The plant alkaloid conophylline inhibits matrix formation of fibroblasts

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Key words: extracellular matrix, fibroblast, TGFβ, hyaluronan, collagen, fibrosis, conophylline, vinca alkaloid, antifibrotic, stellate cell

ABSTRACT

Conophylline is a vinca alkaloid from leaves of the tropical plant Ervatamia microphylla and has been shown to mimic the effect of the growth and differentiation factor activin A on pancreatic progenitor cells. However, whereas activin A stimulates fibrosis of pancreatic stellate cells, conophylline inhibits it, suggesting that this compound may serve as an antifibrotic drug. Here, we investigated the effects of conophylline on human foreskin fibroblasts, especially focusing on extracellular matrix (ECM) proteins. A gene microarray analysis revealed that conophylline remarkably suppressed expression of the gene for hyaluronan synthase 2 (HAS2) and of its antisense RNA, whereas the expression of collagen genes was unaffected. Of note, immunostaining experiments revealed that conophylline substantially inhibits incorporation of versican and collagens into the ECM in cells treated with
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Conophylline inhibits matrix formation of fibroblasts by transforming growth factor β (TGF-β), which promotes collagen synthesis, but not in cells not treated with TGF-β. Moreover, a protein biosynthesis assay disclosed that conophylline decreases collagen biosynthesis, concomitant with a decrease in total protein biosynthesis, indicating that conophylline-mediated inhibition of fibrosis is not specific to collagen synthesis. Conophylline neither affected TGF-β–induced nuclear translocation of SMAD family member 2/3 (SMAD2/3) nor phosphorylation of SMAD2. However, conophylline substantially inhibited phosphorylation of extracellular signal–regulated kinase 1/2 (ERK1/2), suggesting that conophylline inhibits HAS2 expression via TGF-β–mediated activation of the ERK1/2 pathway. Taken together, our results indicate that conophylline may be a useful inhibitor of ECM formation in fibrosis.

INTRODUCTION

Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process (1). There, stromal fibroblasts become activated and transdifferentiated into myofibroblasts, and these cells synthesize high levels of the extracellular matrix (ECM) molecules including collagens. In response to injury including wound healing, fibrosis occurs as scarring (2). When fibrosis occurs in organs such as lung and liver, excessive connective tissue in stroma obliterates the architecture and interferes with their function, leading to serious diseases. In addition, some chemical agents used for disease treatment, such as bleomycin, are known to cause fibrosis as a side effect (3). Therefore, understanding of the mechanisms of fibrosis and its regulation have been general and important issues in the field of clinical medicine.

Conophylline (CNP) is a vinca alkaloid extracted from leaves of the tropical plant Ervatamia microphylla (4). This compound was initially found to mimic the effect of activin A on the differentiation of pancreatic progenitor cells (5). It induces differentiation of pancreatic progenitor cells into insulin-producing β-cells and converts cultured ductal cells to β-cells in vitro (5) and in vivo (6). Interestingly, whereas activin A upregulates the expression of α-smooth muscle actin (αSMA) and collagens of pancreatic stellate cells (PSC) toward pancreatic fibrosis (7), CNP suppresses their expression (5). CNP inhibits progression of non-alcoholic steatohepatitis by inhibiting fibrosis (8). These results suggest that CNP may serve as an anti-fibrosis drug.

Here, we investigated the effects of CNP on behavior of human foreskin fibroblasts (NB1RGB). Our microarray analysis revealed that CNP remarkably suppressed hyaluronan synthase 2 (HAS2) expression, leading to a decrease in
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hyaluronan (HA). CNP inhibited collagen biosynthesis by a decrease in total protein synthesis. Further analysis suggested that CNP inhibits the TGFβ-mediated pathway especially to ERK1/2 but not the Smad2/3 pathway.

RESULTS

Initially, we treated growing and confluent NB1RGB fibroblasts with different concentrations of CNP and examined its cytotoxicity (Figure 1). Under a growing phase, CNP at a concentration of 0.1 µg/mL and higher decreased the cell number, which became apparent as early as day 2 after the treatment (Figure 1B). At day 5, the cells treated with 0.1 µg/mL CNP decreased to ~40% that of non-treated cells (Figure 1C). In contrast, treatment with 0.025 µg/mL CNP in growing phase showed only 20% inhibition of cell proliferation at day 5 (Figure 1C). When cells were plated at confluence and further cultured, the MTT levels slightly increased, indicating that cells slightly proliferated and piled up. When CNP was added to confluent NB1RGB cells, the cell number decreased at 0.025 µg/mL and higher concentration, which became apparent as early as day 2 (Figure 1B). At 0.1µg/mL CNP, the decrease became obvious at day 5 (Figure 1C). By observation under a microscope, cells were viable and attached on the dishes. In addition, both Trypan Blue-exclusion test and alamarBlue® assay showed no clear cytotoxicity (Supplementary Figure S3). These results indicate that CNP inhibits cell proliferation without cytotoxicity at least up to 0.1µg/mL. Therefore, we treated cells with CNP at 0.025 or 0.1 µg/mL in the following experiments.

To detect specific molecules whose expression is affected by CNP treatment, we performed microarray analysis. When total 27,958 genes were analyzed, 39 genes were upregulated >5-fold and 53 genes were downregulated <0.2-fold after 6 hours of CNP treatment in the presence or absence of TGFβ (Figure 2A). Panther classification analysis (PCA) showed that the 22% of CNP-suppressed genes encodes the protein related to heterotrimeric G-protein signaling pathway-Gq alpha and G0 alpha mediated pathway (e.g. BDKRB1, RASGRP1, SSTR1 and BDKRB2) and 11% of them encodes the protein related to inflammation signaling pathway (e.g. IL6 and NFATC4) (Figure 2B). In addition, the PCA by cellular component showed that CNP downregulates expression of membrane-related genes including HAS1 and HAS2 (Figure 2C).

Furthermore, the PCA by molecular function showed that CNP downregulates expression of genes encoded catalytic activity (23%, e.g. DGKI, HAS1, HAS2 and NOX4), receptor activity (23%, BDKRB1, IL7R, IL21R and SSTR1) and binding activity (39%, ADAMTS4, CXCL12 and NFATC4).
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(Figure 2D). Accordingly, the raw fluorescence intensities of both HAS1 and HAS2, which encode hyaluronan synthesizing enzymes, significantly decreased after CNP treatment compared with no treatment (Figure 2E). These results suggest that CNP downregulates expression of HAS genes in NB1RGB fibroblasts.

To validate the microarray results, we performed qRT-PCR. The levels of HAS2 expression dramatically decreased in 3 hours after treatment with CNP at either 0.025 or 0.1 µg/mL (Figure 3A). As HAS2 expression is regulated by its antisense RNA (HAS2 AS), we also examined its expression levels, and found its substantial decreases (Figure 3B). As CNP was initially found to mimic the effect of activin A, we examined whether activin A inhibits HAS2 expression or not. Interestingly, activin A showed little effect on HAS2 expression, suggesting specific inhibition by CNP (Figure 3C).

By erythrocyte exclusion assay, pericellular HA coat was observed in both TGFβ- treated and nontreated cells (Figure 3D, left panels, arrowhead). CNP treatment at either concentration of 0.025 or 0.1 µg/mL abrogated pericellular HA coat, in both TGFβ-treated and nontreated cells (Figure 3D, middle and right panels). When HA amounts in the cell lysate/ECM and conditioned media after three days of culture were measured using ELISA, significant decreases of HA levels in the conditioned media and a trend, not statistically significant ($p = 0.3$, n=6), of decrease in HA deposition, correlated with CNP concentrations, were observed in both TGFβ-treated and non-treated samples (Figure 3E, F). Interestingly, TGFβ slightly increased HA deposition in 3 days of culture (Figure 3F).

Formation of pericellular HA coat requires HA-binding molecules, including versican, TSG-6, pentraxin-3, and Inter-α-trypsin inhibitor (ITI) (9-11). By immunofluorescence staining, versican expression was substantially upregulated by TGFβ-treatment. In both treated and non-treated samples, versican was localized along with fibers, and was not accumulated in the pericellular coat (Figure 3G). Whereas CNP had little effect on versican deposition of cell culture without TGFβ treatment, it substantially inhibited its deposition with TGFβ treatment (Figure 3G, H). Our microarray data revealed little effect of CNP on expression of TSG6 and pentraxin-3, although TGFβ substantially up-regulated expression of TSG6 and down-regulated that of pentraxin 3 (Supplementary Figure S1).

We further mined the transcriptomic data for the effects of CNP on glycolytic pathways genes, including those for production of UDP-sugar precursors. No genes related to HA metabolism, except for HAS1 and HAS2, showed over 3-fold changes of expression (Supplementary Table S1).
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Taken together, a decrease in HA secretion in CNP-treated cells is mainly due to remarkable reduction in HAS expression.

As CNP was reported to inhibit fibrosis of pancreatic tissue (5), we investigated the effects of CNP on collagen secretion and its incorporation into the ECM. By immunofluorescence staining, CNP treatment substantially diminished type I collagen incorporation into the ECM (Figure 4A, upper panels). TGFβ treatment increased collagen incorporation into the ECM. Under this condition, CNP treatment dramatically decreased its incorporation (Figure 4A lower panels). Quantification of the staining intensity confirmed the decreased levels, with statistical significance, of collagen incorporation observed under the microscope (Figure 4B). We also investigated expression of type III collagen, known to be involved in fibrosis. Immunofluorescence staining displayed essentially the same patterns with lower staining levels than type I collagen (Figure 4C, D). Specificity of immunostaining patterns for collagens was confirmed by immunostaining using non-immune IgG in place of primary antibodies (Figure 4E). Immunoblot analysis confirmed that CNP substantially inhibited type I collagen incorporation (Figure 4F). By Sircol™ assay, CNP at both 0.025 and 0.1 µg/mL did not affect collagen levels in the cell lysate and the ECM of cell culture without TGFβ treatment.

When co-treated with TGFβ, CNP inhibited them (Figure 4G). Taken together, these results clearly indicate that CNP suppresses incorporation of collagens into the ECM, which is more remarkable when cells are treated with TGFβ.

Next, we labeled fibroblasts with [3-H] proline and examined the effects of CNP on biosynthesis of collagens and total proteins. The levels of total protein synthesis decreased by treatment with CNP at concentrations of both 0.025 and 0.1 µg/mL by ~40%. TGFβ stimulation up-regulated protein biosynthesis by 50%, and under such conditions, CNP treatment inhibited total protein biosynthesis in a similar manner to that of un-stimulated cells, i.e., by ~35% and 50% at 0.025 and 0.1 µg/mL, respectively (Fig. 4H, left). The ratio of collagen per total proteins in non-stimulated cells was ~2% in both CNP treated and non-treated cells. That in TGFβ-stimulated cells was ~6%, which was unaffected by CNP treatment (Fig. 4H, right). These results suggest that CNP inhibits total protein biosynthesis, and its inhibition is not specific to collagen.

We further mined the transcriptomic data for the effects of CNP on collagen gene expression, which confirmed that CNP has no specific effects on collagen expression (Supplementary Table S2).

The fact that CNP inhibited collagen incorporation more strongly when the cells were treated with TGFβ (Figure 4) suggests that CNP
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Inhibits TGFβ-mediated pathways. TGFβ facilitates differentiation of fibroblasts toward myofibroblasts that actively synthesize collagens. Therefore, we examined whether CNP affects the TGFβ-mediated pathway and the differentiation. When double-stained for total Smad2/3 and αSMA, Smad2/3 was mainly localized in the cytoplasm in all the TGFβ non-treated cells. When treated with TGFβ, Smad2/3 was translocated to the nuclei in all the samples, regardless of CNP treatment. Without TGFβ treatment, CNP did not affect the number of αSMA-positive cells. When treated with TGFβ, the number of αSMA-positive cells increased at 24 h, which decreased by CNP treatment (Figure 5A, B). These results indicate that CNP inhibits TGFβ-mediated differentiation of fibroblasts toward myofibroblasts.

The results shown in Figure 5 suggested that CNP has little effect on Smad2/3 signal transduction. When analyzed by western blot, TGFβ-treatment substantially increased phosphoSmad2, and CNP at both 0.025 and 0.1µg/mL had little effect on phosphorylation of Smad2 (Figure 6A, upper panel and graph), confirming the results of Smad2/3 nuclear translocation (Figure 5A). Next, we investigated other signaling pathways mediated by TGFβ. CNP inhibited phosphorylation of ERK1/2 at both 0.025 and 0.1µg/mL. TGFβ treatment substantially inhibited it, and combined treatment with CNP further inhibited the phosphorylation in a dose dependent manner (Figure 6A, second panel and graph). CNP inhibited phosphorylation of p38 MAPK in a dose dependent manner. TGFβ substantially inhibited it, however, combined treatment with CNP did not affect it (Figure 6A, third panel and graph).

To investigate whether RAS-ERK1/2 pathway was suppressed by CNP treatment, we analyzed the microarray data, using the GSEA software program, v2.2.4, and Molecular Signatures Database (Broad Institute). The representative GSEA enrichment plot and corresponding heatmap image of the indicated gene set for the CNP-treated and untreated cells confirmed suppression of RAS-ERK1/2 pathway by CNP treatment (Figure 6B).

To examine involvement of PI3K/AKT pathway is involved in CNP effects, we analyzed the transcriptomic data, and found that CNP slightly inhibits expression of AKT2 and AKT3, but not AKT1 (Supplementary Table S3). When expression of four representative genes downstream of PI3K/AKT pathway was examined, we observed no remarkable changes by CNP treatment (Supplementary Figure S2). Western blot analysis demonstrated little effect on phosphorylation of Akt by CNP (Figure 6A, lower panel and graph).
DISCUSSION

In this study, we investigated the effects of CNP, vinca alkaloid that mimics activin A function, on fibroblast behavior and formation of the ECM. The interesting features of this study were as follows: (a) CNP dramatically inhibits HAS 1, HAS 2, and HAS2 AS expression, resulting in a decrease in HA secretion. (b) CNP inhibits cell proliferation and biosynthesis of collagens at the same ratio with total protein biosynthesis, (c) CNP inhibits versican incorporation into the ECM, (d) CNP inhibits TGFβ-mediated differentiation of fibroblasts toward myofibroblasts, (e) whereas CNP does not affect Smad2/3 signaling, it inhibits ERK1/2 activation, which may be responsible for CNP action. Taken together, these findings compellingly indicate the inhibitory effects of CNP on ECM formation by fibroblasts.

Our microarray analysis revealed marked decreases in HAS1 and HAS2 expression by CNP treatment among ECM related genes. In addition, the HAS2AS was substantially reduced by the treatment, which was validated by qRT-PCR. The measurement of HA in cell lysate and the ECM, and erythrocyte exclusion assay confirmed its decreased levels by CNP treatment. HAS2 transcription is regulated by a variety of signal transduction pathways involving as many molecules (12-14). Among them, both EGF-growth family factors and G protein-coupled receptors (GPCRs) are known to be major molecules that regulate HAS2 expression. EGFs increase HAS2 expression (13). Early growth response-1 (EGR-1) induces CD44v6, which then sustains ERK signaling, upregulating HAS2 expression (15). GPCRs act via protein kinase A and its downstream CREB1 binds to its response elements in HAS2 promoter, inducing HAS2 transcription (16-19). We have shown that CNP inhibits ERK1/2 phosphorylation. Our microarray data using the GSEA software program confirmed suppression of RAS-ERK1/2 pathway by CNP. G protein-coupled receptors (GPCRs)/protein kinase A/CREB1 cascade upregulates HAS2 transcription (19). Our gene ontology analysis of microarray data shows CNP profoundly inhibits the G protein-mediated pathways (Figure 2B). Therefore, it is likely that CNP inhibits both EGF-mediated and GPCR-mediated pathways, leading to downregulation of HAS2 transcription. Although PI3K/AKT pathway has been reported to upregulate HAS2 expression (14), our microarray analysis did not suggest involvement of this pathway (Supplementary Figure S2, Supplementary Table S3). HAS2AS1 forms a duplex with HAS2 mRNA, and stabilizes the HAS2 transcript (20). In addition, HAS2AS1 binds to O-GlcNAcylated p65 and induces HAS2 transcription (12, 21). Reduced HAS2AS levels by CNP treatment may prompt inhibition of HAS2...
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Incorporation of HA into the ECM involves HA-binding ECM molecules, such as versican. We have shown that TGFβ substantially increases versican expression and CNP inhibits its incorporation into the ECM, without affecting its transcription. Whereas versican deposition decreased to ~60% by CNP treatment, the HA amount in the cells/ECM and the conditioned medium decreased only slightly. This is consistent with our previous results that mouse embryonic fibroblasts (MEFs) with ~20% expression levels of versican exhibit ~85% levels of HA in the ECM (26). These observations suggest that versican is not prerequisite for HA deposition. Interestingly, these MEFs exhibit sustained HA-mediated signaling and attain premature senescence. Similarly, CNP treatment may alter HA-mediated signaling. Pericellular HA coat formation involves HA-binding molecules including TGS6, pentraxin 3 (11), heavy chains of ITI (10), and versican (9). Our microarray analysis revealed that CNP has little influence on expression of TSG6 and pentraxin 3. Therefore, these molecules are unlikely to affect HA coat sizes of CNP-treated fibroblasts. Recently, versican G1 fragment but not full-length versican forms aggregates with HA and heavy chains of ITI in the pericellular matrix (27), which agrees with our observation that intact versican was not accumulated in pericellular matrix.

Whereas CNP was reported to inhibit fibrosis of pancreas (7), our microarray analysis showed no significant differences in mRNA levels of collagens. Our biosynthesis assay revealed that TGFβ upregulates biosynthesis of total proteins including collagens and that CNP inhibits protein biosynthesis both in the presence and absence of TGFβ (Fig. 5A). Although TGFβ treatment increased the ratio of collagens per total proteins, CNP had little effect on the ratio, which agrees with our microarray results.

By both immunostaining and the Sircol™ assay, the inhibition of collagen deposition by CNP was statistically significant only when treated with TGFβ, although there was tendency of inhibition in the absence of TGFβ. In contrast, when treated with TGFβ to facilitate collagen synthesis, CNP substantially inhibited its deposition, suggesting that the inhibitory activity of CNP on collagen levels is mainly exerted via TGFβ-mediated pathway. CNP altered collagen fiber structure from diffuse fine fibers to broader woven fibers with more spaces among fibers. A previous study showed a similar collagen fiber patterns by inhibiting HA deposition, using 4-Methylumbelliferone (4-MU), HA oligosaccharide, and hyaluronidase (28). Formation of woven collagen fibers by CNP treatment may be due to a decrease in HA
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deposition.

We demonstrated that CNP did not affect the Smad2/3 signal transduction pathway, but did both ERK1/2 and p38MAPK pathways. CNP inhibited ERK1/2 phosphorylation in both the presence and the absence of TGFβ, whereas CNP inhibited p38 phosphorylation only in its absence. These inhibition patterns suggest that the inhibitory effects of CNP is operated via ERK1/2 signaling pathways. As ERK1/2 upregulates cell proliferation (29) and HAS2 gene expression by activating its downstream CREB, the inhibitory effects of CNP are likely due to downregulation of ERK1/2 pathway. Crosstalk between TGFβ-signaling and ERK1/2-signaling has been reported (30, 31). EGF treatment has been shown to inhibit basal and TGFβ-induced expression of type I collagen and αSMA (31), contrary to our observations. Two signaling pathways may interact differently, dependent on expression of other molecules, cell types and culture conditions. Previously, CNP was reported to inhibit cell proliferation and collagen secretion via the p38 MAPK pathway in rat pancreatic stellate cells (4, 7, 32), and mainly via JNK and partly via both ERK1/2 and p38 pathways in Lx-2 hepatic stellate cells (33). The effects of CNP may be mediated by different MAPK pathways in various types of fibroblastic cells.

Our results indicate that CNP affects MAPK pathways but not the Smad2/3 pathway, which facilitates COLIA2 transcription. This supports our observation that CNP did not inhibit collagen biosynthesis in a specific manner. CNP was first identified as a compound that shares the receptor with activin A, but interestingly, it inhibits fibrosis whereas activin A facilitates it (5). Whereas activin A binds to its receptor and induces Smad2/3 signal transduction similar to TGFβ (34-37), CNP had little effect on the Smad2/3 pathway. The pathway-specific inhibitory effects of CNP eliminate its competitive inhibition as a ligand with activin A and TGFβ. It may modify the receptor complex or inhibit the function of intervening molecules between TGFβ receptors and MAPK.

In this study, we have demonstrated that CNP inhibits fibroblast proliferation and ECM formation, decreasing incorporation of HA, versican, and collagens, without cytotoxicity. CNP may be used as an inhibitor of ECM formation under pathological conditions, where excess ECM formation is involved in several pathological processes, such as liver and lung fibrosis, keloid, and hypertrophic scar. CNP may be a good tool to control these pathological conditions.

EXPERIMENTAL PROCEDURES

Cell culture
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Human foreskin fibroblasts NB1RGB were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT Japan, and were grown in DMEM containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂ incubator.

Fibroblasts up to three passages were used for all the experiments.

**MTT assay, alamarBlue® assay and trypan blue exclusion test**

NB1RGB cells were plated in 96 wells at growing and confluent phases and cultured for 48 hours. The medium was replaced with new one, CNP (final concentrations of 0.025, 0.1, 0.15, 0.1, 0.2, and 0.3 μg/mL) was added and the cells were cultured for 24, 48, and 120 hours. Then, MTT (Sigma-Aldrich) dye solution was added to each well and incubated for 4 hours, and absorption at 570 nm was determined with an automatic ELISA plate reader (Multiskan; Thermo Electron, Vantaa, Finland) (38). NB1RGB cells at 60% confluence were treated with CNP for days as indicated. AlamarBlue® assay (Invitrogen) was performed according to the manufacturer’s instruction. For trypan blue exclusion test, after staining with 5 mg/mL trypan blue for 1 min, the number of both stained and unstained cells was counted under a microscope.

**Microarray analysis**

Cells were plated at 80% confluence and cultured for 16 hours, CNP at each concentration (0.025, 0.1 μg/mL) was added and the cells were incubated for 1 hour. TGFβ (hereafter, final concentration of 10 ng/mL) was then added, and cells were cultured for 6 hours. After washing with PBS, cells and extracellular matrix were collected and applied to microarray analysis. We performed a comprehensive gene expression analysis and compared the results among TGFβ + and -, and CNP + and – groups, using a Human Gene Expression 4 x 44K Microarray chip (G4845A, Agilent Technologies, Santa Clara, CA, USA), which can examine 27,958 genes. For the analysis of the gene expression profiling, total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction. The quality of the isolated RNA was ascertained using a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific).

The experimental procedure for cDNA microarray analysis was performed according to the manufacturer’s instruction (Agilent Technologies). In brief, cDNA synthesis and cRNA labeling with cyanine 3 (Cy3) dye were performed using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies).

The Cy3-labeled cRNA was purified, fragmented, and hybridized on a Human Gene Expression 4 × 44 K Microarray chip containing 43,377 oligonucleotide probes with the Gene
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Expression Hybridization kit (Agilent Technologies). After incubation at 60 °C for 17 hours, the microarray slide was washed and scanned using an Agilent DNA microarray scanner (Agilent Technologies).

The scanned data were quantified using the Feature Extraction software program (version 11.0.1.1, Agilent Technologies). The signal intensities were then normalized as previously described (39). Gene expression changes were detected as follows: the difference of the normalization value = {averaged normalization value of CNP-treated cells (0.025 µM and 0.1 µM) - untreated cells}, an up-regulated gene was determined by a difference from the normalization value >0.55 (fold change >5.0), a down-regulated gene by a difference from the normalization value < -0.55 (fold change < -0.2). The raw and normalized microarray data have been submitted to GEO database at NCBI (Accession number GSE104813; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106401).

Data mining was performed using a pathway database, REACTOME (https://reactome.org/what-is-reactome).

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Cells were plated in 35 mm-dishes (cell density, $1 \times 10^5$ cells/dish) and were incubated for 24 h at 37°C. Then, CNP was added at the aforementioned concentrations. In other experiments, activin A (R&D Systems) at a concentration of 10 ng/mL was added. After 0, 3, 6 and 12 hours, cells were washed with PBS and collected, and total RNA was extracted with RNeasy Mini Kit™ (QIAGEN), and with 2 µg of total RNA, cDNA was synthesized using SuperScript VILO™ cDNA Synthesis Kit (QIAGEN). qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems) and Power SYBR PCR Master Mix (Life Technologies Japan) according to the manufacturer’s protocol. The oligonucleotides used for PCR were: HAS-f, GTCATGTACACACCTTCAGAGC; HAS2-r, ACAGATGAGGCTGGGTCAAGCA; HAS2AS-f, TCGAATAAAGCTGGAAATGATGC; HAS2AS-r, GATGTCAAAAACCTGAAAGGGAT; GAPDH-f, TGCACCACCAAACCTGCTTAC; GAPDH-r, GGCATGGACTGTGGTCATGAG. The levels of PCR products were determined with StepOnePlus™ equipment (Life Technologies Japan) and analyzed using the accessory software. The relative abundance of transcripts was normalized to the GAPDH mRNA level.

Erythrocyte exclusion assay

Assessment of pericellular HA coat was performed, as described previously (40). Cells were plated in 35 mm dishes (cell density, $3 \times 10^4$
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cells/dish), cultured for 2 days, and treated with CNP and/or TGFβ for additional 2 days. Then, conditioned medium was removed, and glutaraldehyde-fixed rabbit erythrocytes were added. After 15 min, the cells were observed by phase-contrast microscopy.

Quantification of HA levels

Cells were plated in 35 mm dishes at 60% confluence (~cell density, 1 × 10^5 cells/dish) and cultured for 24 hours. Then, after washing twice with PBS, the medium was replaced with DMEM without FBS and incubated for 16 hours. Then, CNP and TGFβ were added in the same manner as above, and the cells were cultured for 3 days. The conditioned medium was collected. After washing with PBS twice, cells together with the extracellular matrix were recovered with CytoBuster Protein™. Hyaluronan was quantified using a HA assay kit (COSMO Bio CO, LTD).

Immunocytochemistry

Cells were plated onto a cover glass in a 6-well plate at 60% confluence and cultured overnight.

After replacing the medium, CNP at the concentration described above was added or not, and cultured for 1 hour. Then, TGFβ was added or not, and cells were cultured for 3 days. After removal of the medium, the cells were fixed with 4% paraformaldehyde for 20 min, rinsed with PBS for 5 min three times, and blocked with 10% BSA for 30 min at RT. After washing with PBS for 5 min three times, the cells were treated with mouse anti-human versican (1:500, 2B1, Seikagaku), rabbit anti-human type I collagen (1:200, LSL, LB-1196) or rabbit anti-human type III collagen (1:200, LSL, LB-1393) overnight at 4 °C. After washing, they were treated with Alexa-594 conjugated goat anti-mouse or goat anti-rabbit antibody for 1 hour at RT. After washing with PBS for 5 min three times, the nuclei were stained with DAPI (WAKO chemical) for 5 min. After washing briefly, the coverslip was flipped over and mounted onto a slide glass. The cells were then observed under a fluorescent microscope (Keyence, BZ-9000).

Sircol™ assay

Cells were plated in 35 mm dishes at 60% confluence (~cell density, 1 × 10^5 cells/dish) and cultured for 24 hours. Then, the medium was replaced with fresh one, and CNP and TGFβ were added in the same manner as above, and the cells were cultured for 3 days. After washing with PBS twice, cells together with the extracellular matrix were recovered, and their collagen concentration was measured using the Sircol™ Assay kit (BioSite) according to the manufacturer's protocol.

Analysis of collagen biosynthesis

Analysis of collagen biosynthesis was carried out as previously described (41). Cells were plated at 80% confluence in a 35-mm dishes (cell
density, $1.3 \times 10^5$ cells/dish) and cultured for 16 hours, until confluence. The medium was replaced with 1 mL DMEM containing 10% FBS and CNP at each concentration (0, 0.025, 0.1 μg/mL), 0.5 mM β-aminopropionitrile (Tokyo Kasei). Two hundred μM L-ascorbyl phosphate magnesium phosphate n-hydrate (Wako Pure Chemicals) was added and incubated for 1 hour. Then, TGFβ (final concentration of 10 ng/mL) was added, and cells were cultured. In the control dishes, cells were cultured in the absence of TGFβ. After 2 hours, cells were labeled with 10 mCi [3-H] proline for 24 hours. Then both cells and conditioned medium were collected, sonicated. Two hundred mL of 0.5% BSA and 50 mL of 0.2% gelatin were added to 400 mL suspension, and the sample was boiled for 10 min. After cooling down on ice, the sample was dialyzed against 5 mM acetate, and lyophilized.

The lyophilized sample was dissolved in 950 mL NaOH, sonicated, and neutralized by 2 M Tris-HCl pH 7.5. The sample was then aliquoted into 100 mL. One was treated with 400 mg/mL collagenase (Advance Biofactures Corporation, Form III) at 37 °C for 4 hours. Another was left untreated by addition of collagenase solvent. Then, 250 mL 0.5% tannic acid-10% trichloroacetate was added, and the sample was placed on ice for 15 min, centrifuged for 5 min and the supernatant was processed to liquid scintillation counting (COL).

Five hundred mL 0.5% SDS-5mM DTT was added to the precipitate, and the sample was sonicated, boiled for 5 min, and processed to liquid scintillation counting (NCP). Collagen biosynthesis (%) was calculated by the formula $100 \times \text{COL}/\text{COL} + 5.4 \times \text{NCP}$.

**Western blotting**

Cells were plated at 60% confluence (cell density, $1 \times 10^5$ cells/dish) for 24 hours, and then medium was replaced with DMEM without FBS, and incubated for 16 hours. CNP was added or not, and after one hour, TGFβ was added or not. After 30 min, cells were collected as samples. After measurement of protein amounts using MicroBCA kit (Thermo Fisher Scientific), samples with the same protein amount were subjected to SDS-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.05% Tween-20 (TBST), treated with indicated antibodies diluted in CanGetSignal™ Solution I overnight at 4 °C. After washed with TBST, the membrane was incubated with a goat anti-rabbit polyclonal antibody conjugated to peroxidase (Cappel, 1:1000) diluted in CanGetSignal™ Solution II for 1 hour. After washing with TBST, the bands were detected with amplified...
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chemiluminescence (ECL) using LAS4000 (GE Healthcare). The band density was measured using ImageJ (NIH). The antibodies used are:
rabbit monoclonal antibodies against total Smad2/3 (1: 1000; CST), phosphoSmad2 (1: 1000; CST), total ERK1/2, phosphoERK1/2 (1: 1000; CST), total p38 (1: 2000; CST), phospho-p38 (1: 2000; CST), and rabbit polyclonal antibodies against phosphoAkt1 (1:1000; CST) and total Akt (1:1000; CST).

For western blot of type I collagen, cells were plated in 35 mm dishes at 90% confluence (cell density, $1.5 \times 10^5$ cells/dish), and cultured for 16 hours. Then cells were treated with or without CNP and TGFβ as above, and cultured for 72 hours. After washing with PBS twice, cells together with the extracellular matrix were collected and applied to western blot analysis, as above. The primary antibody used is rabbit anti-human type I collagen (1:1000, LSL, LB-1196).

Statistical analyses

Data were presented as the means ± standard deviation (S.D.). Statistical analyses were performed by post hoc tests (Bonferroni) using ANOVA. Probability values of 0.05 were considered statistically significant.

Acknowledgments: We would like to thank Ms. K. Ota for technical assistance, and Drs. Nagai and Tsuchimoto for critical reading of the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest regarding the contents of this article.

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FOOTNOTES:

This work was supported in part by a Grant-in-Aid (KAKENHI) for Scientific Research (B, Grant number 25293096 to H.W.), Grant-in-Aid (KAKENHI) for Young Scientists (B, Grant number 15K19561 to A.O.), Grant-in-Aid (KAKENHI) for Scientific Research (C, Grant number 25460395 to S.K.).

The abbreviations used are: ECM, extracellular matrix; CNP, conophylline; αSMA, alpha-smooth muscle
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actin; HAS2, hyaluronan synthase-2; HAS1, hyaluronan synthase-1, HA, hyaluronan; ITI, inter-α-trypsin inhibitor; EGR-1, early growth response-1; GPCRs, G protein-coupled receptors; MEFs, mouse embryonic fibroblasts.

FIGURE LEGENDS

Figure 1. Effects of CNP on the viability of NB1RGB cells. The cells in growing phase (left) and at confluence (right) were treated with CNP at the concentrations as indicated for 24 h (A), 48 h (B), and 120 h (C) and viability was assessed by MTT. The experiment was performed twice, with essentially the same results.

Figure 2. Gene expression analysis. NB1RGB cells were cultured for 16 hours, and treated with CNP or not. After one hour, cells were treated with TGFβ(Tβ) or not, and cultured for additional 6 hours. Next, total RNA from CNP-treated and untreated cells was extracted and subjected to a cDNA microarray analysis using a Human Gene Expression 4 x 44K Microarray chip (Agilent Technologies). (A) Heatmap of upregulated genes (39 genes; fold change, >5.0) and downregulated genes (53 genes, fold change <0.2) after CNP treatment in the presence or absence of TGFβ. The heatmap was constructed using normalized values for each sample and the Treeview software. The corresponding gene names are annotated on the right. (B-D) Gene ontology analyses by using the Panther classification system. The upregulated and/or downregulated genes were classified using PANTHER - Gene List Analysis (www.pantherdb.org/). Pie chart showing the percentages of genes classified into each molecular pathway (B), cellular component (C) and molecular function (D). (E) The graphs show the differential gene expression between CNP-treated cells and untreated cells. Raw fluorescence values obtained by scanning were utilized for comparison of gene expression of HAS2 and GAPDH. *P <0.01 and **P <0.001, significant difference.

Figure 3. Expression of HAS2 and HAS2AS, hyaluronan secretion and coat formation, and versican localization. (A, B) Expression levels, assessed by qRT-PCR, of both HAS2 (A) and HAS2AS (B) of the cells treated with CNP for indicated times are shown (n=3, mean + SD, p < 0.05). (C) Expression levels of HAS2 of the cells treated with activin A are shown (n=3, mean +SD). (D) NB1RGB cells, treated with CNP and TGFβ as indicated, under a particle exclusion assay are shown. (E, F) HA levels in cells/the ECM (E) and conditioned medium (F) measured by ELISA are shown (n=3, mean + SD, p < 0.05). (G)
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Immunostaining patterns of versican in cultured fibroblasts treated with CNP and TGFβ as indicated are shown. (H) Quantification of versican by measurement of the pixels of the immunostaining pictures (mean + SD, of ten fields, $p < 0.05$). The experiments were performed twice (for C, E, F), three times (for A, B, G, H) or four times (for D), with essentially the same results. The representative results are shown.

**Figure 4. Incorporation into the ECM and biosynthesis of collagen by NB1RGB cells treated with CNP and TGFβ.** (A) Immunofluorescent staining for type I collagen. Cells were cultured for 72 h in the presence of CNP and TGFβ as indicated, and then immunostaining was performed. (B) Quantification of collagen by measurement of the pixels of the immunostaining pictures (mean + SD, of ten fields, $p < 0.05$). (C) Immunofluorescent staining for type III collagen. Cells were cultured and treated as above, and then immunostaining was performed. (D) Quantification of collagen by measurement of the pixels of the immunostaining pictures (mean + SD, of ten fields, $p < 0.05$). (E) Negative control of immunostaining, where non-immune rabbit IgG was used in place of primary antibody. Picture was taken with longer exposure. (F) Western blot for type I collagen. Cells were treated with CNP and TGFβ as above, and cells/ECM were collected and applied to western blot. The bands of alpha 1 and alpha2 chains of type I collagen are pointed ($\alpha_1$, $\alpha_2$). The number below the panel indicates the level of band density with non-treated sample as 100. (G) Quantification of collagen by Silcol™ assay (n=3, mean + SD, $p < 0.05$). (H) Biosynthesis of total proteins and collagens. Cells were treated with CNP and/or TGFβ, and then labeled with [3H]-proline for 24 h. Radioactivity incorporated in the cells was measured. Total protein levels (left, n=3, mean + SD, $p < 0.05$) and collagen synthesis levels (%) of total proteins (right) are shown. The experiments were performed twice (for G), three times (for C, D, F, H) or five times (for A, B, E), with essentially the same results. The representative results are shown.

**Figure 5. Differentiation toward myofibroblasts and Smad2/3 nuclear translocation.** Cells were cultured for 24 h in the presence of CNP and TGFβ as indicated, and immunostaining for Smad2/3 (red) and αSMA (green) was performed, and nuclei were stained with DAPI (blue) (A). Note that Smad2/3 nuclear translocation by TGFβ is not affected by CNP treatment. (B) The ratio of αSMA-positive cells per total cells are indicated by a graph (B, mean + SD, of ten fields, $p < 0.05$). The immunofluorescence staining was performed five times, with essentially the same results. The representative picture panels are shown.
Figure 6. Signal transduction pathways affected by CNP. (A) Immunoblot panels for Smad2/3, ERK1/2, p38 MAPK, and Akt, together with their phosphorylation forms are shown. The line in the pAkt and tAkt panels indicates a splice where the same image was reordered so that the data are presented in the same order as the other panels in this figure. Right graphs are quantification data of their band density. Each experiment was performed five times, with essentially the same results. Statistical analysis was performed using the results of all the experiments. (B) Suppression of RAS-ERK signaling by CNP. The GSEA was conducted using the GSEA software program, v2.2.4, and Molecular Signatures Database (Broad Institute). All raw data were formatted and applied to all GO gene sets (C5). Representative GSEA enrichment plot and corresponding heatmap image of the indicated gene set is shown for the CNP-treated and untreated cells, respectively. Genes contributing to the enrichment are shown in rows, and the samples are shown in columns on the heatmap. Expression is shown as a gradient from high (red) to low (blue). FDR, false discovery rate; NES, normalized enrichment score.
Figure 1

A

Optical Density (570 nm)

CNP (µg/mL)

Growing phase  Confluent phase

B

Optical Density (570 nm)

CNP (µg/mL)

Growing phase  Confluent phase

C

Optical Density (570 nm)

CNP (µg/mL)

Growing phase  Confluent phase
Figure 2

A

B

Pathway

Up-regulated genes [FC > 5.0]

Down-regulated genes [FC < 0.2]

C

Cellular Component

Up-regulated genes

Down-regulated genes

D

Molecular Function

Up-regulated genes

Down-regulated genes

E

HAS1

HAS2

GAPDH

Raw fluorescence value

None CNP

None CNP

None CNP

None CNP
Figure 4

A

CNP (μg/mL) 0 0.025 0.1

TGFβ

(-)

(+)  

B

CNP (μg/mL) 0 0.025 0.1

TGFβ

(-)

(+)  

C

D

E

F  

G

H

Ratio of type I collagen α1 chain
Figure 5

A

CNP (µg/mL) 0 0.025 0.1

αSMA

Smad2/3

Merge

αSMA

Smad2/3

Merge

B

αSMA positive ratio (%)

CNP (µg/mL) 0 0.025 0.1

TGFβ (-) 0 0.025 0.1

TGFβ (+) 0 0.025 0.1

** **
Figure 6

A

|          | TGFβ (-) | TGFβ (+) |
|----------|----------|----------|
| CNP (μg/mL) | 75kDa | 75kDa |
| 0 | 0.025 | 0.1 |
| 75kDa | 50kDa | 50kDa |
| 0 | 0.025 | 0.1 |

Phospho-Smad2

Smad2/3

Phospho-ERK1/2

ERK1/2

Phospho-p38

p38

Phospho-Akt

Akt

B

Enrichment plot:
GO_REGULATION_OF_RAS_PROTEIN_SIGNAL_TRANSDUCTION

NES: -1.90
FDR: 4.32%

CNP (+) vs CNP (-)

| Gene | NES | FDR |
|------|-----|-----|
| PLEKHG5 |     |     |
| RAPGEF1 |     |     |
| RASA1 |     |     |
| FOXM1 |     |     |
| GPR56 |     |     |
| NGEF |     |     |
| ABR |     |     |
| SPRY1 |     |     |
| ITPKB |     |     |
| ARHGEF2 |     |     |
| F2R |     |     |
| ARHGEF4 |     |     |
| VAV3 |     |     |
| ARHGDHB |     |     |
| RASGRF2 |     |     |
| SSX2IP |     |     |
| CUL3 |     |     |
| NRG1 |     |     |
| NRG1 |     |     |
| ARHGEF5 |     |     |
| DGKI |     |     |
| RASGRP1 |     |     |
The plant alkaloid conophylline inhibits matrix formation of fibroblasts
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*J. Biol. Chem.* published online October 30, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.005783

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