Biochemical and Biophysical Research Communications 420 (2012) 124–129

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ROCK inhibitor prevents the dedifferentiation of human articular chondrocytes

Emi Matsumoto, Takayuki Furumatsu *, Tomoko Kanazawa, Masanori Tamura, Toshifumi Ozaki

Department of Orthopaedic Surgery, Science of Functional Recovery and Reconstruction, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikatacho, Kitaku, Okayama 700-8558, Japan

ARTICLE INFO

Article history:
Received 18 February 2012
Available online 1 March 2012

Keywords:
Chondrocyte
Dedifferentiation
Redifferentiation
ROCK inhibitor
SOX9
Type II collagen

ABSTRACT

Chondrocytes lose their chondrocytic phenotypes in vitro. The Rho family GTPase ROCK, involved in organizing the actin cytoskeleton, modulates the differentiation status of chondrocytic cells. However, the optimum method to prepare a large number of un-dedifferentiated chondrocytes is still unclear. In this study, we investigated the effect of ROCK inhibitor (ROCKi) on the chondrogenic property of monolayer-cultured articular chondrocytes. Human articular chondrocytes were subcultured in the presence or absence of ROCKi (Y-27632). The expression of chondrocytic marker genes such as SOX9 and COL2A1 was assessed by quantitative real-time PCR analysis. Cellular morphology and viability were evaluated. Chondrogenic redifferentiation potential was examined by a pellet culture procedure. The expression level of SOX9 and COL2A1 was higher in ROCKi-treated chondrocytes than in untreated cells. Chondrocyte morphology varied from a spreading form to a round shape in a ROCKi-dependent manner. In addition, ROCKi treatment stimulated the proliferation of chondrocytes. The deposition of safranin O-stained proteoglycans and type II collagen was highly detected in chondrogenic pellets derived from ROCKi-pretreated chondrocytes. Our results suggest that ROCKi prevents the dedifferentiation of monolayer-cultured chondrocytes, and may be a useful reagent to maintain chondrocytic phenotypes in vitro for chondrocyte-based regeneration therapy.

1. Introduction

Articular cartilage has a poor intrinsic capacity for healing, and cartilage lesion from trauma or degeneration can result in morbidity and functional impairment [1]. Cartilage repair strategies that include arthroscopic debridement, bone marrow stimulation, osteochondral mosaicplasty, and autologous chondrocyte implantation are available for the treatment of symptomatic chondral and osteochondral lesions [2]. In chondrocyte-based approaches for the treatment of cartilage defects, several issues involved in a loss of chondrocytic phenotype during culture, which is called dedifferentiation, and requiring two surgeries (chondral tissue harvest and cultured cell implantation) are considered [3]. Monolayer-cultured articular chondrocytes leads to a process of dedifferentiation whereby the cells acquire a fibroblastic morphology and lose their chondrocytic properties [4–7]. The expression of chondrocyte-specific genes, such as α1(II) collagen (COL2A1), aggrecan, SRY-type high-mobility-group box (SOX) 9, and SOX 5/6, is gradually downregulated during cell multiplication in monolayer culture conditions [4–7]. Several authors have reported that three-dimensional culture systems, such as cultures in agarose, alginate beads, and the other scaffolds, recover the chondrogenic phenotype of dedifferentiated chondrocytes and induce their redifferentiation in vitro [4–6]. However, three-dimensional culture is not a cell-proliferating method to prepare a sufficient number of chondrocytes. Since primary chondrocytes that can be isolated from the native cartilage is limited, a monolayer culture technique that stimulates cellular proliferation without inducing their dedifferentiation or hypertrophic differentiation is important for a clinical application of chondrocyte-based therapy. Several biochemical factors such as fibroblast growth factor (FGF)-2, insulin, insulin-like growth factor, and bone morphogenetic protein (BMP)-2 synergistically promote chondrocyte proliferation in a three-dimensional atelocollagen gel culture [8,9]. After the cell number-increasing step (FGF-2/insulin-supplemented monolayer culture), the combination of BMP-2, insulin, and triiodothyronine has the optimal effect on chondrocyte redifferentiation in the sequential gel culture system [9]. However, ex vivo three-dimensional culture systems accompanied by continuous growth factor treatments are expensive and complicated. In this study, we investigated the effect of a reasonable reagent, ROCK inhibitor (ROCKi), on the status of cellular dedifferentiation, redifferentiation, and proliferation in monolayer-cultured human articular chondrocytes.

The Rho family GTPases including RhoA, Rac1, and Cdc42 are members of the Ras superfamily of small GTPases [10,11]. Rho GTPases regulate various biological functions such as cell motility and gene expression by organizing the actin cytoskeleton [12]. ROCK, one of the RhoA downstream effectors, has an important role in inducing stress fiber formation and assembly of focal adhesions

* Corresponding author. Fax: +81 86 223 9727.
E-mail address: matino@md.okayama-u.ac.jp (T. Furumatsu).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.02.127

[11,13]. However, the effect of RhoA/ROCK signaling is still controversial on chondrogenic property and cellular behavior of cultured chondrocytes. RCOK inhibition causes cortical actin organization and cell rounding, and reduces the number of actin fibers in chondrogenic ATDC5 cells [14]. In addition, ROCKi treatment increases glycosaminoglycan synthesis and the expression of chondrogenic marker genes, such as Col2a1, aggrecan, Sox9, and Sox5/6, in ATDC5 cells [14,15]. On the other hand, RhoA overexpression in ATDC5 cells results in increased proliferation and a delay of hypertrophic differentiation [16]. Inhibition of ROCK signaling partially rescues the effects of RhoA overexpression in chondrocytic cells [16]. In the present study, we investigated cellular behavior of ROCKi-treated articular chondrocytes and evaluated the role of ROCK signaling in chondrocyte dedifferentiation during monolayer culture condition.

2. Materials and methods

2.1. Cells and cell culture

Institutional Review Board approval and informed consent were obtained before beginning all experimental studies. Articular cartilage was obtained at notchplasty in patients suffering from anterior cruciate ligament injury ($n = 4$). Patients were 13, 18, 19, and 21 years of age. Articular chondrocytes were prepared by collagenase (Sigma, St. Louis, MO) digestion [17]. Attached cells (passage 0) were maintained with Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, South Logan, UT), and penicillin/streptomycin (Sigma). Monolayer-cultured chondrocytes between passage 1 and 7 were used. ROCK inhibition was performed by a ROCK-specific inhibitor Y-27632 (Wako). ROCKi-supplemented media were changed every 3 days.

2.2. Cell proliferation assay

Cell proliferation assays were performed as described [18]. Cells were incubated in ROCKi-supplemented media for 48 h prior to addition of WST-1 (Roche, Mannheim, Germany). Untreated chondrocytes used as controls. The optical density (OD, 450–630 nm) was measured, and the mean value derived from 5 wells was evaluated.

2.3. RT-PCR and quantitative real-time PCR

RNA samples were obtained from monolayer- and pellet-cultured chondrocytes. Total cellular RNAs were isolated using ISOGEN reagent (Nippon Gene, Toyama, Japan). RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The cDNAs underwent PCR amplification in the presence of specific primers using Taq DNA polymerase (TaKaRa, Ohtsu, Japan). For all the RT-PCR fragments, the reaction was allowed to proceed for 28–32 cycles. The following primer sets were used: COL2A1, aggrecan, SOX9, SOX5, SOX6, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [19–22], 5′-ccc ttt ttg ctg cta gta tcc ttg-3′ and 5′-cta cag ctg atg gtc ccg gt-3′ for α1(X) collagen (COL10A1). Quantitative real-time PCR analyses were performed using FastStart DNA Master SYBR Green I kit (Roche) [23]. The cycle number crossing the signal threshold was selected in the linear part of the amplification curve. Amplification data of G3PDH were used for normalization. These assays were run in triplicate, and relative mRNA levels were normalized with the level of untreated chondrocytes for every sample.

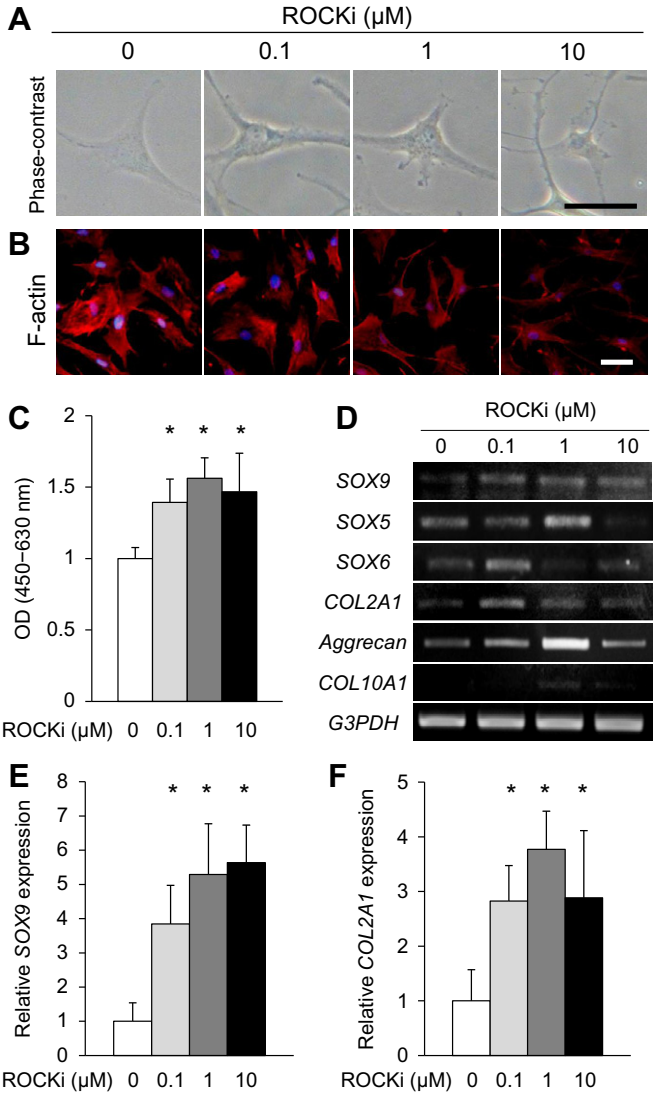

Fig. 1. ROCKi influences cellular morphology, proliferation, and gene expression of primary chondrocytes. (A) Phase-contrast microscopic images of human articular chondrocytes (passage 1) in the presence or absence of ROCKi (0, 0.1, 1, and 10 μM, 48 h). ROCK inhibition transformed cell shape to small and round, chondrocyte-like morphology. (B) Stress fibers were detected by phalloidin regents (F-actin, red). Cellular morphology and stress fiber formation were modified by ROCKi treatments (48 h). (C) Cellular proliferation was increased up to 1.5-fold levels of untreated chondrocytes by ROCKi treatments (0.1, 1, and 10 μM). OD (450–630 nm) was measured at 48 h after ROCKi administration. (D) Chondrogenic gene expressions of SOX9, SOX5/6, COL2A1, and aggrecan were affected by 1-week-treatment of ROCKi in monolayer-cultured chondrocytes (passage 1). The expression of COL10A1, a hypertrophic differentiation marker, was not influenced by ROCKi treatment. (E and F) Real-time PCR analyses revealed that ROCKi treatment increased the expression of SOX9 and COL2A1 in human articular chondrocytes. Bars, 50 μm. $^{*}p < 0.05$ compared with each untreated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Chondrocyte redifferentiation, histological analysis, and Western blot

To assess the redifferentiation ability of dedifferentiated chondrocytes after monolayer culture, pellet-cultured chondrocytes (5×10^5 cells/pellet) were maintained in ROCKi-free chondrogenic induction media [DMEM-high glucose supplemented with 10% FBS, dexamethasone (0.1 mM, Sigma), ascorbate (0.17 mM, Sigma), ITS Premix (1%, BD Biosciences, Bedford, MA), and transforming growth factor-β3 (10 ng/ml, R&D, Minneapolis, MN)] for 2 weeks