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Suppression of osteogenic differentiation in mesenchymal stem cells from patients with ossification of the posterior longitudinal ligament by a histamine-2-receptor antagonist

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ABSTRACT

Mesenchymal stem cells (MSCs) in ossification of the posterior longitudinal ligament (OPLL) patients have a high propensity toward osteogenesis. Histamine receptor H2 (H2R) antagonists (H2 blockers) like famotidine decrease ossification in patients, by an unclear mechanism. To confirm that MSCs express H2R and to clarify how H2 blockers suppress osteogenic differentiation, we used spinal-ligament MSCs from patients with OPLL or with cervical spondylotic myelopathy (CSM) (control). The MSCs were treated with 10, 30, or 100 nM famotidine for 7 or 21 days. Flow cytometry revealed that cells from both groups expressed MSC surface markers CD44, CD90, and CD105 (> 97.5%) but not CD34 or CD45 (< 2.5%). Immunoblotting showed that the MSCs from both groups expressed H2R, but those from OPLL patients expressed it at higher levels. Real-time qPCR indicated the *H2R* expression was significantly suppressed by 30 nM famotidine for 7 days or by 30 or 100 nM for 21 days. However, histidine decarboxylase, a key enzyme in histamine production, did not change significantly after famotidine addition. Famotidine treatment at 100 nM for 21 days significantly suppressed mRNA expression of the osteogenic markers *osteocalcin* (*OCN*), *bone morphogenetic protein 2* (*BMP2*), and *runt-related transcription factor 2* (*RUNX2*) only in OPLL-derived MSCs. Immunoblots showed that famotidine suppressed BMP2 and OCN in the OPLL group and H2R and RUNX2 in both groups. These results suggest famotidine inhibits osteogenic differentiation in OPLL-derived MSCs by acting as an H2R antagonist, but also by decreasing H2R expression, and support the clinical use of famotidine to treat OPLL.

1. Introduction

Ossification of the posterior longitudinal ligament (OPLL) of the cervical spine is a common musculoskeletal disease that involves heterotopic ossification (Ehara et al., 1998; Nakamura et al., 1999). Risk factors for the formation and progression of OPLL include diabetes mellitus and genetic, hormonal, environmental, and lifestyle factors (Inamasu et al., 2006). The ossification pathophysiology is thought to involve mesenchymal stem cells (MSCs) derived from the posterior longitudinal ligament that differentiate inappropriately into osteogenic cells (Asari et al., 2012; Naraghi et al., 1996). We previously showed that mesenchymal stem cells (MSCs) in human spinal ligaments localize to the perivascular area and the collagenous matrix (Chin et al., 2013). MSCs from OPLL ligaments have a higher ossification potential than those from non-ossified spinal ligaments (Harada et al., 2014). Unmethylated WNT5A and GDNF genes are reported to promote osteogenic features (Chiba et al., 2015).

OPLL can be treated conservatively with non-steroidal anti-inflammatory drugs (NSAIDs) or indomethacin, by localized radiation therapy, or surgically (Furukawa, 2008). The surgical removal of the ossified lesion is difficult and has a high incidence of complications (Li and Dai, 2011). While drugs such as bisphosphonate and P2 purinoceptor Y1 (P2Y1) antagonists are reported to prevent the OPLL ossification process (Furukawa, 2008), it is still important to develop new drug therapies to halt the progression of OPLL.

Histamine plays a pivotal role in a number of processes, including inflammation, allergic reactions, gastric acid secretion, and neurotransmission. Histamine is a diamine derivative of histidine, and is produced under the control of a single enzyme, histidine decarboxylase (HDC). Histamine exerts its functions by binding one of four known G protein-coupled receptors: histamine receptors H1-H4 (H1R-H4R), which elicit different downstream signaling effects (Jutel et al., 2009). H2R is present on parietal cells, in human bone-marrow MSCs, and in

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TT-D6 cells derived from the Achilles tendon of transgenic mice. H2R antagonists (H2 blockers) such as famotidine, cimetidine, and ranitidine have been used clinically to treat digestive-system diseases. Recently, H2 blockers were found to inhibit osteogenic differentiation in tendon-calcifying cells (Yamamoto et al., 2012). Oral famotidine treatment suppresses OPLL progression in a mouse model (Maeda et al., 2015). However, it is not clear how H2 blockers suppress ossification.

This study was designed to confirm that H2R is present in MSCs derived from OPLL patients and to clarify how famotidine suppresses osteogenic differentiation.

2. Materials and methods

2.1. Samples

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine. All subjects gave informed, written consent before participating in the study. The ligamentum flavum was collected aseptically during cervical spine surgery from nine patients with OPLL and five patients with cervical spondylotic myelopathy (CSM, n = 6). The ligamentum flavum in OPLL patients (n = 8) showed no evidence of ossification when collected. Table 1 shows the gender, age, and clinical diagnosis for all patients who provided tissue samples for this study.

2.2. Cell isolation and culture

MSCs were isolated as previously described (Asari et al., 2012). Briefly, the collected samples were washed with phosphate-buffered saline (PBS), minced, and digested with 3 mg/ml Collagenase Type V (Wako Pure Chemical Industries, Osaka, Japan) in α-modified Eagle's Medium (α-MEM; Invitrogen, Carlsbad, CA, USA) at 37 °C for 3 h. Nucleated cells were plated in plastic dishes at a density of 5×10^5 cells/95-mm dish. Cells were maintained in α-MEM Complete Culture Medium (Invitrogen, USA) with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), 100 U/ml penicillin G sodium, and streptomycin sulfate (Invitrogen, USA).

2.3. Flow cytometry

MSCs isolated from OPLL and CSM were stained with fluorochrome-conjugated antibodies by resuspending them in PBS containing a mouse anti-human CD34 antibody coupled with PE, anti-CD44 coupled with V450, anti-CD45 coupled with FITC, anti-CD90 coupled with APC, or anti-CD105 coupled with PerCP (BD Biosciences, San Jose, CA, USA. The cells were analyzed using a FACSAria II (BD Biosciences). Data were analyzed using BD FACSDiva software v6.1.3 (BD Biosciences).

2.4. In vitro famotidine treatment for osteogenic induction

MSCs were plated on 35-mm plates at 8×10^4 cells/well and cultured in histamine-free normal medium or an osteogenic differentiation medium consisting of conditioned medium plus histamine-free α-MEM supplemented with 50 mg/ml L-ascorbic acid and 10 mM β-glycerophosphate, as previously reported (Sakaguchi et al., 2005). For famotidine (Wako, Osaka Japan) treatment, cells isolated from all OPLL and CSM samples were cultured under osteogenic differentiation conditions with famotidine (10, 30, or 100 nM), under the osteogenic differentiation conditions only, or in normal medium only (control) for 7 or 21 days.

Table 1
Age, gender, and clinical diagnosis of all subjects who provided tissue samples.

Sex/age	Diagnosis	Sex/age	Diagnosis
M/77	CSM	M/71	C-OPLL
M/66	CSM	F/68	C-OPLL
F/77	CSM	F/57	C-OPLL
M/74	CSM	M/55	C-OPLL
F/59	CSM	M/51	C-OPLL
F/78	CSM	M/55	C-OPLL
		M/63	C-OPLL
		M/67	C-OPLL

2.5. Real-time qPCR

Real-time qPCR was used to detect differences in the mRNA expression of human and *osteogenic-related osteocalcin* (*OCN*), *osteopontin* (*OPN*), *bone morphogenetic protein 2* (*BMP2*), *runt-related transcription factor 2* (*RUNX2*), *alkaline phosphatase* (*ALP*), *osterix (OSX),* and *t*wo kinds of histamine receptors: *histamine receptor H1* (*H1R*), and *histamine receptor H2* (*H2R*). We also examined the histidine decarboxylase (*HDC*) expression to confirm that MSCs have a potential to produce histamine and that famotidine acted as a histamine antagonist or inverse agonist; Table 2 shows the PCR primers used. GAPDH was used to normalize the RNA expression levels of the markers shown. The data were compared with osteogenic induction only (control). Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Real-time PCR was performed with Power SYBR Green PCR Master Mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data were analyzed as previously described (Tanaka et al., 2011).

2.6. Immunoblot analysis

To prepare whole-cell lysates, cell pellets were dissolved directly in sample buffer (Thermo, Carlsbad, CA, USA) and sonicated to shear the DNA. After protein quantification, 2-mercaptoethanol (final concentration, 1%) and bromophenol blue (final concentration, 0.01%) were added to each sample and the mixtures were incubated at 95 °C for 5 min (RUNX2) or at room temperature for 30 min. The proteins were loaded (15 micrograms per lane) and separated by 2–20% SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 1% skim milk in PBS with 0.1% Tween 20 (TBST) or 1% BSA-TBST (for H2R blots) and then blotted with an anti-H2R (1:1000, GeneTex, USA), anti-RUNX (1:1000, Abcam, USA), anti-BMP2 (1:0000, Abcam, USA), anti-OCN (1:1000, Abcam, USA), or anti-lamin B (actin, for untreated cells) antibody diluted by Can Get Signal (Toyobo, Japan) overnight. The membranes were washed with TBST, incubated with horseradish-peroxidase-conjugated secondary antibodies (U87MG cells for H2R, sample control from Santa Cruz for RUNX2, HeLa cells for BMP2, and 293T cells for OCN) for 1 h at room temperature. The membranes were stripped for 10 min at room temperature and blocked with 1% skim milk TBST for 30 min at room temperature. Anti-lamin B was diluted 1:500 with Can Get Signal at 4 °C overnight before use. The membranes were incubated with secondary antibodies (anti-goat-IgG-HRP, 1:500) for 1 h at room temperature and visualized using ImmunoStar Chemiluminescent Reagent (Wako Pure Chemical).

2.7. Statistical analysis

All data were expressed as the mean ± S.E.M. Experimental data were compared by ANOVA using Tukey's test. A P value < 0.05 was considered statistically significant. Statistical calculations were performed with SPSS version 22.0 (SPSS, Chicago, IL, USA).