Original Article

Effect of 7-ketocholesterol on Hyaluronic Acid Synthase 2 in Human Aortic Smooth Muscle Cells

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ABSTRACT

Background: Hyaluronic acid (HA) is a component of the soft extracellular matrix in the arterial wall, and is involved in the elasticity of the arterial wall; however, its regulation and role in the progression of atherosclerosis remain unclear. We investigated the effects of 7-ketocholesterol (7KCHO), an oxysterol found in the atheromatous lesion, on the synthesis of HA in the cultured human smooth muscle cells (SMCs).

Methods: The cell count was done using a hemocytometer. Messenger ribonucleic acid (mRNA) and protein "hyaluronic acid synthase 2 (HAS2)" was measured by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot, respectively. Production of the intracellular reactive oxygen species (ROS) was measured by fluorescence-activated cell sorting.

Results: Treatment with 7KCHO increased ROS and decreased HA production in SMCs. N-acetyl cysteine, an antioxidative compound, restored the 7KCHO-induced decrease in the HA production. No changes were observed in the mRNA and protein expression of HAS2 by 7KCHO treatment.

Conclusions: These results suggest that HA production is decreased by 7KCHO treatment through stimulation of ROS. The 7KCHO-induced decrease of HA production may be involved in the loss of elasticity and the enhancement of arterial stiffness.

Toho J Med 4 (2): 74–80, 2018

KEYWORDS: hyaluronic acid, smooth muscle cell, 7-ketocholesterol, reactive oxygen species

1. Introduction

Hyaluronic acid (HA) is a component of the soft extracellular matrix, and is mainly found at the inner side of the artery. HA is normally produced at the plasma membrane by one or more of the three hyaluronic acid synthases (HAS 1, 2, and 3).[1, 2)] In atherosclerosis, HA is produced by vascular smooth muscle cells (SMCs) in the neointima.[3, 4)] Evidence from certain studies on atherosclerosis reveals that HA promotes vascular SMC proliferation and migration, and that HA is accumulated during neointimal hyperplasia in association with SMC proliferation.[5)] From these studies, it was hypothesized that interstitial HA in the plaque matrix promotes neointimal expansion. Alternatively, HA plays an important role in tissue hydrodynamics and viscoelasticity, and is involved in the reduction of shear stress and maintenance of arterial elasticity.[6)] Nagy et al.[7)] reported that the inhibition of HA synthesis acceler-

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DOI: 10.14994/tohojmed.2017-011

Received Oct. 23, 2017: Accepted Jan. 26, 2018
Toho Journal of Medicine 4 (2), June 1, 2018.
ISSN 2189–1990, CODEN: TJMOA2

ated atherosclerosis. Considering the properties and functions of HA in hydrodynamics and viscoelasticity, Et-Taouil et al.[8] reported that high-sodium diet affected the structural and functional characteristics of large arteries in the spontaneous hypertensive rats and decreased the HA content accompanied by lowered systemic arterial compliance. HA content in the cerebral artery is reported to decrease with aging, and decreased HA is presumed to cause increased arterial wall stiffness.[9] Thus, contradictory hypotheses exist regarding the role of HA in the arterial wall: first, HA promotes arteriosclerosis, and second, HA improves the arterial compliance and protects against the progression of arteriosclerosis. Therefore, HA is deeply involved in the progression of arteriosclerosis; however, the regulatory mechanism of HA production has not been completely investigated.

In the arterial wall, the SMCs produce HA from UDP-glucuronic acid (UDP-GlcUA) and UDP-*N*-glucosamine (UDP-GlcNAC), forming a high molecular weight glycosaminoglycan chain.[10] The conspicuous feature of arteriosclerosis is the accumulation of cholesterol in the arterial wall as atheroma. Within the atheromatous lesion, oxidized cholesterol is generated.[11] Oxidized cholesterols such as 7-ketocholesterol (7KCHO) are reported to work as injurious substances causing cell death or inflammatory reaction in the arterial wall.[12] In this study, we examined the effect of 7KCHO on HA production, and the mechanism involved.

Among the three HAS, HAS2 mainly produces high molecular weight HA that is involved in the tissue hydrodynamics and viscoelasticity of the arterial wall.[6] We thus investigated the effect of 7KCHO on the expression of HAS2 to elucidate how HA synthesis is regulated by 7KCHO.

2. Materials and Methods

2.1. Cells and reagents

7KCHO ($C_{27}H_{44}O_2$) and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cultured human aortic SMCs (FC-0015, cell strain number 01293) were purchased from Kurabo Industries (Osaka, Japan) and were grown in Humedia-SG2 (HSG2) culture medium (Kurabo Industries, Osaka, Japan) supplemented with 5% fetal bovine serum (FBS; GE Healthcare Life Sciences, UT, USA).

2.2. Cell culture

Human aortic SMCs (passage 7-8) were seeded at a density of 10×10^4 cells/well in a 6-well plate and incubated in HSG2 for 24 h. Thereafter, the medium was switched from HSG2 medium to Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% FBS for 48 h. Cultured SMCs were treated with 7KCHO at indicated concentrations for various durations (1, 24, and 48 h). For studying the effect of reactive oxygen species (ROS), the cells were incubated with NAC for 1 h before 7KCHO treatment.

2.3. Cell number

Cells were seeded in triplicate in 12-well microplates at a density of 5×10^4 cells/well. After culturing for 24 h in HSG2 medium, the medium was switched to DMEM with 5% FBS and 7KCHO (25, 50, or 100 μM) was added. The 7KCHO-treated cells were incubated for 24 h. After culturing, the cells were trypsinized and suspended in PBS. The cell count in the single-cell suspensions was measured using a hemocytometer.

2.4. Measurement of HA

Cells were seeded and the medium was switched to DMEM with 5% FBS after 48-h culture, as described above. The cells were treated with 7KCHO and incubated. Then the cells were trypsinized and disrupted by sonication. The supernatants were assayed for HA concentration using the Quantikine® Hyaluronan ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (GloMax®-Multi Microplate Reader, Promega Corporation, Fitchburg, WI, USA).

2.5. Measurement of radical oxygen species

Human SMC cultures were incubated with 50 μM 2', 7'-dichlorodihydrofluorescein diacetate (Invitrogen Corp., Waltham, MA, USA) for 30 min, washed, trypsinized, and suspended in phosphate buffered saline (PBS). Thereafter, 20,000 cells were counted and the intracellular ROS were measured by fluorescence-activated cell sorting (FACS Callibur; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at excitation wavelength of 488 nm and emission wavelength of 650 nm.

2.6. Reverse transcription polymerase chain reaction (RT-PCR) for hyaluronic acid synthase 2 (HAS2) messenger ribonucleic acid (mRNA)

Total RNA was extracted from SMCs using the RNeasy Kit (Qiagen, Hilden, Germany), and reverse transcribed using the TaKaRa RNA PCR Kit (AVM) (Takara Bio Inc., Shiga, Japan) and a GeneAmp® PCR System 9700 (Applied Biosystems, Waltham, MA, USA). The HAS2 gene was

amplified on a Step One Plus Real Time PCR System (Applied Biosystems, Waltham, MA, USA) using the TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA). The following human TaqMan gene expression assays were used: HAS2 (Hs00193435_ m1), and the housekeeping gene human 18S rRNA (4319413E). The cycling conditions were: 20 s at 95℃, followed by 40 cycles of 1 s at 95℃ and 20 s at 60℃. The amount of HAS2 mRNA was normalized by dividing the averaged sample value by the averaged 18S rRNA value.

2.7. Western blot analysis of HAS2

Western blot analysis was used to determine the relative protein expression of HAS2 in human SMCs. Cells were suspended in a lysis buffer containing 10 mmol/l tris (hydroxymethyl) aminomethane hydrochloride [Tris-HCl (pH 7.5)], 150 mmol/l NaCl, 0.5% Triton R X-100 (The Dow Chemical Co., Midland, MI, USA), 0.5 mmol/l phenylmethyusulfonyl fluoride, and 1 mmol/l EDTA at 4℃ for 4 h. After centrifugation at 12,000 rpm, the supernatants were collected as samples. Samples were diluted in equal volumes of electrophoresis sample buffer containing 100 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, and 5% b-mercaptoethanol. After boiling for 5 min, the samples were electrophoresed on 10% SDS-polyacrylamide gel. Proteins were transferred onto PVDF membranes (Immobilon-P, Merck Millipore, Billerica, MA, USA). After blotting, the membranes were washed with Tris buffer saline [TBS; 100 mM Tris-HCl (pH 7.5), 0.9% NaCl], blocked with 5% bovine serum albumin in TBS, and then briefly washed in TBS containing 0.1% Tween-20 (TTBS). The membranes were incubated with primary antibodies to HAS2 (goat polyclonal IgG diluted1: 200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or actin (mouse monoclonal IgG diluted 1: 1000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4℃ overnight. After washing with TTBS, the blot was incubated with antibody against goat IgG (1:1000) for HAS2 or against mouse IgG (1:1000) for actin at room temperature for 1 h and was then washed with TTBS. The proteins were visualized by photo detection.

2.8. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using Student's t-test, ANOVA, or two-way factorial ANOVA. All analyses were performed using JMP version 8.0 (SAS Institute, NC, USA). *P* values <0.05 were considered as statistically significant.

3. Results

3.1. Effects of 7KCHO on HA synthesis in human SMCs

We investigated the effect of 30 μM 7KCHO (as there is no difference between the effects of 0 μM and 25 μM 7KCHO; Fig. 1B) on HA synthesis in human aortic SMCs (Fig. 1A). In the untreated SMCs, HA synthesis increased in a time-dependent manner. Treatment with 7KCHO significantly inhibited the HA synthesis at 24 and 48 h after treatment compared to the untreated control and to 1 h treatment.

The dose-effect of 7KCHO on HA synthesis and cell numbers were examined after treatment for 24 h (Fig. 1B and C). 50 and 100 μM 7KCHO significantly decreased the HA production compared to no 7KCHO treatment (Fig. 1 B); however, the numbers of human SMCs did not change after 7KCHO treatment at all the concentrations tested (Fig. 1C).

3.2. Effects of 7KCHO and NAC on ROS production in the human SMCs

Flow cytometric analysis was performed to measure ROS in human SMCs. The histograms in Fig. 2A compare the amount of ROS in SMCs under various treatments. In SMCs exposed to 7KCHO, the peak indicated a rightward shift from the control, and the shift increased in a dose-dependent manner (Fig. 2A). These results indicated a dose-dependent stimulation of the intracellular ROS production by 7KCHO. In contrast, cotreatment with NAC shifted the peak leftward from the 7KCHO-induced peak, and the shift increased depending on the dose of NAC (Fig. 2B). These results indicated that NAC inhibited the 7KCHO-stimulated increase in ROS.

3.3. Effect of 7KCHO and NAC on the HA synthesis and HAS2 mRNA and protein expression in SMC

Furthermore, we investigated the effect of NAC on 7KCHO-induced inhibition of HA synthesis in human SMCs. Decreased production of HA induced by 7KCHO (30 μM) was recovered by NAC treatment in a dose-dependent manner (Fig. 3A).

To investigate the mechanism of 7KCHO-induced inhibition of HA synthesis, we measured both mRNA and protein expression of HAS2, a key enzyme of HA synthesis. Treatment with 7KCHO alone or together with NAC did not change the mRNA or protein expression of HAS2 in human SMCs (Fig. 3B and C).

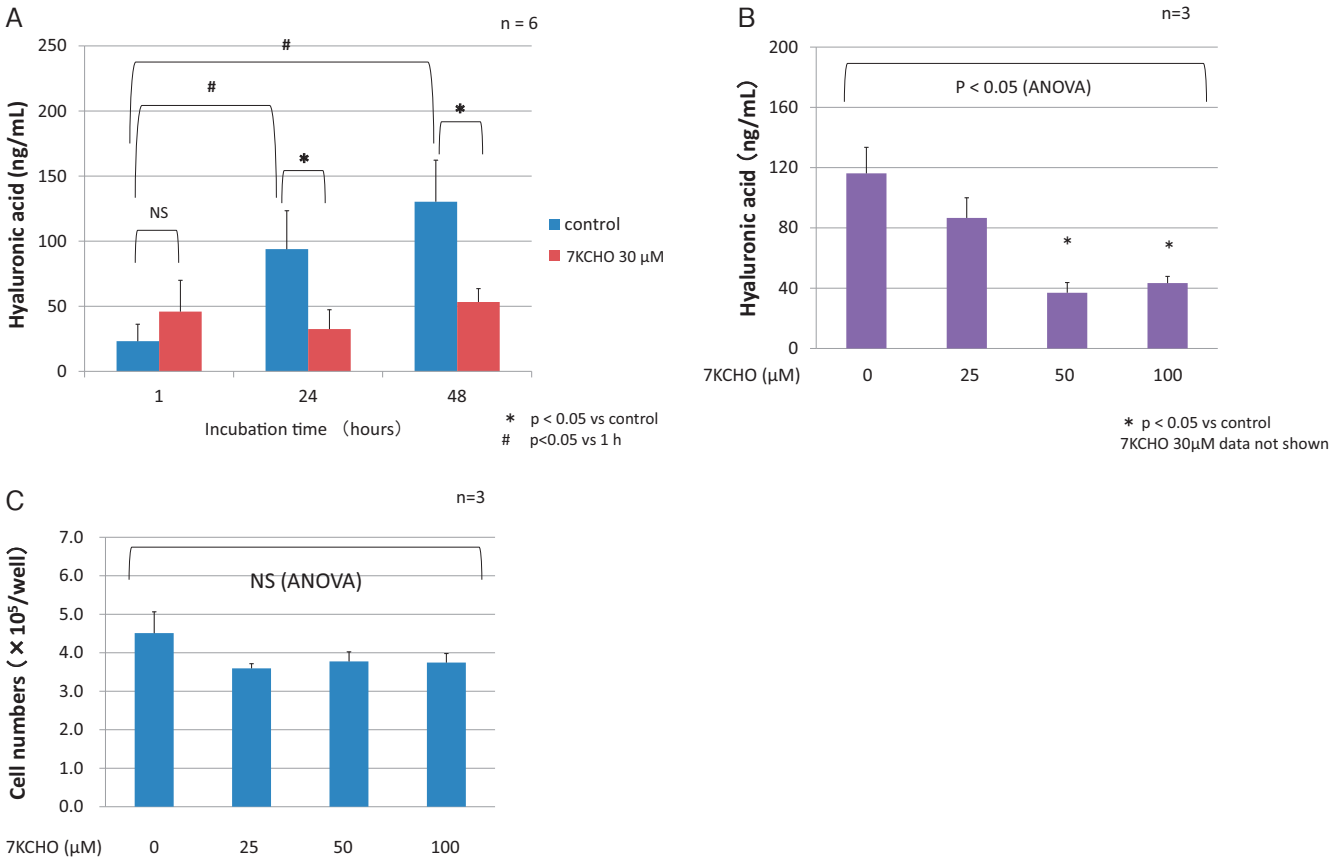

Fig. 1 Effect of 7-ketocholesterol (7KCHO) on hyaluronic acid (HA) production (A and B) and cell number (C) in the human SMC cultures.

A. SMCs were treated with 30 μM 7KCHO for 1, 24, or 48 h, and the HA concentrations in the supernatants of sonicated cells were measured. Values are expressed as mean ± SD ($n = 6$). *$p < 0.05$ vs. control, #$p < 0.05$ vs. 1 h, by two-way factorial ANOVA. B. SMCs were treated with 25, 50, or 100 μM 7KCHO for 24 h. HA concentrations were measured as described above. Values are expressed as mean ± SD ($n = 3$). *$p < 0.05$ vs. control, by ANOVA followed by Student's t-test. C. SMCs were treated with 25, 50, or 100 μM 7KCHO for 24 h. Cell numbers were determined using a hemocytometer. No significant changes were detected.

4. Discussion

As discussed earlier, HA is crucial in maintaining the viscoelasticity of the arterial wall. The arterial elasticity decreases with aging[12]; one possible reason is the decrease in the amount of HA.[9]

We attempted to elucidate the regulatory mechanism of HA production related to the progression of arteriosclerosis, using cultured human SMCs. We investigated the effect of 7KCHO, one of the most toxic oxidized cholesterol in the atheromatous lesion, on HA production in the cultured SMCs. 7KCHO dose-dependently inhibited the production of HA in the cultured SMCs as presented in Fig. 1 A and B; however, it did not change the cell numbers of SMC as presented in Fig. 1C. These results suggest that 7KCHO in the atheromatous lesion inhibits the synthesis of HA in the arterial wall. Thus, the process of the progression of arteriosclerosis is speculated as follows. In atheromatous lesion, the deposited cholesterol is oxidized and 7KCHO is generated, which inhibits the synthesis of HA, leading to loss of arterial wall viscoelasticity and an increase in the arterial stiffness.

To investigate the mechanism by which 7KCHO inhibits the production of HA, we studied the role of the antioxidant NAC in the cultured SMCs, and the results are presented in Fig. 2A and B. Treatment of SMCs with 7KCHO generated ROS, and addition of NAC inhibited the 7KCHO-stimulated ROS generation. Moreover, NAC restored the 7KCHO-induced decrease in the HA production, as presented in Fig. 3A. These results suggest that 7KCHO stimulates the ROS production and the ROS generated decreased HA synthesis.

Furthermore, the effects of 7KCHO and NAC on the expression of HAS2 mRNA and protein expression were

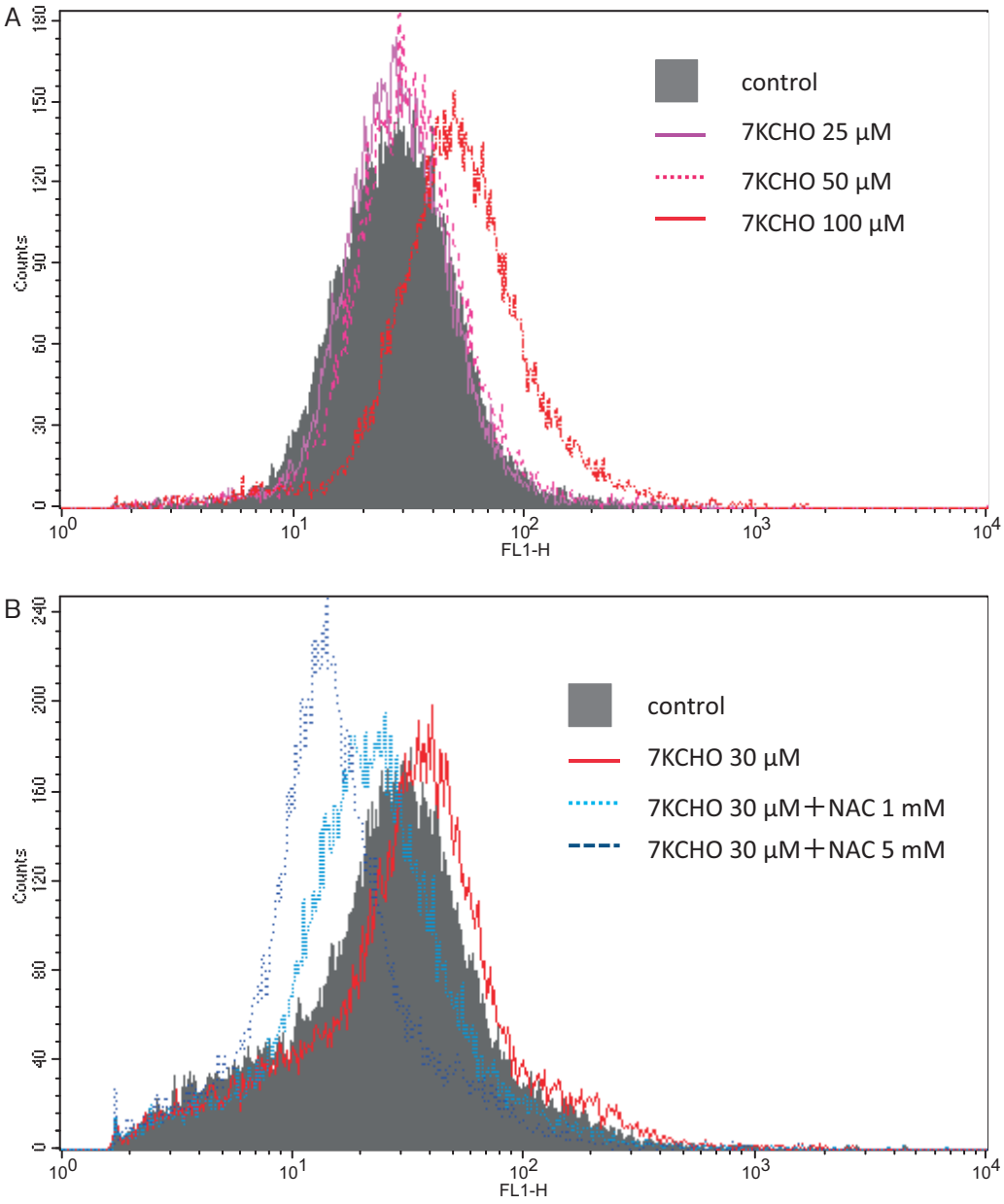

Fig. 2 ROS in 7KCHO exposed human smooth muscle cell (SMC) cultures.
A. SMCs were incubated with 25, 50, or 100 μM 7KCHO. Thereafter, 20,000 cells were counted and ROS was measured by fluorescence-activated cell sorting. 7KCHO treatment causes a leftward swift of the peak. B. SMCs were incubated with 30 μM 7KCHO alone or together with 1 or 5 mM NAC. Cotreatment with NAC causes a rightward shift of the peak.

studied. As presented in Fig. 3B, the addition of 7KCHO and/or NAC did not change the HAS2 mRNA or protein expression, indicating that the decreased HA synthesis was not due to a decrease in the expression of HAS2, but presumably due to the interference with the reaction process or posttranslational process of this enzyme. The influencing HA synthesis is the posttranslational modification of HAS proteins and the traffic of HAS to the plasma membrane. In particular, on phosphorylation, ubiquitination, and O-GlcNACylation of HAS, the posttranslational modifications can modify its enzymatic activity. The traffic of HAS to the plasma membrane may also be coupled to its posttranslational modifications; however, insufficient data is available regarding this.[2] HA synthesis consumes large quantities of UDP-GlcUA and UDP-GlcNAC, the substrates for HAS enzymes. The cellular concentration of

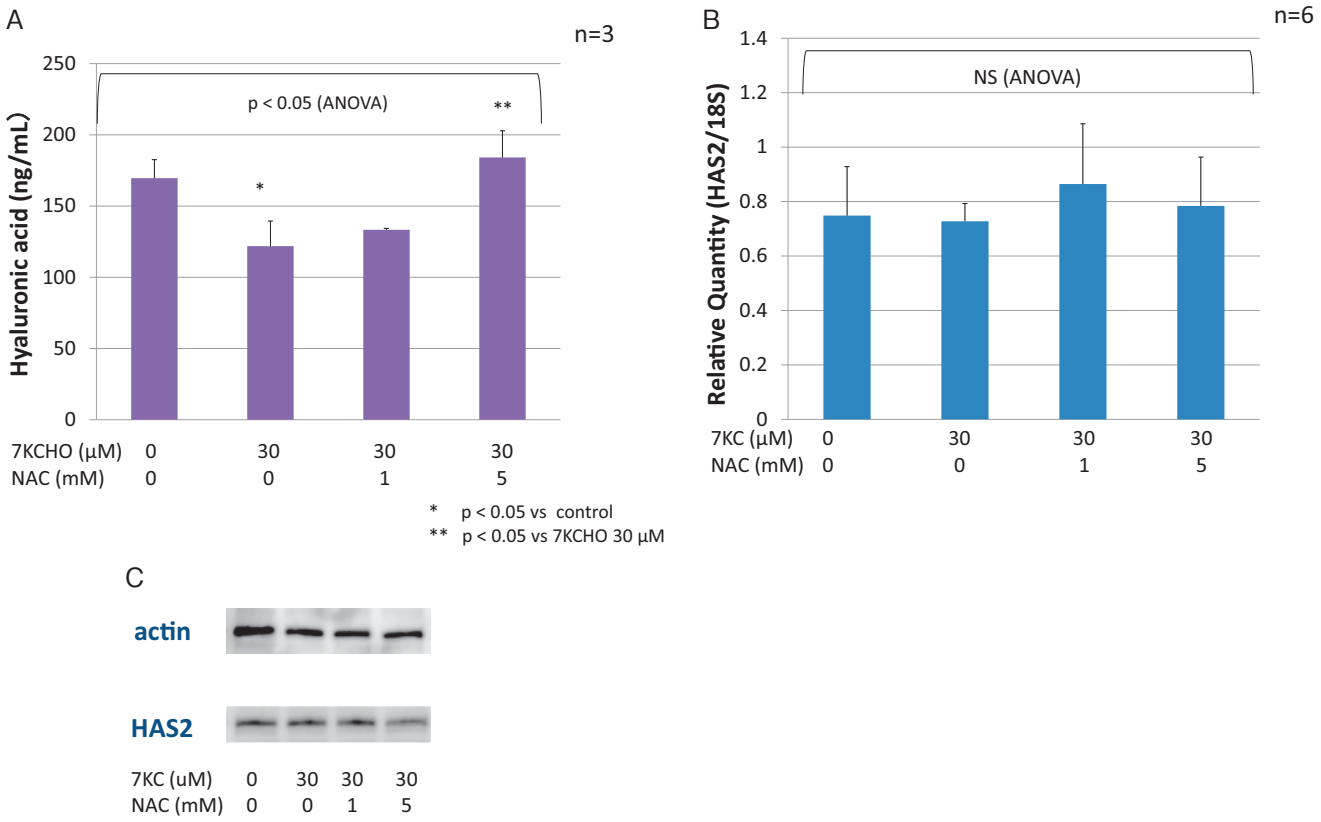

Fig. 3 Effects of cotreatment with 7KCHO and NAC on HA secretion (A) and mRNA (B) and protein (C) expression of HAS2 in the human SMC cultures.
A. Arterial SMCs were treated with 30 μM 7KCHO alone or together with NAC 1 or 5 mM for 24 h, and then sonicated. HA concentrations in the supernatants were measured. Values are expressed as mean ± SD ($n = 3$). *$p < 0.05$ vs. control, **$p < 0.05$ vs. 30 μM 7KCHO, by ANOVA followed by Student's t-test. B. Arterial SMCs were treated with 30 μM 7HCHO alone, or together with NAC 1 or 5 mM for 1 h. Expression of HAS2 mRNA in SMC was detected by RT-PCR. Values are expressed as mean ± SD ($n = 6$). C. Arterial SMCs were treated as described in A. Expression of HAS2 protein in SMCs was detected by Western blot analysis. No differences were detected.

either of these UDP-sugars can become limited in the HA synthesis.[13]

Considering the role of HA in the progression of arteriosclerosis, HA has been reported to promote vascular SMC proliferation and migration,[5] and it was hypothesized that the interstitial HA in the plaque matrix promotes the progression of arteriosclerosis expansion. The contradictory roles of HA in the progression of arteriosclerosis may be due to the heterogeneity in the molecular size of HA. Relatively low molecular HA (2×10^5 to 2×10^6 Da) is involved in the proliferation of SMCs, whereas high molecular HA ($>2 \times 10^6$ Da) is involved in the viscoelasticity of the tissue.[6] Therefore, we studied HAS2 that synthesizes high molecular HA.

This study has several limitations. First, we studied only HA synthesis. Further studies should investigate the degradation of HA. Although we examined the mRNA expression of hyaluronidase 1 in the SMCs, the enzyme was not detectable. Second, the *in vivo* models were not studied to verify the pathophysiological effects.

5. Conclusion

Treatment with 7KCHO inhibited the synthesis of HA in the cultured SMCs, presumably by stimulating ROS production. 7KCHO may inhibit the HA production by interfering with the reaction process or posttranslational processing. The present findings suggest that the 7KCHO-induced inhibition of HA production in atheromatous lesion may be involved in the enhancement of arterial wall stiffness and progression of arteriosclerosis.

Conflicts of interest: Ichiro Tatsuno received payments for lectures given to Takeda Pharmaceutical Co., Ltd. and Mochida Pharmaceutical Co., Ltd.

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