and matrix synthesis is thus inhibited and the consequent erosion of the cartilage accelerated [Sandell and Aigner, 2001]. Mast cell histamine is another potent mediator with stimulatory effects on chondrocytes, as well as synoviocytes and endothelial cells. It is of special interest that human chondrocytes express both H_1R and H_2R, their stimulation resulting in increased MMP-13 and cyclic AMP production, respectively [Taylor and Woolley, 1987; Tetlow and Woolley 2002]. In contrast to the published data regarding H_1R and H_2R, very little is known about the expression of H_4R on chondrocytes and thus became the focus of the present study.

The molecular mechanisms of cartilage destruction in OA remain largely unknown, particularly the early events. No proven disease-modifying therapy is currently available, and the goal of contemporary management of patients with OA remains control of pain and improvement in function and health-related quality of life with avoidance of toxicity due to symptomatic therapy [Dreier, 2010]. A great deal of information on OA has come from research into artificial arthroplasty; however, such studies focus on the end of the process. To contribute to the potential therapeutic exploitation of new drug targets for OA, more studies are needed that focus on the beginning of the process. Recent research attention has centered around endochondral ossification signals as the mechanism involved in the progression of OA [Kawaguchi, 2008]. Experimental mice models of OA revealed that COL X and MMP-13 were significantly induced during OA progression. Furthermore, expression of COL X, a specific marker of hypertrophic chondrocytes, appeared in the early-phase of cartilage degradation, followed by MMP-13 production in hypertrophic chondrocytes [Kamekura et al., 2006]. These findings suggest that articular chondrocytes undergo hypertrophic differentiation in response to joint instability, and the hypertrophic chondrocytes express MMP-13, which may degrade the cartilage matrix. Therefore, in the present study, we used the cell line ATDC5, a widely used in vitro model of chondrogenic differentiation, to focus on the expression of H_4R during differentiation into hypertrophic chondrocytes. It has previously been shown that, from the early stage of differentiation, ATDC5 cells express the chondrocyte marker genes COL II and aggrecan. Further differentiation to hypertrophic chondrocytes induces the production of COL X. Hypertrophic chondrocytes also express MMP-13, alkaline

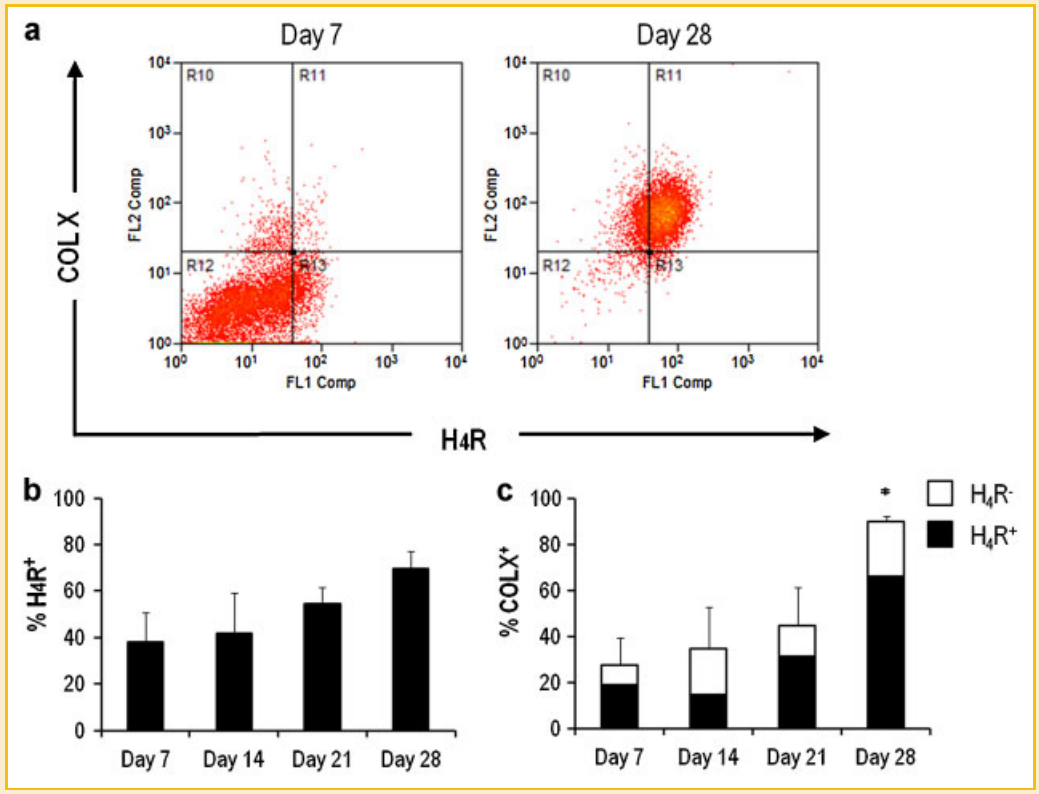

Fig. 4. Expression of histamine H_4 receptor (H_4R) and type X collagen (COL X) protein on ATDC5 cells. Cells were cultured in 100 mm dishes for 7, 14, 21, and 28 days then expression of H_4R and COL X were examined using flow cytometry. (a) Representative scatter plots, (b) quantification of the percentage of H_4R^+ cells, and (c) quantification of the percentage of COL X^+ cells. Data are expressed as mean ± SEM of COL X^+ cells ($n = 3$). $^*P < 0.05$, Dunnett's test compared with day 7. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

phosphatase, vascular endothelial growth factor, and osteopontin. COL X, MMP-13, and alkaline phosphatase are well-established markers of the overt hypertrophic stage of late chondrocyte differentiation [Dreier, 2010]. In the present study, ATDC5 chondrocytes differentiated into hypertrophic chondrocytes after at least 21 days in culture. Moreover, we found that these chondrocytes express HDC and H_4R, especially during the hypertrophic stage of late chondrocyte differentiation. Flow cytometric analysis revealed that the majority of the hypertrophic chondrocytes express H_4R.

Consistent with previous studies in human articular chondrocytes [Woolley, 2003], we have shown in the present study that ATDC5 chondrocytes express HDC, indicating that ATDC5 could produce endogenous histamine. Under these conditions, H_4R is a particularly important receptor isoform because the affinity of the endogenous ligand histamine for H_4R is 1,000- to 10,000-fold higher than for H_1R and H_2R [Lim et al., 2005]. The expression of HDC and histamine secretion after de novo synthesis has been reported in many cells such as macrophages, dendritic cells, and T cells, which also express H_4R [Thurmond et al., 2008]. Histamine can have varying and counteracting effects on these cells depending on its concentration, the number of receptors, and the affinity of the receptors for histamine. The levels of histamine receptors may change during different stages of cell development or under pathophysiologic conditions. For example, H_1R increases on in vitro differentiation of monocytes into macrophages [Triggiani et al., 2007], and inflammatory stimuli can upregulate the expression of H_4R in monocytes [Dijkstra et al., 2007]. Therefore, there is the possibility that H_4R may be involved in hypertrophic differentiation of chondrocytes in an autocrine manner. However, further investigation is needed to elucidate the role of the H_4R receptor in the differentiation of chondrocytes.

In conclusion, we have shown, for the first time, the expression of H_4R in ATDC5 chondrocytes. Our results support the supposition that H_4R in ATDC5 chondrocytes may be involved in the differentiation of chondrocytes into hypertrophic cells, which are abnormally observed in a joint lesion in OA. Additional studies are required to demonstrate the role of individual histamine receptor isoforms in mediating the actions of histamine in chondrocyte differentiation.

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