A Novel Mechanism for the Inhibition of Hyaluronan Biosynthesis by 4-Methylumbelliferone

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Running Title: Inhibition of HA synthesis mediated by MU

Key words: hyaluronan, biosynthesis, synthase, 4-methylumbelliferone, UDP-glucuronosyltransferase

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SUMMARY

Specific inhibitors of hyaluronan (HA) biosynthesis can be valuable therapeutic agents to prevent cancer invasion and metastasis. We have previously found that 4-methylumbelliferone (MU) inhibits HA synthesis in human skin fibroblasts and in group C Streptococcus. In this paper, the inhibition mechanism in mammalian cells was investigated using rat 3Y1 fibroblasts stably expressing HA synthase 2. Exposure of the transfectants to the inhibitor resulted in significant reduction of HA biosynthesis and matrix formation. The evaluation of HAS transcripts and analysis of cell-free HA synthesis demonstrated the posttranscriptional suppression of HAS activity by MU. Interestingly, the posttranscriptional suppression of HAS activity was also observed using p-nitrophenol, a well known substrate for UDP-glucuronyltransferases (UGT). We investigated whether the inhibition was exerted by the glucuronidation of MU using both HPLC and TLC analyses. The production of MU-GlcUA was consistent with the inhibition of HA synthesis in HAS transfectants. MU-GlcUA was also detected at a similar level in control cells, suggesting that the glucuronidation was mediated by an endogenous UGT. Elevated levels of UGT significantly enhanced the inhibitory effects of MU. In contrast, the inhibition by MU was diminished to the control level when an excess of UDP-GlcUA was added to the cell-free HA synthesis system. We propose a novel mechanism for the MU-mediated inhibition of HA synthesis involving the glucuronidation of MU by endogenous UGT resulting in a depletion of UDP-GlcUA.
INTRODUCTION

There are a considerable number of reports showing that the biosynthesis of hyaluronan (HA) is elevated in disorders such as fibroses of organs, diseases associated with inflammation, and some types of tumors including mesothelioma and Wilm’s tumor (1-5). For instance, accumulation of HA is associated with the progression of atherosclerosis (6). During the progression of hepatitis, HA derived from the Ito cells accumulates in the liver, causing fibrosis and eventually cirrhosis of the liver (7). Since HA is directly associated with liver fibrosis it has long been utilized as a marker for the diagnosis of chronic hepatitis (8). Also, an exponential increase of HA in the endocervical canal at inappropriate stage of pregnancy can result in a miscarriage (9). Recent genetic approaches showed that overproduction of HA accelerated tumor growth and is associated with cancer metastasis (10-14).

HA is a non-sulfated linear glycosaminoglycan composed of thousands of repeating units of GlcNAc-β(1→4)-GlcUA-β(1→3) (15). In vertebrates, this molecule is a ubiquitous component of the extracellular matrix and play critical roles in dynamic functions such as embryonic development, tissue regeneration and cell migration (16). Both eukaryotic and prokaryotic HA synthases (HAS) catalyze the transglycosylation from both UDP-GlcUA and UDP-GlcNAc donors (17). Following the first cloning of a HAS gene from Streptococcus in 1993, three distinct mammalian isoforms, HAS1, HAS2, HAS3 have been identified and characterized from mouse, human, and other species (17). Considerable progress in understanding HA biosynthesis and its biological functions has been made in recent years.

Identification of a specific inhibitor for HA biosynthesis would not only help elucidate the functions of HA but would also have applications in clinical medicine for the treatment of diseases caused by elevated levels of this glycosaminoglycan. Over the past few decades many
researchers have attempted without success to discover specific inhibitors of HA synthesis in mammalian cells (18-22). 4-Methylumbelliferone (MU, 7-hydroxy-4-methyl-2H-1-benzopyran-2-one) was previously found to inhibit HA synthesis in cultured human skin fibroblasts but had no effect on the synthesis of any other glycosaminoglycan (23, 24). Since then MU has been used as an inhibitor of HA synthesis in many studies on the functions of HA, although its precise mechanism has not been established in mammalian cells (10, 25, 26).

For many years MU has been used safely in human medicine as a cholagogue by oral administration (27). The clinical application of MU for controlling HA synthesis could potentially prevent malignant alteration of cancer cells and fibrosis of organs. It would therefore be helpful to clarify the inhibition mechanism of MU in mammalian cells. The information may also be useful in developing new compounds which are more effective inhibitors and/or display lower cytotoxicity than MU. A possible phospholipid-dependent inhibition mechanism of MU was found using group C Streptococcus in our preceding paper (28). We suggested that MU treatment may inhibit HAS activity by altering the distribution of cardiolipin species surrounding HAS in the plasma membrane. However, the change in the distribution of cardiolipin cannot by itself account for the observed inhibition of HA synthesis. Furthermore the proposed mechanism of inhibition involving cardiolipin might be specific for Streptococcus, since mammalian cells have low levels of cardiolipin in the plasma membrane. Indeed the effect of cardiolipin on enzymatic activity is distinct between mammalian HAS1 and streptococcal HAS (29), suggesting that the MU-mediated inhibition is quite complex. In this study we demonstrate a UGT-dependent inhibition mechanism for HA synthesis in mammalian cells using transfectants that express mouse HAS2. We propose the inhibition of HA synthesis is due, in part, to a depletion in the pool of UDP-GlcUA, a common substrate of HAS and UGT.
EXPERIMENTAL PROCEDURES

Materials and Reagents. MU was purchased from Wako Pure Chemicals (Osaka, Japan). 

$p$-Nitrophenol ($p$NP), $p$NP-sugars ($p$NP-Glc, $p$NP-GlcUA, $p$NP-GlcNAc), and MU-sugars (MU-Glc, MU-GlcUA, MU-GlcNAc) were from Sigma (St. Louis, MO). MU and MU-sugars were dissolved in dimethylsulfoxide (DMSO), $p$NP and $p$NP-sugars were dissolved in ethanol, and the final concentration of these vehicles in the culture medium and reaction mixtures for the cell-free HA synthesis were adjusted to 0.1%. UDP-$[^{14}C]$ GlcUA (313 mCi/mmol) and $[^{14}C]$ $p$NP (70 mCi/mmol) were from ICN Biomedicals, Inc. (Irvine, CA) and American Radiolabeled Chemicals Inc. (St. Louis, MO), respectively. UDP-GlcUA, dithiothreitol (DTT) and ATP were from Nakarai Tesque (Kyoto, Japan). UDP-GlcNAc, bovine liver β-glucuronidase and glutaraldehyde-stabilized sheep erythrocytes were from Sigma (St. Louis, MO). Streptomyces hyaluronidase was obtained from Seikagaku Corporation (Tokyo, Japan). Hyaluronic Acid “Chugai” quantitative test kit for the sandwich binding protein assay was purchased from Chugai Pharmaceutical (Tokyo, Japan) (30). Recombinant human UGT1A6 and UGT1A7 proteins were from Calbiochem (La Jolla, CA) and their expression vectors were reported previously (31).

Cell Culture and Transfection. Stable transfectants were established by transfection of mouse HAS2 and control vector into rat 3Y1 cells as described previously (32). Cells were routinely cultured in DMEM containing 10% FCS and 400 µg/ml of G418 at 37 °C. pFLAG-HAS2 and human UGT1A6 expression vector were transiently transfected into COS-1 cells by electroporation as described previously (32). The cells were cultured in DMEM containing 10% FCS and 2 mM L-glutamine at 37 °C.
**Particle exclusion assay.** 3Y1-HAS2 cells plated at $1 \times 10^3$ cells in a 35 mm dish were cultured for 2 days and then further cultured for a day with or without 300 µM MU. An aliquot of glutaraldehyde-stabilized sheep erythrocytes ($3 \times 10^8$) in PBS was then added to the culture medium. After 15 min, the culture was observed using an inverted phase-contrast microscope (OLYMPUS IMT-2) (33).

**Quantitative Analyses of the HAS Transcripts.** The relative level of HAS expression in the HAS2 transfectants was determined by real-time quantitative RT-PCR as described previously (11). The gene-specific PCR primers and probes were designed from the mouse HAS2 and rat HAS sequences using the Primer Express software (Applied Biosystems, Foster City, CA). The sequences of the various oligonucleotides were: the forward primer for mouse HAS2 was 5’-CCTCGGAATCACAGCTGCTTATA-3’, and the reverse primer was 5’-CTGCCACTTTACTGCTGAATA-3’; the probe for mouse HAS2 was 5’-TCGCATCTCATCATAACAAAGCCTCTTTG-3’; the forward primer for rat HAS1 was 5’-GGAGATGTGAGAATCCTTAACCCTC-3’, and the reverse primer was 5’-TGCTGGCTCAGCCAACGAAGGAA-3’; the probe for rat HAS1 was 5’-CAGAGCTACTTTTCAGCTGTGCTCTGCATC-3’; the forward primer for rat HAS2 was 5’-CCTCGGAATCACAGCTGCTTATA -3’, and the reverse primer was 5’-CTGCCCATGACTTCACTGAAGA -3’; the probe for rat HAS2 was 5’-TCACACCTCATCATCCAAAGCCTCTTTG -3’; the forward primer for rat HAS3 was 5’-GGTACCATCAGCGAAGCAGC-3’, and the reverse primer was 5’-GAGGAGAATGTTCCAGATGCG-3’; the probe for rat HAS3 was 5’-TGGCTACCGGACTAAGTATACAGCAGC-3’. Total RNA was isolated from subconfluent...
rat 3Y1 cells expressing mouse HAS2 using the RNeasy Mini kit (Qiagen, Valencia, CA). Two hundred nanograms of total RNA was used for real time RT-PCR and subsequent analysis. The reaction master mix was prepared to give final concentrations of 1x TaqMan EZ buffer, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.6 mM dUTP, 6 mM manganese acetate, 0.01 U/µl uracil N-glycosylase, 0.1 U/µl rTth DNA polymerase, 200 nM primers and 100nM TaqMan probe. The conditions for RT-PCR were as follows; 1 cycle at 50 °C for 2 min, 1 cycle at 60 °C for 30 min, 1 cycle at 95°C for 5 min, 50 cycles at 95 °C for 20 s, and 60 °C for 1 min. Fluorescent signals generated during PCR amplifications were monitored in real time using the 7700 sequence detector system (Applied Biosystems) and analyzed with the sequence detector 1.7 program (Applied Biosystems). The relative amount of GAPDH mRNA was measured using the TaqMan rodent GAPDH detection reagents (Applied Biosystems). The amount of HAS mRNA was divided by the amount of GAPDH mRNA in each sample. The normalized values were designated as the "relative expression coefficient" in this study. Standard curves were generated by serial dilution of total RNA isolated from HAS2 transfectants.

**Determination of the HA concentration by ELISA-like Assay.** Cells were plated at a density of 1 x 10^5 and 8 x 10^5 cells/well in a 6-well plate (defined as exponentially growing phase and confluent phase, respectively). The cells were cultured with various concentrations of MU for 24 h. HA released into the culture medium was quantified by an ELISA-like assay using HA binding protein (HABP) according to the manufacture’s instructions for the Hyaluronic Acid “Chugai” quantitative test kit (30). The quantity of HA was expressed per live cell number. The viability of cells was assessed by trypan blue staining.
HA Synthase Assay. HAS activity was monitored in the cell-free HA synthesis system using UDP-[\(^{14}\)C] GlcUA and UDP-GlcNAc as donors and a membrane-rich fraction of the transfectants as an enzyme source as described previously (32). Briefly, the HAS transfectants were washed, harvested, and disrupted by sonication in 10 mM Hepes-NaOH, pH 7.1, 0.5 mM dithiothreitol containing 0.25 M sucrose. Suspensions of the disrupted cells were ultracentrifuged in a Beckman TLS rotor at 43,000 rpm for 1 h to give the high-speed pellet. The crude membrane fractions prepared from HAS transfectants were resuspended with standard reaction mixture (0.1 ml of 25 mM Hepes-NaOH, pH 7.1, 5 mM DTT, 15 mM MgCl\(_2\), 0.1 mM UDP-GlcNAc, 2 \(\mu\)M UDP-GlcUA, and 0.2 \(\mu\)Ci of UDP-[\(^{14}\)C]GlcUA) and incubated at 37°C for 1 h. Depending on the type of experiment, MU, pNP and their sugar derivatives were added to the reaction mixture. Alternatively, the membrane fractions were preincubated at 37°C for 1 h with 300 \(\mu\)M of MU or 300 \(\mu\)M of MU-GlcUA in the preincubation mixture (0.1 ml of 25 mM Hepes-NaOH, pH 7.1, 5 mM DTT, 15 mM MgCl\(_2\), 0.1 mM UDP-GlcUA) and then HA synthesis was initiated by adding 2 mM UDP-GlcNAc and 0.2 \(\mu\)Ci of UDP-[\(^{14}\)C]GlcUA. The reaction was terminated at the indicated time by addition of SDS to 2\% (w/v). The mixtures were then spotted onto Whatman no. 3MM paper, and the paper was developed in 1 M ammonium acetate (pH 5.5) and ethanol (65:35 V/V) for 3 days. The origin, containing the synthesized polymers, was removed and the amount of radioactivity in the high molecular mass HA was determined by liquid scintillation counting.

Agarose Gel Electrophoresis of HA. The size distribution of radiolabeled HA synthesized in the cell-free reaction was analyzed by agarose gel electrophoresis (0.5\% gel) as described previously.
The synthesized HA was incubated at 37°C for 1 h with or without 1 TRU of *Streptomyces* hyaluronidase prior to loading on the gel. After drying the gel, the radioactive HA was detected using a BAS 5000 Bio-Imaging Analyzer (Fuji Film Co. Tokyo, Japan).

**HPLC analysis of the MU-sugar derivative.** MU-sugar derivatives, from culture conditioned medium and from cell lysate, were analyzed by HPLC using a TSK-gel ODS-120T (15 cm x 4.6 mm I.D.) column. HPLC conditions were identical to those described by Zimmerman *et al.* (34). Eluted fractions were monitored by detecting fluorescence (excitation 325 nm, emission 380 nm) using a fluorescence spectrophotometer HITACHI F-1050 (Tokyo, Japan). Cells were washed with PBS three times and then disrupted in a solution of 2% SDS-PBS by sonication for 2 min (4 bursts of 30 sec) using a sonifier (model UR-20P, Tomy Seiko, Tokyo, Japan). The cell lysate was then obtained by centrifugation at 105,000 g for 30 min. The supernatants were used for HPLC analysis. For TLC analysis in Fig. 3 and for mass spectrometry, a peak corresponding to MU-GlcUA was collected by HPLC as described below. A fraction containing MU-GlcUA was prefractionated from culture supernatant using a Sep-Pac Plus C18 Cartridge (Waters, MA) prior to HPLC. Briefly, the culture supernatant was loaded onto the cartridge and then the cartridge was washed with water. Bound materials containing MU-GlcUA were eluted with 100% methanol and concentrated using a vacuum evaporator centrifuge (Iwaki, Tokyo, Japan), and analyzed by HPLC. A peak of MU-GlcUA was collected and desalted using a Sep-Pac Plus C18 Cartridge. After drying the residue was dissolved into methanol and analyzed by TLC or mass spectrometry.

**TLC analysis of the MU-sugar or pNP-sugar derivative.** Radiolabeled MU-sugar or pNP-sugar
derivatives produced in the cell-free HA synthesis system was treated with or without 2 U of β-glucuronidase at 37°C for 1 h and then separated by TLC. TLC was performed on a silica 60 TLC plate (MERCK, Darmstadt, Germany) using 1-butanol/ethanol/water (5:3:2, v/v/v) as the mobile phase (35). After drying the plate, radioactive spots were detected using a BAS 5000 Bio-Imaging Analyzer. The radiolabeled product derived from the incubation of pNP and UDP-[14C]GlcUA according to the standard assay method of UGT (36) was used for determining the mobility of pNP-GlcUA. Fluorescent spots containing MU or MU-sugar were detected by ultraviolet irradiation using a transilluminater. Authentic MU-GlcUA was used as a standard.

Mass spectrum measurements. Mass spectra were obtained on a PE-Sciex API-100 single-quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source described previously (37). The mass spectrometer was operated in the negative mode. Samples dissolved in 50% isopropanol were ionized by electrospray ionization (ESI) and continuously infused into the ESI chamber at a flow rate of 5 μl min⁻¹.

RESULTS

Inhibitory effect of MU on HA production and HA matrix formation of the HAS2 expressing cells. MU was originally found to inhibit HA synthesis and HA matrix formation of cultured human skin fibroblasts (23, 24). The recent discovery that MU inhibits HA synthesis of group C Streptococcus in a phospholipid-dependent fashion provided new insight into understanding the mechanism of inhibition (28). However, the inhibition of HA synthesis by MU cannot be explained by this mechanism alone, particularly in mammalian cells. In this study, we examined the effects of MU on HA synthesis using HAS2 overexpressing cells in order to clarify which
step of HA synthesis is the target for the inhibition. The HAS2 overexpressing cells, named 3Y1-HAS2, were established from rat 3Y1 fibroblasts by transfection with HAS2 cDNA as described previously (32). Formation of pericellular HA matrices and HA production were significantly inhibited in 3Y1-HAS2 cells by treatment with MU as observed previously in cultured human skin fibroblasts (23, 24) (Fig. 1). The formation of pericellular HA matrices was inhibited by ~30% during exposure to 300 μM MU compared with the non-treated control. The level of inhibition was even more apparent at higher concentrations of MU (data not shown). HA accumulation in the conditioned medium was dose-dependently decreased by MU treatment both in the exponentially growing and confluent phases of 3Y1-HAS2 cells (Fig. 1E).

Transcription of the mouse HAS2 transgene and the endogenous HAS genes in the transfectants was assessed by real-time RT-PCR using mouse and rat specific HAS primers and probes. No obvious change in the transcriptional levels of these HAS genes was observed at the low or moderate concentrations of MU, whereas the level of the endogenous HAS2 gene was inhibited during exposure to a high dose of MU (Table I). These results suggest that MU inhibits HAS activity posttranscriptionally at the low or moderate concentrations of MU. In contrast, a high dose of MU inhibits HA synthesis by suppressing HAS functions both transcriptionally and posttranscriptionally.

**Effect of MU on HA synthesis and chain elongation in a cell-free HA synthesis system.** Cell-free HA synthesis was examined using the membrane-rich fraction of 3Y1-HAS2 cells. As shown in Fig. 2A, HAS activity was inhibited by MU in a dose-dependent manner and the inhibition reached up to 50% of the non-treated control at 300 μM MU. The inhibitory effect was observed at an early stage of HA synthesis and reached a plateau 30 min after treatment with MU (Fig. 2B).
The size distribution of HA synthesized in the cell-free system was also determined by agarose gel electrophoresis (Fig. 2C and D). The decrease in the molecular size of HA was caused by MU treatment in both a dose- and time-dependent manner. This data suggests MU inhibits HA synthesis by suppressing HAS function posttranscriptionally. Furthermore, the MU-mediated inhibition of HA synthesis may be caused by direct inhibition of HAS activity.

MU has been used as a substrate to measure the activity of UGTs, which are involved in the detoxification of phenolic compounds in mammalian cells, particularly in the liver (38). Since HAS possesses a glycosyltransferase activity for UDP-GlcUA within its polypeptide (17), we initially hypothesized that the enzyme is able to transfer GlcUA to MU from UDP-GlcUA and the glucuronidation of MU competitively inhibits chain elongation of HA. We therefore tested the effect of pNP, another acceptor for UGTs (39), on HAS activity. As shown in Fig. 2A, HAS activity was indeed inhibited by pNP.

Production of MU-GlcUA in the culture medium and in the reaction supernatant of the cell-free HA synthesis. The effects of both MU and pNP prompted us to investigate whether the glucuronidation of these compounds is involved in the inhibition of HAS activity. The production of MU-GlcUA was analyzed by HPLC using conditioned medium from 3Y1-HAS2 and 3Y1-Mock cells which were cultured for 24 h in the presence of various concentrations of MU. In the presence of MU, two major peaks appeared with a retention time of approximately 7 min and 22 min (Fig. 3A and B). The peak 2 at 22 min corresponded to that of the MU standard, whereas the peak 1 at 7 min was identified as MU-GlcUA since this coincided with that of an authentic standard sample and was sensitive to treatment with β-glucuronidase. Interestingly, a similar level of MU glucuronidation was observed in the control 3Y1-Mock cells (Fig. 3B) which
express low levels of endogenous HAS2 and HAS3. This suggests that glucuronidation was mediated by the endogenous UGT activity expressed in the 3Y1 host cells. The MU derivative in peak 1, collected from the HPLC fraction, migrated on TLC at the same position as authentic standard MU-GlcUA (Fig. 3C). To estimate the molecular weight of the MU derivative, the peak 1 observed on HPLC was collected and subjected to ion-spray mass spectrometry. The negative-ion mass spectrum of the peak 1 from 3Y1-HAS2, shown in Fig. 4, had a [M-H]\(^{-}\) ion peak at m/z, 351. Therefore, the molecular weight of the MU derivative in peak 1 was determined to be 352, which was consistent with the calculated mass for MU-GlcUA (C\(_{16}\)H\(_{16}\)O\(_{9}\); \(M_r = 352\)). The molecular ion at m/z, 351 was also determined when the peak 1 from 3Y1-mock was used. The combined results of HPLC, TLC, and ion spray mass spectrometry confirmed that the MU-derivative in peak 1 is MU-GlcUA (chemical structure is shown in Fig. 4). When 3Y1-HAS2 cells were cultured with various concentrations of MU, maximal production of MU-GlcUA was observed at 200 \(\mu\)M of MU (Fig. 3D and E) both in the culture conditioned medium and in the cell lysate. Most MU-GlcUA was secreted into the culture medium (Fig. 3D). The production of MU-GlcUA in the cell lysate was 60 fmoles/10\(^4\) cells at 200 \(\mu\)M of MU, which was much less than that in the culture conditioned medium; 17.0 nmoles/10\(^4\) cells. The secretion may be mediated by ATP-binding cassette transporters of the multidrug resistance protein (MRP) family as demonstrated in the other cell systems (40). It was difficult to estimate the ratio of the UDP-GlcUA consumed for production of MU-GlcUA, to total intracellular UDP-GlcUA. It seems, however, reasonable to assume that the amount of UDP-GlcUA, which had been consumed to produce MU-GlcUA, is equal to (or more than) the amount of the produced MU-GlcUA. Total amounts of secreted and intracellular MU-GlcUA suggested that 22.8\% and 9.4\% of loaded MU was converted to MU-GlcUA at the concentrations of 30 \(\mu\)M and 300 \(\mu\)M, respectively.
Transglycosylation of GlcUA to MU or pNP was confirmed by TLC analysis of the cell-free reaction mixture. When UDP-[\textsuperscript{14}C] GlcUA was used to monitor the glucuronidation, radiolabeled spots corresponding to authentic MU-GlcUA or pNP-GlcUA used as standards were produced by endogenous UGTs in the presence of MU or pNP, respectively (Fig. 5A and C). When UDP-GlcUA and [\textsuperscript{14}C] pNP were added to the cell-free HA synthesis, radiolabeled spots corresponding to pNP-GlcUA was also detected on TLC (Fig. 5B). The radiolabeled spots disappeared after treatment with \( \beta \)-glucuronidase, showing that GlcUA was transferred from UDP-GlcUA to MU or pNP in the cell-free HA synthesis. Our experiments suggest that HAS does not mediate the glucuronidation of MU or pNP, since MU-GlcUA and pNP-GlcUA were detected using both 3Y1-HAS2 and 3Y1-Mock cells.

**Effect of MU-GlcUA on the HAS activity.** MU and pNP derivatives of xylosides, MU-Xyl and pNP-Xyl, have been shown to act as artificial primers for the initiation of glycosaminoglycan synthesis in cultured mammalian cells (41, 42). These derivatives behave as native primers. Then the idea that MU-GlcUA and pNP-GlcUA might affect the initial step in HA elongation has struck us. To examine this possibility, MU-GlcUA or pNP-GlcUA was added to the cell-free HA synthesis. As expected, HAS activity was inhibited by MU-GlcUA or pNP-GlcUA in a dose-dependent manner (Fig. 6A and B). However, the inhibitory activity of these compounds was far less than that of aglycon, MU and pNP, suggesting that they do not directly inhibit HA synthesis. Surprisingly, similar results were also obtained using other sugar derivatives of MU or pNP, i.e. MU-GlcNAc, MU-Glc, pNP-GlcNAc, and pNP-Glc. Indeed when any of the pNP sugar derivatives were incubated with UDP-[\textsuperscript{14}C] GlcUA in the cell-free HA system, the \textit{de novo} production of radiolabeled pNP-GlcUA was detected by TLC (Fig. 6C). However, radiolabeled
pNP-GlcUA was absent when the pNP sugar derivatives were incubated without the membrane fraction (data not shown). The de novo production of radiolabeled pNP-GlcUA could be due to the liberation of pNP or MU from its respective sugar derivative, and the transfer of GlcUA to the free aglycon from UDP-GlcUA by the endogenous UGT activity. Therefore the inhibition of HAS activity may be dependent on the repeated glucuronidation of the free aglycon liberated from the sugar derivatives.

**UGTs enhanced the inhibition of HAS activity by MU.** Although our results suggested that MU-GlcUA did not directly inhibit HAS activity, the production of MU-GlcUA by the endogenous UGTs seemed to be important for inhibition. We therefore investigated whether the inhibitory effect of MU and pNP was related to glucuronidation. To test this hypothesis, we examined the effect of recombinant UGT proteins on the inhibition by MU or pNP. Increased glucuronidation of pNP was observed by TLC when a recombinant UGT1A6 protein was added to the reaction mixture of cell-free HA synthesis (Fig. 7B). Under the same conditions used in the TLC analysis, the recombinant UGT significantly enhanced the inhibitory effect of pNP on HA synthesis (Fig. 7A). In contrast, UGT did not affect the HAS activity in the absence of pNP regardless of whether it was active or not. Almost the same degree of inhibition was observed by adding UGT1A7, which is one of the isoforms, in the presence of pNP or MU (Fig. 7C). These results suggested that the inhibition of HAS activity was directly related to extent of glucuronidation mediated by UGT.

The link between the inhibition of HAS and glucuronidation was further confirmed using the transfectants overexpressing a human UGT1A6 isoform (Fig. 8). The UGT1A6 and/or HAS2 expression plasmids were transiently transfected into COS cells and the HAS activity was then
measured in the presence or absence of 100 µM MU. COS cells do not express any UGT activity, as only a trace amount of MU-GlcUA was detected by TLC in the case of the membrane-rich fraction from the Mock or HAS2 transfectants (Fig. 8B). However, a large amount of MU-GlcUA was detected when UGT1A6 was expressed in the COS cells (Fig. 8B). The system is therefore suitable for investigating the effects of MU glucuronidation on the HAS activity. When COS cells co-transfected with both UGT1A6 and HAS2 cDNA were treated with MU, HAS activity was only 30% that of the untreated control value (Fig. 8A). However, for the membrane-rich fraction from transfectants expressing HAS2 alone, only a slight decrease in HAS activity was observed in the presence of MU (Fig. 8A). HA production into the conditioned medium was also measured when these transfectants were cultured with or without MU. The results were similar to those obtained using the cell-free system (Fig. 8C). Taken together, these results suggest that the inhibition of HA synthesis by MU or ρNP is caused by the UGT-dependent glucuronidation of these compounds.

**Effect of UDP-GlcUA on the inhibition of HAS activity by MU.** The inhibition of HA synthesis may be caused by a depletion in the pool of UDP-GlcUA due to UGT-mediated MU glucuronidation. If this hypothesis is correct, we would expect HAS activity to recover after addition of an excess amount of UDP-GlcUA to the cell-free HA synthesis, even in the presence of MU. Indeed we did observe the recovery of HAS activity following addition of UDP-GlcUA in a dose-dependent manner (Fig. 9A). UDP-GlcUA was then added to the reaction mixture 1 h after the initiation of HA synthesis. The HAS activity inhibited by the MU treatment was partially rescued when the UDP-GlcUA concentration increased (Fig. 9B). We also determined whether HAS inhibition was enhanced by pretreatment of the membrane fraction with MU prior
to the initiation of the HA synthesis. If the kinetic lag shown in Fig. 2B reflects the duration for the depletion of UDP-GlcUA in the reaction mixture or formation of MU derivative inhibitor, then the preincubation would inhibit HAS activity more completely. However, time-dependent changes in the HAS activity revealed that the kinetic lag still remained and the effect was minimal at an early incubation time (Fig. 10). Furthermore, the pretreatment of membranes with MU-GlcUA had a lesser effect on HAS activity than that of MU (Fig. 10), suggesting that the HAS activity is not inhibited irreversibly by MU-GlcUA. This was also confirmed by the experiment where HAS activity was measured under the MU-GlcUA-free conditions using membrane fractions pretreated with 300 µM MU-GlcUA (data not shown). Similar result was also obtained when the membrane fractions were pretreated with 300 µM MU (data not shown).

Taken together, the results suggest that the glucuronidation event followed by the subsequent reduction of UDP-GlcUA concentration affects the HAS activity at the late stage of HA elongation.

DISCUSSION

In this study we present a novel mechanism for the inhibition of HA synthesis involving the UGT-dependent glucuronidation of MU. Although MU has been widely used as an inhibitor of HA synthesis (10, 25, 26), the exact inhibitory mechanism is not clear, especially in mammalian cells. A major problem in the elucidation of this inhibitory mechanism has been the complexity of the HA synthetic system that is regulated by three related mammalian HAS isoforms, HAS1, HAS2, and HAS3 (17). To reduce this complexity we initially established three rat 3Y1 cell lines, each expressing a distinct HAS isoform, to test whether MU inhibited HA production and matrix formation in a similar way to that observed for human skin fibroblasts (23, 24). When the cells
were cultured in the presence of MU, a decrease in both HA production and matrix formation was observed in all three transfectants, and in particular the HAS2 transfectant (data not shown). Therefore, we adopted HAS2 expressing cells to further elucidate the inhibition mechanism of MU in this study.

We had previously observed growth suppression of human skin fibroblasts when treated with a high concentration of MU. HA synthesis is known to be down-regulated in growth-arrested cells (43). Our recent study also suggested that HAS gene expression is regulated via signaling cascades linked to cell proliferation (44). Therefore we investigated whether the antiproliferative effect of MU lowers the expression of HAS and subsequently HA synthesis. At low and moderate concentrations of MU, however, we could not detect any significant change in the transcriptional levels of endogenous HAS genes in the rat 3Y1 transfectants, despite the significant decrease in HA synthesis. The fact that MU inhibited HA synthesis, even in the transfectants in which HAS expression is driven by an exogenous promoter, suggested the posttranscriptional inhibition of HA synthesis. However, we could not rule out the possibility that MU down-regulated the promoter activity of the endogenous HAS genes since they were moderately suppressed at high concentrations of MU.

To avoid the influence of growth suppression, we examined the effect of MU on HAS activity in a cell-free system. The HAS activity in the crude membrane fraction from the HAS2 transfectants was inhibited by MU treatment. The result is consistent with the idea that MU inhibits HA synthesis posttranscriptionally and in a growth-independent fashion. Interestingly, a similar result was obtained when \( pNP \) was tested for the ability to inhibit HAS activity in the cell-free HA synthesis. This led us to examine whether these two compounds, known to be good substrates for UGT, can act as acceptors for glucuronyltransferase of HAS. Substantial amount of glucuronidation of MU was observed in the conditioned medium and in the supernatant of the
cell-free HA synthesis. Intriguingly, a considerable amount of glucuronidation was also observed in the control cells expressing very little HAS, suggesting that MU was glucuronidated by an endogenous UGT other than HAS. Monosaccharide derivatives of MU and pNP have been shown to act as artificial primers for the initiation of glycosaminoglycan biosynthesis in cultured mammalian cells (41, 42). In plants, the lipid-sugar conjugate has been shown to act as a primer in the synthesis of cellulose (45). We considered the possibility that MU-GlcUA may act as an artificial initiation primer for HA synthesis thereby decreasing the efficiency of HA-polymerization, the primary role of HAS. However, this hypothesis seemed unlikely since the inhibition rate of MU-GlcUA was less than that of MU. Moreover, no elongation products of MU-GlcUA were detected in the conditioned medium or in the supernatant of the cell-free HA synthesis (data not shown). Alternatively, previous studies have shown that MU and pNP derivatives of xylosides, MU-Xyl and pNP-Xyl, significantly inhibited the biosynthesis of HA as well as sulfated glycosaminoglycans (23, 46). As for the mechanism, it was suggested that free pNP, which is generated by the enzymatic hydrolysis of pNP-Xyl mediated the inhibitory effect (47). In the current study, we also detected a significant liberation of pNP from its sugar derivatives. These results together with our finding that glucuronidation of the free aglycons occurred in consistent with the inhibition rate of HA synthesis support the importance of the glucuronidation in the inhibition.

Elevating the level of MU-GlcUA, by addition of exogenous recombinant UGT, promoted the inhibition of HA synthesis in the cell-free system. Furthermore, when COS cells overexpressing UGT and HAS2 were treated with MU, the inhibitory effects were enhanced both in terms of HAS activity and HA production. Excess glucuronidation of MU by UGT could deplete the pool of UDP-GlcUA, which is a common substrate for HAS and UGT. As shown in Fig. 9A, the inhibition of HA synthesis was reduced to the control level when an excess of UDP-
GlcUA was added to the in vitro reaction mixture. Thus, it is conceivable that the cellular concentration of UDP-GlcUA could be an important factor in the inhibitory action of MU. To clarify the mode of action, we determined whether the HAS inhibition was enhanced by pretreatment of the membrane fraction with MU or MU-GlcUA prior to initiating HA synthesis. Time dependent changes in HAS activity demonstrated that pretreatment with MU exerted little effect on the inhibition of HA synthesis at the initiation step (Fig. 10). However, the inhibition of HAS activity after MU treatment was partially rescued by increasing the UDP-GlcUA concentration at the later time points (Fig. 9B). Taken together, the results suggest that the glucuronidation event followed by the subsequent reduction of UDP-GlcUA concentration predominantly affects the chain elongation after the lag period of HAS inhibition. Consistent with this idea, the chain elongation of HA was reduced by MU after the lag period as shown in Fig. 2. This could be rationalized by assuming that the $K_m$ value for UDP-GlcUA of HAS enzymes is altered in a manner depending on the chain length of synthesizing HA.

The glucuronidation event may affect HAS activity to a greater extent in the cells expressing elevated levels of UGTs. Indeed MU significantly lowered HAS activity in rat 3Y1 cells expressing endogenous UGT, in contrast to COS cells which do not express any UGT. Altering the cellular level of UDP-GlcUA would be expected to mirror these observations. Depletion of UDP-GlcUA in the cellular pools may affect the biosynthesis of other GlcUA-containing glycosaminoglycans such as heparan sulfate and chondroitin sulfate. Previous studies, however, indicated that the action of MU had no affect on glycosaminoglycan biosynthesis in human skin fibroblasts (23, 24). We considered several possible explanations related to the selective inhibition of the synthesis by MU. MU may specifically target HAS due to its cellular localization which is different from other glycosyltransferases, EXTs (48, 49) and chondroitin synthases (50-52) involved in the biosynthesis of heparan sulfate and chondroitin sulfate,
respectively. All the glycosaminoglycans, with the single exception of HA, are synthesized at the intracellular Golgi network. HA is synthesized on the inner side of the plasma membrane by a membrane-associated HAS (17). The cell-free HA synthesis revealed that UGT activity is present in the membrane preparations. This is consistent with previous observations in which most UGTs are present in the endoplasmic reticulum (ER) and nuclear membrane (53).

Glucuronidation of MU may result in a differential reduction in the local concentration of UDP-GlcUA near the plasma membrane and in the Golgi and ER. Alternatively, this observation could be rationalized by assuming that the $K_m$ value for UDP-GlcUA of these enzymes varies markedly. In previous work, however, the $K_m$ values for UDP-GlcUA were similar among recombinant HAS2 (32), chondroitin synthases (51, 52) and UGT1A6 (54). Another explanation for the selective inhibition would be the preferential effects of MU on the chain elongation of a large molecular-mass of HA (over 1 x $10^6$ Da) as shown in Fig. 2.

One might expect a common inhibition mechanism for MU in both the mammalian cells and Streptococcus, since they synthesize HA with exactly the same structure using homologous enzymes (17). A possibility of posttranscriptional inhibitory mechanism was also speculated from the results in group C Streptococcus that MU did not affected the expression level of HAS (28). On the other hand, we speculated that MU inhibits HA synthesis of group C Streptococcus in a cardiolipin-dependent fashion without significant change in HAS activity in the cell-free HA system. The discrepancy between this result and the observations reported in this paper may be due to a different level of MU-GlcUA in the Streptococcus. Indeed, the production of MU-GlcUA was not detected in either the conditioned medium or in the cell extract of Streptococcus. Furthermore, the effect of cardiolipin on enzymatic activity is distinct between mammalian HAS and streptococcal HAS (29). Although the mechanism of action of MU is complex, our results suggest that glucuronidation of MU is partially account for the inhibition of HA synthesis in
mammalian cells.

Specific inhibitors targeting HA biosynthesis may serve as useful drugs to prevent the malignant alteration of cancer or the fibrosis of organs. Clinical application of MU, based on the inhibition of HA synthesis, is feasible since this compound has been used safely for many years as a cholangiolytic in human medicine (27). Nevertheless it is important to avoid the use of a very high dose of MU thereby reducing the possibility of cytotoxicity. The information presented in this report will be useful in the development of new drugs with improved efficacy. In particular, other acceptors for UGTs should be potent inhibitors of HAS activity. Further investigation will be undertaken in this regard.
ACKNOWLEDGEMENTS

We thank Drs. Andrew P. Spicer (Texas A and M University) and John A. McDonald (University of Utah) for providing mouse HAS2 cDNA. We also thank Dr. Minoru Okayama (Kyoto Sangyo University, Kyoto, Japan) for his helpful comments. This study was supported by grants from the CREST of JST (Japan Science and Technology Agency), the preparatory grant for the research at the Division of Matrix Glycoconjugates, Research Center for Infectious Disease, Aichi Medical University, grants-in-aid for Young Scientists (B) and grants-in-aid for Scientific Research on Priority Areas from the Japan’s Ministry of Education, Culture, Sports, Science and Technology (Nos. 14780480 and 15040203), Culture, Sports, Science and Technology, grant-in-aid for Scientific Research (B) from Japan Society for the Promotion of Science (No. 15370041), the Aichi Cancer Research Foundation, grant-in-aid from the Tokyo Biochemical Research Foundation, special research funds from Seikagaku Corporation, and the Karoji Memorial Fund for Medical Research (A) and (B) in Hirosaki University.

FOOTNOTES

1The abbreviations used are: HA, hyaluronan; MU, 4-methylumbelliferone; pNP, p-nitrophenol; GlcUA, glucuronic acid; GlcNAc, N-acetylglucosamine; Glc, glucose; HA synthase, HAS; DMSO, dimethylsulfoxide; HABP, hyaluronan binding protein; PBS, phosphate buffered saline; DTT, dithiothreitol; UGT, UDP-glucuronosyltransferase; ESI, electrospray ionization.
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FIGURE LEGENDS

Figure 1. HA production and matrix formation of HAS2 transfectants after MU treatment.
A-D. Pericellular HA matrices surrounding 3Y1-HAS2 transfectants were visualized by the particle exclusion assay and photomicrographs were taken under inverted phase-contrast microscope at x300 (A, B) and then magnified further (C, D). The 3Y1-HAS2 cells were cultured for 24 h with (B, D) or without (A, C) 300 µM of MU. E. The HA contents in the conditioned medium from exponentially growing (open circle) and confluent cultures (closed circle) were measured by ELISA-like assay at 24 h after the treatment of 3Y1-HAS2 transfectants with various concentrations of MU. Data represent average of two independent experiments.

Figure 2. Effects of MU or pNP on HAS activity and size distribution of synthesized HA. A. A membrane-rich fraction from 3Y1-HAS2 cells were incubated with various concentrations of MU (closed circles) or pNP (open circle), and then HAS activity was calculated as a percentage relative to that of the non-treated sample. B. HAS activities were measured at various periods after the treatment of 3Y1-HAS2 transfectants without (closed circle) or with 300 µM MU (closed triangle). C. The same reaction supernatants were digested with (+) or without (-) 10 TRU Streptomyces hyaluronidase and then the size distributions of HA were analyzed by 0.5% agarose gel electrophoresis. Radiolabeled HA was detected by autoradiography. Standard HA samples with known molecular size were used to determine the size distributions of newly synthesized HA. D. The time-dependent effect of MU on the chain elongation was assessed as above. Plus and minus indicate samples treated with or without 300 µM MU, respectively.
Figure 3. HPLC and TLC analyses of MU derivatives found in the culture conditioned medium. 3Y1-HAS2 (A) and 3Y1-Mock (B) cells were incubated without (a) or with 1000 µM MU (b, c) for 24 h. The conditioned medium was analyzed before (a, b) and after digestion (c) with 1 U of β-glucuronidase by HPLC on Tsk-gel ODS 120-T. Peaks 1 and 2 corresponded to MU-GlcUA and MU used as authentic standards, respectively. C. Peak 1 in the HPLC, with a retention time coinciding with that of an authentic MU-GlcUA standard, was collected and analyzed by TLC. Lanes: 1, standard MU-GlcUA; 2, Peak 1 from 3Y1-HAS2 cells; 3, Peak 1 from 3Y1-Mock cells; 4, standard MU. D. 3Y1-HAS2 cells were treated with various concentrations of MU for 24 h and the production amount of peak 1 in the conditioned medium (closed circle) and in the cell lysate (open circle) were expressed per viable cell number. E. HPLC profiles of MU derivatives from 3Y1-HAS2 cells treated with various concentrations of MU for 24 h.

Figure 4. Chemical structure of MU-GlcUA and ion-spray mass spectrum of MU derivative. Peak 1 from 3Y1-HAS2, eluted at the same position of authentic standard MU-GlcUA in the HPLC (Fig. 3) was collected and analyzed by ion-spray mass spectrometry. The conditions were described under “Experimental Procedures”.

Figure 5. TLC analysis of pNP- or MU-GlcUA produced in the supernatant of the cell-free HA synthesis. The membrane-rich fractions from 3Y1-Mock or 3Y1-HAS2 cells were incubated in the presence of UDP-[14C] GlcUA and pNP (A), or UDP-GlcUA and [14C] pNP (B), or UDP-[14C] GlcUA and MU (C), and the reaction supernatants were analyzed by TLC on silica 60 plate as described in the “Experimental Procedures”. Arrow 1 and 2 show the positions corresponding
to those of the standard pNP- or MU-GlcUA, respectively. Minus and plus show samples before and after treatment with 2 U β-glucuronidase.

**Figure 6. Effects of MU- and pNP-sugars on HAS activity.** The effects of various sugar derivatives of MU (A) or pNP (B) were assessed by cell-free HA synthesis. Various concentrations of MU (closed circle), MU-GlcUA (closed diamond), MU-GlcNAc (closed square), MU-Glc (closed triangle), pNP (open circle), pNP GlcUA (open diamond), pNP-GlcNAc (open square), and pNP-Glc (open triangle) were added in the membrane-rich fractions prepared from HAS2 transfectants, and then the HAS activities were calculated as percentages relative to that of the non-treated sample. Data represent average of three independent experiments ± SD.

C. Membrane-rich fractions from 3Y1-HAS2 cells were incubated with 100 µM pNP-sugars and UDP-[14C] GlcUA, and the production of pNP-GlcUA was analyzed on TLC. Radiolabeled spots corresponding pNP-GlcUA (arrow) were detected by autoradiography.

**Figure 7. Enhanced inhibition of HAS activity by recombinant UGT proteins.** A, Partially purified recombinant human UGT1A6 was added together with 100 µM pNP in the cell-free HA synthesis, and then the HAS activities were calculated as percentages relative to the non-treated sample. Inactivated UGT1A6 was obtained by boiling the active enzyme. Data represent average of three independent experiments ± SD. *p < 0.05. B. Membrane fractions from 3Y1-HAS2 cells were incubated with 100 µM pNP and UDP-[14C] GlcUA, and the production of pNP-GlcUA was analyzed on TLC. Radiolabeled spots corresponding pNP-GlcUA (arrow) was detected by autoradiography. C. Partially purified UGT1A6 or UGT1A7 was added together
with 100 μM pNP or MU in the cell-free HA synthesis system, and then the HAS activities were calculated as percentages relative to the non-treated sample. Membrane fractions prepared from insect cells infected with baculovirus vector alone were used as a control MF. Data represent average of three independent experiments ± SD. *p < 0.05.

Figure 8. Overexpression of human UGT1A6 enhanced the inhibition of HAS activity and HA synthesis by MU. **A.** Membrane-rich fractions from COS transfectants expressing UGT1A6 and/or HAS2 were incubated with or without 100 μM MU, and then the HAS activities were calculated as percentages relative to the control. **B.** Membrane-rich fractions from COS transfectants were incubated without (open bar) or with 100 μM MU (solid bar) and UDP-[14C] GlcUA, and the production of MU-GlcUA was analyzed on TLC. Radiolabeled spots corresponding to MU-GlcUA (arrow) were detected by autoradiography. Plus and minus indicate samples in the presence or absence of MU, respectively. **C.** The HA contents in the conditioned medium of COS transfectants expressing UGT1A6 and/or HAS2 were measured by ELISA-like assay at 24 h after the treatment with 300 μM MU. Data represent average of three independent experiments ± SD.

Figure 9. Excess amount of UDP-GlcUA diminished the effect of MU on HAS activity. **A.** Various concentrations of UDP-GlcUA were added together with 100 μM MU in the membrane-rich fractions from COS transfectants co-expressing UGT1A6 and HAS2, and then the HAS activities were calculated as percentages relative to that of the non-treated sample. The inhibitory
effect of MU was diminished to the control level by an excess amount of UDP-GlcUA. B. The membrane-rich fractions from COS transfectants co-expressing UGT1A6 and HAS2 were preincubated for 1 h with (+) or without (−) 300 µM MU in the reaction buffer as described in “Experimental Procedures”. Additional UDP-GlcUA was then supplied at the concentration of 0.1 mM to the reaction mixture and further incubated at 37 °C for 1 h. The inhibition of HAS after treatment with MU was partially rescued at the increased concentrations of UDP-GlcUA. Data represent average of three independent experiments ± SD. *p < 0.05.

Figure 10. Pretreatment of the membrane fractions with MU or MU-GlcUA. The membrane fractions isolated from 3Y1-HAS2 transfectants were preincubated for 1 h with 0.1 mM UDP-GlcUA in the presence of 300 µM MU or MU-GlcUA. The HA synthesis was then initiated by addition of a saturated concentration of UDP-GlcNAc, and time-dependent changes in the HAS activity were monitored by the incorporation of radioactivity into the HA fraction. A slight inhibition of HAS activity was observed at 15 min and became more apparent thereafter. The effect of MU-GlcUA was less than that of MU itself. Data represent average of two independent experiments.
### TABLE I

Relative HAS expression in HAS2 transfectants after MU treatment.

| MU (µM) | mouse HAS2 (%) | rat HAS2 (%) | rat HAS3 (%) |
|---------|----------------|--------------|--------------|
| 0       | 100            | 100          | 100          |
| 10      | 130.7 ± 19.7   | 94.7 ± 25    | 98.5 ± 13.9  |
| 30      | 129.1 ± 34.8   | 86.1 ± 10.7  | 93.1 ± 13.7  |
| 100     | 137.4 ± 11.6   | 105.0 ± 28   | 93.9 ± 10.3  |
| 300     | 93.3 ± 29.6    | 77.1 ± 4.3   | 90.4 ± 2.1   |
| 1000    | 104.8 ± 28.4   | 48.6 ± 8.2   | 97.4 ± 10.6  |
Fig. 2

A. Graph showing the effect of MU or p-NP (μM) on HAS activity (%).

B. Graph showing the effect of time (min) on HAS activity (%).

C. Table and gel image showing the relationship between MU (μM), HAase, and Mr (x 10⁵).

D. Gel image showing the effect of time (min) on HAS2 activity.
Fig. 5
Fig. 6

A

HAS activity (%)

MU-sugar (μM)

MU
MU-GlcUA
MU-GlcNAc
MU-Glc

B

HAS activity (%)

pNP-sugar (μM)

pNP
pNP-GlcUA
pNP-GlcNAc
pNP-Glc

C

0.1% EtOH
pNP
pNP-GlcUA
pNP-GlcNAc
pNP-Glc
A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone
Ikuko Kakizaki, Kaoru Kojima, Keiichi Takagaki, Masahiko Endo, Reiji Kannagi, Masaki Ito, Yoshihiro Maruo, Hiroshi Sato, Tadashi Yasuda, Satoka Mita, Koji Kimata and Naoki Itano

J. Biol. Chem. published online June 9, 2004

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