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ORIGINAL ARTICLE

Hyaluronan synthesis inhibitor 4-methylumbelliferone affects extracellular matrix components in cultured human skin fibroblasts

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Abstract: Hyaluronan (HA) is a ubiquitous glycosaminoglycan comprising *N*-acetylglucosamine and glucuronic acid and is involved in matrix formation and cell maintenance. 4-Methylumbelliferone (MU) exhibits anti-tumor activities by inhibiting HA synthesis. In this study, we aimed to elucidate the association between HA and numerous matrix molecules.

Materials and Methods: We created HA-knock down extracellular matrix (ECM) by culturing human skin fibroblasts (HSF) in the presence of MU (0.5 mM) for 3 days after confluence. Following culture in the presence or absence of MU, the cell layer and culture medium were collected and ECM molecules were measured using enzyme-linked immunosolvent assay, and the [^{35}S]-sulfate uptake method.

Results: Proteoglycans and type I collagen were released into the medium as HA decreased. However, type I collagen, but not proteoglycans, was immediately replenished. Fibronectin and CD44 in the cell layer decreased, but were not replenished, as HA decreased. On contrary, laminin, cadherin, and vitronectin were not affected by HA decrease.

Conclusions: HA knockdown by MU treatment elucidated the dynamic interactions between HA and ECM components. Our findings suggest that HA interacts with other ECM components through various ways, including strong interactions for ECM network formation and loose interactions for signal transduction.

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Key words: hyaluronan; hyaluronan synthesis inhibitor; 4-methylumbelliferone; extracellular matrix.

Introduction

Hyaluronan (HA) is a linear polysaccharide composed of thousands of repeating disaccharide units comprising an *N*-acetyl-D-glucosamine and D-glucuronic acid[1, 2]. HA is found in the extracellular matrix (ECM) and on the surface of many cell types. HA also interacts with proteoglycan (PG) and other substances in the ECM. Consequently, HA forms the core of networks in the ECM. Despite this knowledge, details of its interactions with other ECM components require further investigation. Reportedly, HA exhibits various dynamic functions, such as in cell growth, angiogenesis, wound healing, malignant cell growth, and metastasis[1, 2]. In particular, the level of HA, which forms the microenvironment of cells, is increased in cancer tissues[3-5].

We have previously reported that 4-methylumbelliferone (MU) inhibits HA synthesis in cultured human skin fibroblasts (HSF)[6, 7]. The mechanisms underlying this inhibition in bacteria as prokaryotic cells[8], cultured HSF as eukaryotic cells[9], and others[10-12] have been reported.

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Abbreviations: DMSO, dimethyl sulfoxide; ECM, extracellular matrix; HA, hyaluronan; HSF, human skin fibroblast; MU, 4-methylumbelliferone; PG, proteoglycan GlcUA, glucuronic acid

Moreover, MU presents anti-tumor and anti-cancer-cell activities against melanoma, pancreatic cancer, and mesothelioma, among others[13-22]. However, the mechanism underlying these activities via the inhibition of HA synthesis has not been elucidated.

Endo *et al.* have preliminarily reported that MU inhibits HA synthesis in cultured HSF, resulting in a decrease in the level of HA in the ECM and the release of PG and other substances into the culture medium[23]. This decrease in HA results in changes in the ECM, which was the primary focus of analysis in this research.

Materials and Methods

Chemicals

MU was purchased from Wako Pure Chemical Co. (Osaka, Japan). Eagle's minimum essential medium was purchased from Nissui Seiyaku (Tokyo, Japan). HA-binding protein was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan). Anti-procollagen type I antibody (monoclonal antibody of the C-terminal domain), anti-fibronectin antibody (monoclonal antibody of the cell adhesion domain), anti-laminin antibody (monoclonal antibody of the *N*-terminal of B2), anti-E-cadherin antibody (monoclonal antibody of the Ca^{2+} binding domain), and anti-vitronectin antibody (monoclonal antibody of the C-terminal domain) were purchased from Takara Shuzo Co. (Kyoto, Japan). Anti-CD44 antibody (anti-extracellular *N*-terminal domain of the human CD44 monoclonal antibody) was purchased from Bender MedSystems (Vienna, Austria). [^{35}S]-Sulfate (specific activity, 1,600 Ci/mM) was purchased from ICN Radiochemicals (St. Louis, MO, USA).

Culture of HSF

HSF (passages 4–7) were cultured to confluence in Eagle's minimum essential medium containing 5% fetal bovine serum at 5% CO_2/95% air in a 20-mm dish at 37°C, as reported previously[6].

MU was added to the culture medium at 0.5 mM, as previously reported[6], and the cells were then cultured for 72 h. MU was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium was adjusted to 0.1%. Cells cultured in medium containing 0.1% DMSO and without MU were used as controls. Data are expressed as mean values from five replicates.

Following culture, the cell layer and culture medium were separately collected. The culture medium was recovered as is and was used as the culture medium fraction. The cell layer was harvested with a cell scraper, solubilized in 0.1% Triton X-100, and used as the cell layer fraction. Phenylmethanesulfonyl fluoride and ethylenediaminetetraacetate were added as proteinase inhibitors in both the fractions.

Measurement of nucleic acid contents and total protein contents in the cell layer

Using cell layer fractions, UV absorption for nucleic acid was measured at a wavelength of 260 nm, and total protein was measured using the Bradford method[24].

Determination of HA levels

HA level was quantified by enzyme-linked immunosolvent assay using HA-binding protein[25] using in both the cell layer and culture medium fractions.

Assay of ECM components

Using the cell layer and culture medium fractions, type I collagen, fibronectin, CD44, laminin, vitronectin, and cadherin were quantified by enzyme immunoassays[26]. Briefly, 96-microtiter wells coated with monoclonal antibodies for antigens were washed with phosphate-buffered saline. They were then incubated with 100 μL of samples for 1 h at 37°C and with peroxidase-conjugated monoclonal antibody for 1 h at 37°C.

After washing the wells, 3,3',5,5'-tetramethylbenzidine was added, and the absorbance at 492 nm was measured with a Bio-Rad EIA reader (Bio-Rad Lab. Inc., Hercules, CA, USA).

Separation of PG

HSF were cultured to confluence in a 20-mm dish. The cells were then incubated for 72 h in culture medium containing 0.5 mM MU in the presence of 50 μCi/mL [^{35}S]-sulfate. Following incubation, the culture medium was collected and the cell layer was washed twice with phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) and solubilized with 4 M guanidine HCl. PG (5 mg) as a carrier was added to both the fractions, and four volumes of ethanol saturated with NaCl were added. The resultant precipitates were collected, and gel filtration was performed using a PD-10 column. Radioactive fractions as PG fractions were collected, and the radioactivity was measured using a liquid scintillation counter.

Electron microscopy

Electron micrographs of cultured HSF were acquired. HSF were cultured to confluence and then incubated in the presence or absence of 0.5 mM MU for 72 h. The cell layer was prefixed with 2.5% glutaraldehyde (pH 6.8) containing Alcian blue 8GX and osmiated in 0.1 M cacodylated buffer (pH 8.6). Ultrathin sections, 70–80 nm thick, were double-stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (JEOL LEM-2000EX) at an accelerating voltage of 80 or 100 kV[27, 28].

Results

Effects of MU on HSF culture

HSF at passages 4–7 were cultured to confluence in Eagle's minimum essential medium. MU (0.5 mM) was added to the culture medium, and the cells were incubated for 72 h as previously reported[6]. In phase-contrast micrographs of the cell layer cultured for 72 h, little difference was observed between the cells cultured in the presence or absence of 0.5 mM MU, as reported previously[6].

Electron microscopy of the cell layer after 72 h of culture in the presence of 0.5 mM MU revealed that there was little extracellular space between the cells and that the ECM was less dense in the cell layer treated with MU compared with that in control cells (Figure 1A–D).

The ECM structure in the control cells indicated the existence of much more water. In the cell layer cultured in the presence of MU, the quantity of water decreased and the ECM structure was not maintained. The suppression of HA synthesis by MU led to the narrowing of intercellular spaces and a decrease in water levels.

Changes in the levels of nucleic acids and proteins after MU treatment

Next, we investigated changes in the ECM components of HSF cultured in the presence of 0.5 mM MU using the cell layer and cultured medium fractions. There was no change in the levels of nucleic acids and proteins in the cell layer after 72 h of culture (Figure 2B and 2C) even in the presence of various concentrations of MU in the culture medium (Figure 2A). Therefore, the narrowing of the intracellular spaces was not caused by a decrease in nucleic acid and protein levels. Moreover, MU had no effect on cell viability until it reached a concentration of 2.0 mM in the culture medium.

Changes in the extracellular components by MU treatment

HA

HA levels were estimated by an ELISA like assay. When the concentration of MU in the culture medium increased to 0.5 mM, HA levels in both the cell layer and culture medium fractions decreased, as reported previously[6] (Figure 3A and 3B). In the cell layer fraction in the

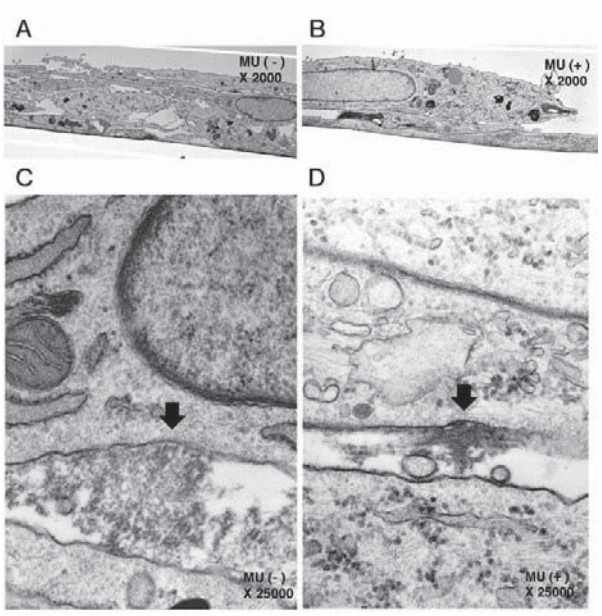

Figure 1 Electron microscopy of the cell layer cultured in the presence of MU

Human skin fibroblasts were cultured to confluence. The medium was then replaced with medium containing 0.5 mM MU, and cells were incubated for 72 h. Next, the cell layer was fixed with Alcian blue, and electron microscopy was performed. Ultrathin sections (70–80 nm thick)were double-stained by uranyl acetate and lead citrate. Magnification: ×2000 (A, B)and ×25,000 (C, D). Cells cultured without (A, C)and with MU (B, D). ↓, extracellular matrix

absence of MU (control), HA levels constantly increased until approximately 40 h, and then the HA synthesis reached a plateau. Meanwhile, HA was released from the cell layer to the culture medium until 48 h.

In contrast, in the presence of MU, no change in HA levels was observed in the cell layer fraction, and only little HA was released to the culture medium. These results suggested that HA synthesis was suppressed by MU.

After 72 h of culture in the presence of MU, HA levels reduced to approximately 60% in the cell layer and approximately 30% in the culture medium fractions compared with the levels in the control cells, as reported previously (Figure 3C and 3D). These data suggested that the narrowing of intracellular space was mainly caused by HA reduction in the presence of MU (Figure 1).

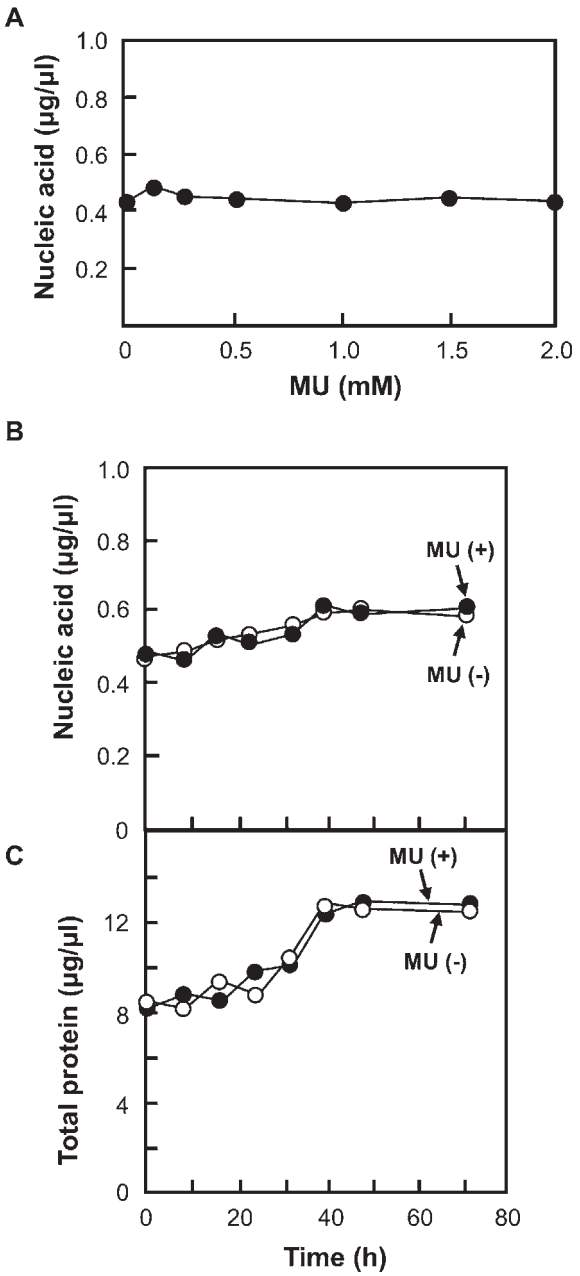

Figure 2 Effect of MU on levels of nucleic acids and proteins in the cell layer and time course of levels of nucleic acids and proteins in the presence of MU

Confluent human skin fibroblasts were cultured in medium containing various concentrations of MU for 72 h (A) and in medium containing 0.5 mM MU for 72 h. The levels of nucleic acids (B) and proteins (C) in the cell layer were then measured. ○, culture without MU; ●, with 0.5 mM MU.

PG

The measurement of PG in the cell layer is extremely difficult because of its low level. Thus,

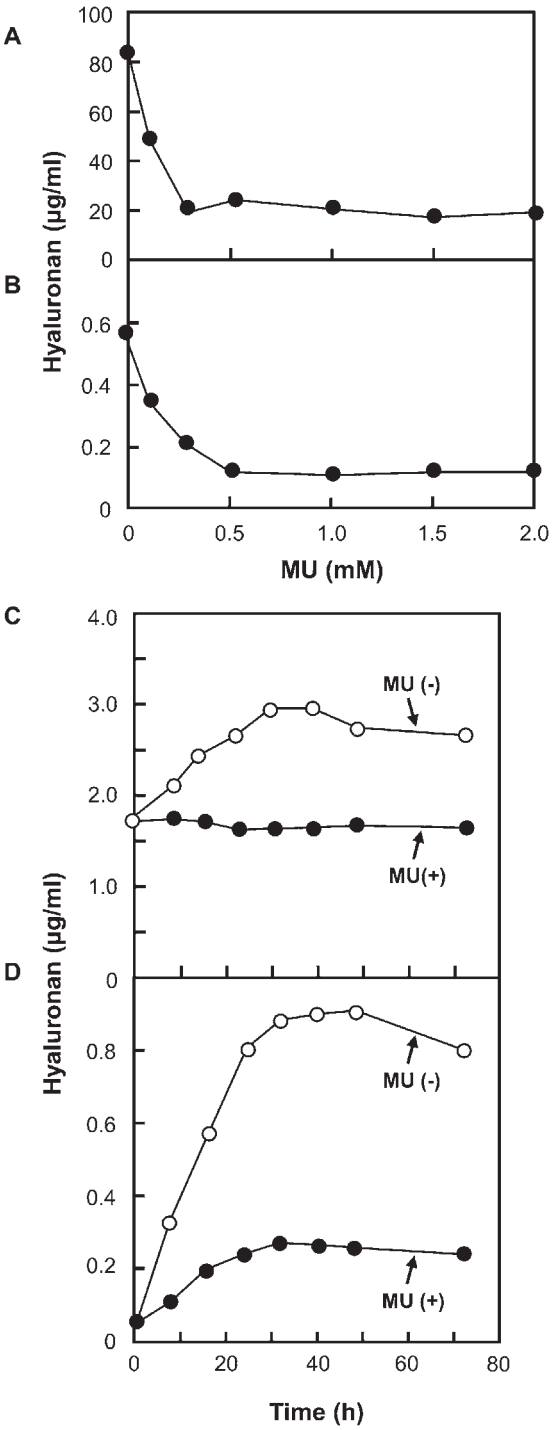

Figure 3 Effect of MU concentration in the culture medium on HA synthesis and time course of HA synthesis in the presence of MU

Confluent human skin fibroblasts were cultured in the medium containing various concentrations of MU for 72 h. HA levels in the cell layer (A)and medium (B)were measured by HA-binding protein assay. Confluent human skin fibroblasts were cultured in the medium containing 0.5 mM MU for 72 h. HA levels in the cell layer (C)and medium (D) were measured by HA-binding protein assay. ○, culture without MU; ●, with 0.5 mM MU.

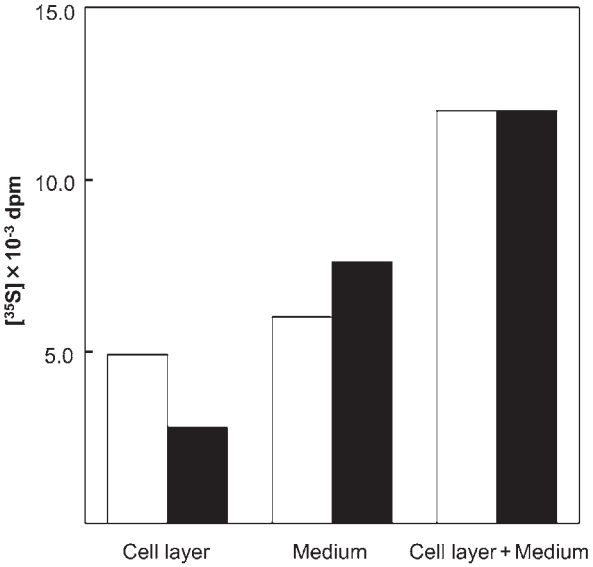

Figure 4 Time course of [^{35}S]-sulfate uptake into the proteoglycan fraction

Confluent human skin fibroblasts were cultured in medium containing 0.5 mM MU for 72 h. The [^{35}S] -sulfate uptake in the cell layer and medium was measured using a liquid scintillation counter. □, without MU; ■, incubation in the presence of 0.5 mM MU.

PG synthesis was estimated using [^{35}S]-sulfate uptake (Figure 4). [^{35}S]-Sulfate uptake into the PG fraction in the cell layer cultured in the presence of MU was approximately 75% of that in control cells. On contrary, [^{35}S]-sulfate uptake into the PG fraction was 1.4 times higher in the culture medium of cells cultured in the presence of MU than in the culture medium of control cells. There was no difference in total [^{35}S]-sulfate uptake calculated by summing the levels in the cell layer and culture medium regardless of MU treatment. These findings suggested that MU has no direct influence on PG synthesis. However, there was a decrease in PG in the cell layer treated with MU that was released from the cell layer into the culture medium, which is a binding partner of PG in the ECM.

Type I collagen

Even after reaching confluence, type I collagen synthesis continued in the control cell layer (Figure 5). Moreover, the release of type I

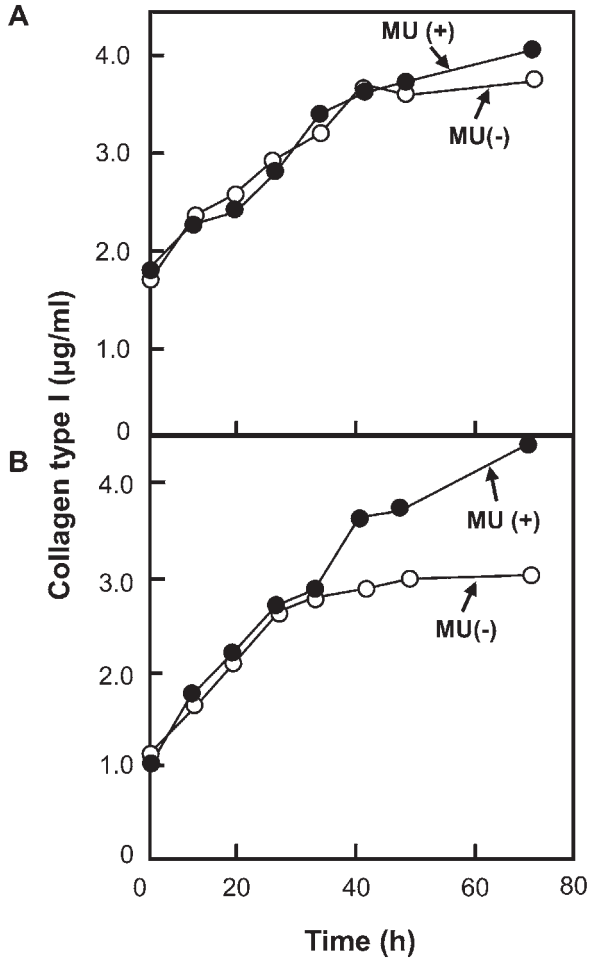

Figure 5 Time course of type I collagen concentration in the cell layer and medium cultured without and with 0.5 mM MU

After incubation in the presence of 0.5 mM MU, type I collagen levels in the cell layer (A)and medium (B)were measured by enzyme immunoassay. ○, without MU; ●, incubation in the presence of 0.5 mM MU

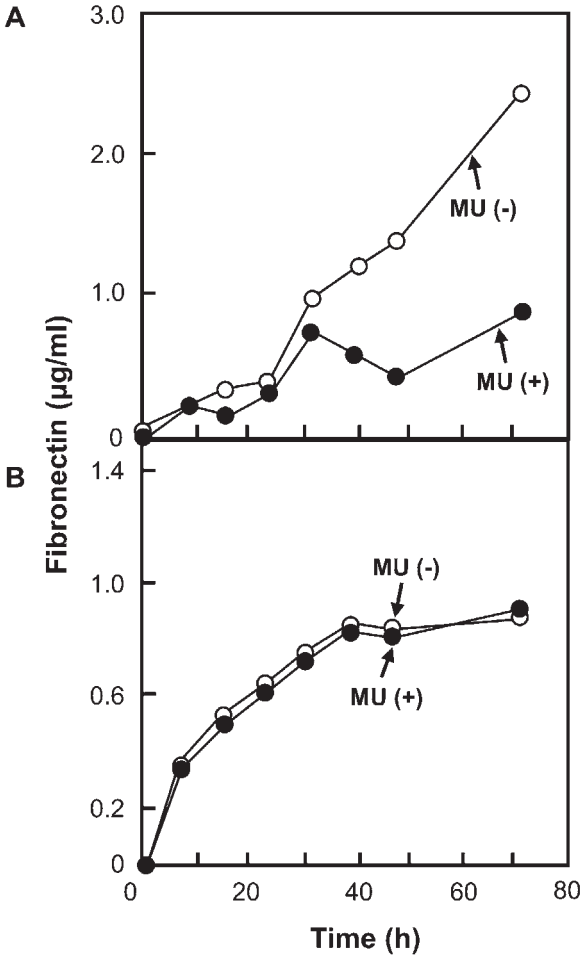

Figure 6 Time course of fibronectin concentration in the cell layer and medium cultured without and with 0.5 mM MU

After incubation in the presence of 0.5 mM MU, fibronectin levels in the cell layer (A)and medium (B)were measured by enzyme immunoassay. ○, without MU; ●, incubation in the presence of 0.5 mM MU

collagen into the culture medium continued and then reached a plateau after approximately 40 h. The synthesis of type I collagen was very similar to that of HA, which continued even after reaching confluence. However, type I collagen synthesis continued in the cell layer regardless of MU treatment. Conversely, the release of type I collagen into the culture medium continued regardless of the decrease in HA.

Therefore, while HA synthesis in the cell layer was suppressed by MU, type I collagen synthesis was not affected by MU treatment, and the release of type I collagen into the culture medium increased. These findings suggest that type I collagen interacts with HA in the ECM and is released into the culture medium accompanying the HA decrease as an interaction partner.

Changes in cell surface components induced by MU treatment

Fibronectin

The synthesis of fibronectin continued even after cultured cells had reached confluence.

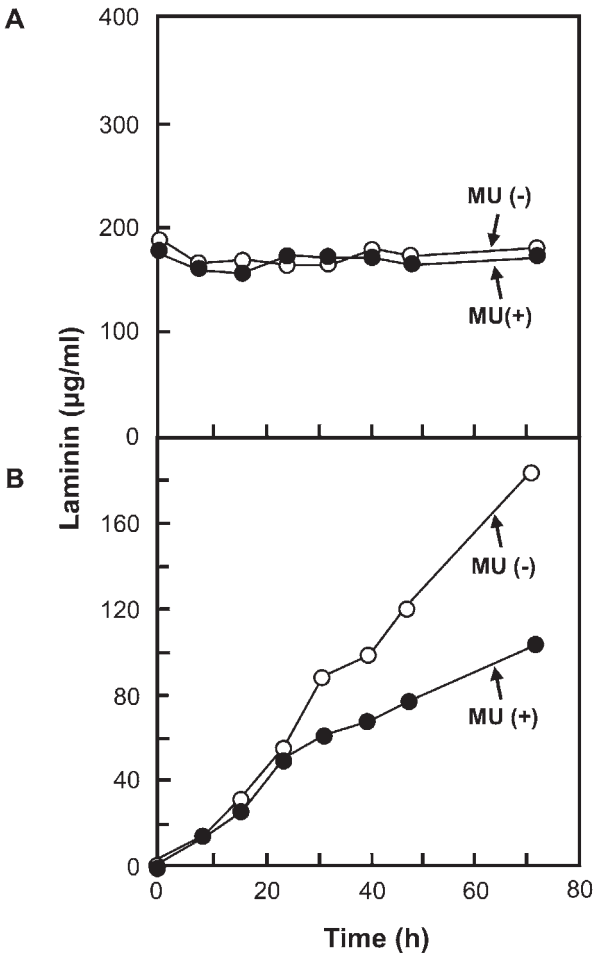

Figure 7 Time course of laminin concentration in the cell layer and medium cultured without and with 0.5 mM MU

After incubation in the presence of 0.5 mM MU, laminin levels in the cell layer (A)and medium (B)were measured by enzyme immunoassay. ○, without MU; ●, incubation in the presence of 0.5 mM MU

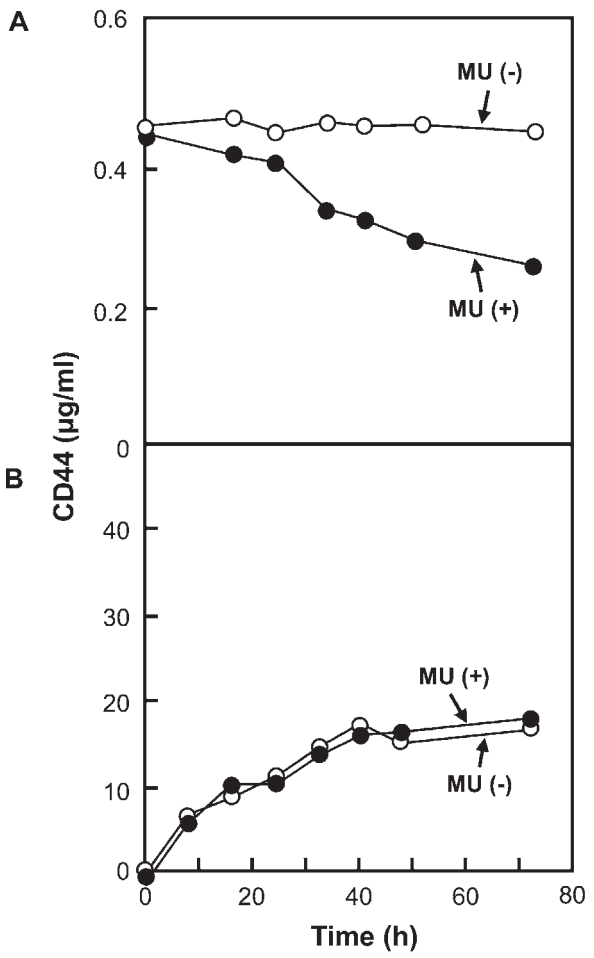

Figure 8 Time course of CD44 concentration in the cell layer and medium cultured without and with 0.5 mM MU

After incubation in the presence of 0.5 mM MU, CD44 levels in the cell layer (A)and medium (B) were measured by enzyme immunoassay. ○, without MU; ●, incubation in the presence of 0.5 mM MU

However, only approximately 1% of the fibronectin was released into the culture medium (Figure 6). Moreover, the release reached a plateau at approximately 40 h.

Fibronectin levels in the cell layer markedly decreased after treatment with MU. Regardless of MU treatment, no difference was observed in terms of the release of fibronectin into the culture medium.

Laminin

After reaching confluence, no change was observed in terms of laminin in the cell layer regardless of MU treatment (Figure 7). However, little laminin was released in the culture medium regardless of MU treatment. In the presence of MU, laminin levels in the culture medium began to decrease from 24 h. After 72 h of culture in the presence of MU, laminin level was reduced to 55% of that in control cells.

Changes in transmembrane components induced by MU treatment

CD44

In the absence of MU, CD44 level was constant and did not increase after the cultured cells had reached confluence (Figure 8). In the presence of MU, CD44 level was reduced to 66% of that in control cells. CD44 level in the culture medium was no more than 5% of that in the cell layer. Little CD44 was released into the culture medium and reached a plateau at approximately 40 h.

Cadherin and vitronectin

In this study, cadherin and vitronectin levels in the cell layer and culture medium were also estimated, but showed no significant changes were observed regardless of MU treatment (data not shown).

Discussion

We have previously shown that MU is a powerful inhibitor of HA synthesis[6, 7]. Additionally, MU exerts anti-tumor effects through the inhibition of HA synthesis in melanoma[13, 14] and pancreatic cancer[15-17]. Many studies have focused on these anti-tumor activities of MU and have demonstrated that it affects the proliferation, invasion, and metastasis of many types of tumor cells[19-22, 29-33]. HA surrounding cancer cells is part of the tumor microenvironment and is associated with the proliferation, invasion, and metastasis of cancer cells.

The mechanism underlying the anti-tumor effects of MU remains unclear. We have previously reported that MU treatment disturbs the cell wall, where HA synthases takes place in bacteria[8]. In mammalian cells, MU treatment has been reported to cause a decrease in UDP-GlcUA, which is required for HA synthesis[9, 10]. Other mechanisms of MU have also been reported in recent years, such as the targeting of UDP-glucose dehydrogenase, which involves UDP-GlcUA biosynthesis.[11, 12].

Animal models in which HA synthases are knocked out are available for the elucidation of the physiological function of HA. However, the methods have not been completely established because the knockout of some HA synthases is lethal[34]. To overcome this obstacle, MU treatment allows us to knock down HA in the tissue through the inhibition of HA synthesis[23]. Therefore, HA knockdown by MU treatment provides a new avenue to investigate the biological functions of HA and to clarify the roles of HA in the tumor microenvironment. In this study, HA knockdown was induced by MU treatment, after which the changes in ECM components were investigated. Elucidating the interactions between HA and other components in the ECM should help us clarify the mechanism through which HA affects the proliferation, invasion, and metastasis of cancer cells.

Our study demonstrated that MU-induced HA knockdown did not affect HSF gross morphology and cell number when assessed using an inverted microscope, as reported previously[6]. Electron microscopy analysis of the cell layer cultured in the presence of MU indicated no change in the nucleus and cytoplasm, intercellular spaces, which constitute the ECM, became narrow, and the portion with deep electron density was partially reduced.

We then assessed changes in the ECM components in the presence and absence of MU. The levels of both nucleic acids and proteins were not affected by MU treatment. Thus, the narrowing of the intercellular space was not due to a decrease in protein levels.

The effects of the inhibition of HA synthesis by MU were similar to those described previously[6]. When HA levels in the cell layer decreased after MU treatment, [^{35}S]-uptake into the PG fraction in the cell layer also decreased, and at the same time, [^{35}S]-uptake into the PG fraction in the culture medium increased. Overall, total levels of [^{35}S]-uptake into the cell layer and culture medium were almost equal regardless of the MU treatment. The level of PG syn-

thesized in the cell layer was constant regardless of the amount of HA. Therefore, when HA level was decreased by MU treatment, only PG, as a binding partner of HA, was released into culture medium. HA binds PG through the G1 domain in the core peptide of PG[35].

MU stimulates the synthesis of a small PG[36]. However, this could not be confirmed because the method used in this study does not allow us to discriminate between small and large PGs.

Similarly, when HA decreased in the cell layer following MU treatment, type I collagen was released in the culture medium and the loss of type I collagen was quickly compensated. Direct binding between HA and type I collagen has not been reported. However, a new binding system between PG and collagen has been reported as cluster binding[37]. Therefore, future studies are warranted to clarify the interaction among HA, PG, and collagen for the formation of the ECM network.

Fibronectin, which binds to HA and is expressed on the cell surface[38], decreased in the cell layer, reflecting the effect of MU treatment. However, in the culture medium, the decrease in fibronectin was very low. Thus, the synthesis and degradation of fibronectin may not always be affected by HA.

HA strongly interacts with laminin, which is also expressed on the cell surface[39]. Our study showed that the synthesis of laminin was interrupted regardless of MU treatment once the cells reached confluence. The synthesis of laminin was not affected by the MU-induced inhibition of HA synthesis. Similarly, the synthesis of cadherin and vitronectin, which are components of the ECM, was not affected in both the cell layer and the culture medium following MU treatment.

The synthesis of CD44, which strongly interacts with HA[40], was interrupted after cultured cells had reached confluence and decreased when the HA levels decreased. However, CD44 levels in the culture medium were not affected by MU treatment.

In conclusion, our findings suggest that the effects of decrease in HA levels on other ECM components are more complicated than expected. Further, HA might interact with other ECM components through various mechanisms, including strong interactions for ECM network formation and loose interactions for signal transduction. In addition, some ECM components may not directly interact with HA, but rather bind to other components, which directly interact with HA. Therefore, such components may be indirectly affected by HA. The elucidation of more detailed interactions among HA and other ECM components will help us clarify the roles of HA in the ECM in relation to the proliferation, invasion, and metastasis of cancer cells.

Declaration of interest

There are no conflicts of interest to declare. The authors alone are responsible for the content and writing of this article.

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