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ORIGINAL ARTICLE

EFFECTS OF HYALURONAN SYNTHASE INHIBITORS, 4-METHYLUMBELLIFERONE AND 4-METHYLESCULETIN ON HUMAN PLEURAL MALIGNANT MESOTHELIOMA CELLS (NCI-H2052)

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Abstract Hyaluronan (HA) is a major component of the pericellular matrix, and is implicated in cell adhesion, invasion, and tumor metastasis. We have reported that 4-methylumbelliferone (MU) inhibits HA synthesis by cultured skin fibroblasts, melanoma cells, and pancreatic cancer cells. We focused in the present study, on mesothelioma which has an extremely poor prognosis, and in which no effective therapy has yet been established. We investigated dealing with this neoplasm whether MU and 4-methylesculetin (ME), a MU derivative, are able to inhibit HA synthesis by the mesothelioma cell line NCI-H2052. MU inhibited HA synthesis by about 20%, and ME by about 40%, in comparison with the control group. MU inhibited the adhesion of NCI-H2052 cells by about 30%, and ME by about 50%, compared with the untreated control. MU inhibited cell locomotion by about 30%, and ME by about 40%. It is suggested through these results suggest that MU and ME inhibit HA synthesis, adhesion, and locomotion by human mesothelioma cells and weaken their pericellular matrix, and that the inhibitory effect of ME on HA synthesis is stronger than that of MU. It is presumed that both MU and ME may have potential as new therapeutic or prophylactic medicines against mesothelioma.

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Key words: hyaluronan; mesothelioma; 4-methylumbelliferone; 4-methylesculetin; NCI-H2052.

原 著

ヒアルロン酸合成抑制剤である4－メチルウンベリフェロンおよび4－メチルウンベリフェロン誘導体である4－メチルエスクレチンの胸膜悪性中皮腫に対するヒアルロン酸合成阻害効果の検討

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抄録 ヒアルロン酸（HA）は，細胞外マトリックスを構成する主要成分であり，細胞の接着能や遊走能，腫瘍の転移にも関与している．我々は4－メチルウンベリフェロン（MU）がHA合成阻害を来たし，癌の転移を抑制する効果があることを示した．今回我々は，悪性中皮腫に注目し，MUとMU誘導体である4－メチルエスクレチン（ME）の悪性中皮腫に対するHA合成阻害効果の検討をした．MUはNCI-H2052培養培地中のHAをcontrol群より約80%，MEは約60%に抑制した．MUおよびME存在下に培養したNCI-H2052の接着能はcontrol群よりそれぞれ70%，50%に阻害した．遊走能はそれぞれ70%，60%に阻害された．in vitroでMUおよびMEは悪性中皮腫細胞においてHA合成阻害効果を示し，MEでよりHA合成阻害効果がみられた．その結果，悪性中皮腫患者の今後の治療の一助となることが期待された．

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キーワード： ヒアルロン酸；悪性中皮腫；4－メチルウンベリフェロン；4－メチルエスクレチン；NCI-H2052.

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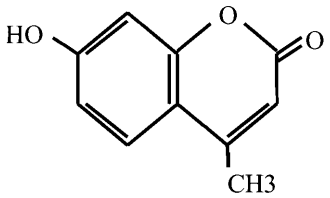
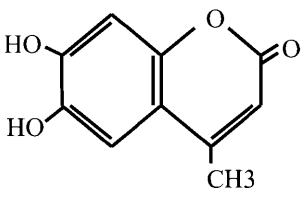


4-methylumbelliferone = MU
(7-Hydroxy-4-methyl coumarin)



4-methylesculetin = ME
(6,7-Dihydroxy-4-methyl coumarin)

Fig. 1 The structure of MU and ME.

Introduction

Malignant mesothelioma is a devastating neoplasm that originates from the mesothelial cells of the pleural and peritoneal cavities as well as the pericardium, and has a strong etiological relationship with asbestos exposure. Its incidence has been rising worldwide since 1990, and is expected to peak in most industrialized countries in 2020. Malignant mesothelioma has a very poor prognosis, and it is resistant to therapy[1]. Efforts are being made worldwide to establish new forms of therapy, so far with limited success.

Hyaluronan (HA) is a high-molecular-mass polysaccharide composed of repeated β-1,4-GlcUA-β-1-3-GlcNAc disaccharide units[2]. It is present in many tissues as a major component of the extracellular matrix, and is crucial for various physiological processes[3,4]. Previous studies showed that the amounts and composition of glycosaminoglycan undergo dramatic changes in various neoplastic tissues[5,6]. The elevated level of HA in malignant tumors is associated with the cell proliferation and migration. HA has been implicated in malignant transformation and tumor progression, and is associated with the degree of differentiation of various invasive tumors[7] and promotion of tumor angiogenesis[8]. HA has been implicated either directly or indirectly in a variety of cell characteristics such as adhesion, cell motility, growth, and migration, which are important early steps in cancer metastasis[9-11]. It is natural to come to the hypothesis through these studies that HA might be a promising target for cancer therapy.

Nakamura et al. reported that 4-methylumbelliferone (MU) (Fig. 1) inhibits HA synthesis in cultured human skin fibroblasts[12,13]. Kudo et al. reported that MU reduced both the adhesion and invasion of B16F10 melanoma cells[14] and increased the efficacy of gemcitabine against KP1-NL pancreatic cancer cells, exerting a potentially useful anticancer effect[15].

4-Methylesculetin (ME) (Fig. 1) is a MU derivative bearing two hydroxy groups, unlike MU itself, which has one hydroxy group. We have studied the structure, and effect on HA synthesis, of MU derivatives bearing hydroxy groups and methyl groups with coumarin at various positions, and found that ME had an inhibitory effect on hyaluronan, similar to that of MU[16]. We have also reported that ME inhibits pancreatic cancer growth and metastasis through inhibition of HA synthesis in vitro, and that ME may prolong the survival time of mice with end-stage pancreatic cancer[17].

Several studies have shown that malignant mesothelioma was in most cases associated with elevated amounts of HA, which has increased the malignant properties of malignant mesothelioma

cells[18]. We investigated in the present study whether inhibiting the HA synthesis could decrease the proliferative and metastatic ability of NCI-H2052 malignant mesothelioma cells.

Material and Methods

Materials

4-Methylumbelliferone was purchased from Wako Pure Chemicals (Osaka, Japan). 4-Methylesculetin was purchased from Tokyo Kasei Corporation (Tokyo, Japan). AlamarBlue was purchased from Biosource International (Camarillo, CA, USA). A hyaluronan measuring kit was purchased from Seikagaku Corporation (Tokyo, Japan).

Cells and culture conditions

The human pleural malignant mesothelioma cell line NCI-H2052 was obtained from the American Type Culture Collection (ATCC) Global Bioresource Center. The cells were routinely maintained as monolayer cultures in RPMI-1640 (Nissui, Tokyo Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1 mM sodium pyruvate at 37℃ in a mixture of 5% CO_2 and 95% humidified air.

Cell growth assay

Proliferation was determined using AlamarBlue assay[19]. Ninety microliters of medium containing 6.25×10^2 cells was seeded into 96-well plates (Iwaki, Tokyo, Japan). Serial dilutions of MU and ME dissolved in dimethyl sulfoxide (DMSO) were added to a final volume of 100 μl after overnight incubation. The concentration of DMSO in the medium did not exceed 0.1%. Cell numbers were assessed after 24, 48, 72, 96 and 120h. At each time point, 10 μl of AlamarBlue dye was added to each well. After incubation for 3 h, plates were read on a microplate fluorescence reader (Fluoroscan II, Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 544 and 590 nm.

Analysis of HA synthesis in culture supernatant

NCI-H2052 cells suspended at a density of 1.525×10^4/ml of culture medium were seeded into a 100-mm culture dish and allowed to adhere for 24 h. Cells were treated with 10 μM MU or ME dissolved in DMSO. Control culture received 0.1% DMSO. After 72, 96 and 120 h of incubation, the medium was removed and centrifuged to remove the cells. Hyaluronan in the supernatant was measured with the HA measuring kit in accordance with the manufacturer's protocol.

Visualization of cell surface HA

Cell surface HA was visualized using a particle-exclusion assay[20]. NCI-H2052 cells suspended at a density of 1.56×10^3 /ml of culture medium were seeded into a 35-mm culture dish (Iwaki, Tokyo, Japan) and allowed to adhere for 24 h. The cells were then treated with 10 μM MU and ME dissolved in DMSO. After 96 h of incubation, the medium was removed and the cells were washed with phosphate-buffered saline twice. Then 0.75 ml of a glutaraldehyde-fixed sheep erythrocyte (Sigma, UK) suspension was added to each dish. The pericellular HA matrix was visualized by phase-contrast microscopy, and the cells were photographed with a digital camera.

Adhesion assay

NCI-H2052 cells were preincubated with or without MU and with or without ME for 96 h, and then suspended at 5×10^5 cells/ml in culture medium. Two milliliters of the cell suspension was added to a 35-mm culture dish and incubated for 1 h at 37℃. Any non-adherent cells were removed by two gentle washings with PBS. The adherent cells were removed using trypsin, resuspended in medium, and counted with a

hemacytometer. The effect of MU and ME on cell adhesion was expressed as the relative adhesion (% of control) and was calculated as: relative adhesion = (number of adherent cells in experimental dishes) / (number of adherent cells in control dishes) × 100%[14].

Locomotion assay

The locomotion assay was performed using a Matrigel Invasion Chamber (BD Bioscience, Franklin Lakes, NJ). NCI-H2052 cells that had been preincubated with MU and ME were suspended at 1×10^6 cells/ml in culture medium without FBS, and 500 μl of the cell suspension was placed in the upper chamber. Culture medium containing 10% FBS was added to the lower chamber. After incubation for 5 h at 37 ℃, the filters were stained with hematoxylin and the cells migrating through the membrane were counted as: relative invasion = (number of cells migrating under experimental conditions) / (number of cells migrating under control conditions) ×100%[14].

Statistical analysis

Statistical comparison was made using the two-tailed Student's t test, and a value of $p < 0.05$ was accepted as indicating significance.

Results

Cell growth assay

The malignant mesothelioma cells were seeded in 96-well plates and incubated in the presence of various concentrations of MU and ME (0-1000 μM) to determine whether MU and ME have any direct effect on the cell growth. Their growth was assessed with the AlamarBlue assay. Neither MU nor ME at 10 μM inhibited the proliferation of the NCI-H2052 cells at 72, 96, or 120 h (Fig. 2).

Analysis of cultured cells for HA synthesis

We had previously shown that MU inhibits HA synthesis in cultured dermal fibroblasts, Streptococcus equi FM100, B16F10 melanoma cells, and KP1-NL pancreatic cancer cells, and that ME also inhibits HA synthesis in KP1-NL pancreatic cancer cells[12, 14, 16, 17, 20].

Neither MU nor ME at 0 − 10 μM inhibited cell growth for 72, 96, or 120 h. Therefore, MU and ME at 10 μM −the maximal concentration− were added to the cell cultures. First, after 72 h, HA in the cell culture was measured to determine the inhibitory effect of 10 μM MU and ME on HA synthesis.

After 72 h, MU was found not to have inhibited HA synthesis in comparison with the untreated control group. However, after 96 and 120 h, MU had lowered the level of HA synthesis about 20% in comparison with the untreated control group, though statistically insignificant (Fig. 3).

On the other hand, ME did not inhibit HA synthesis in comparison with the untreated control group after 72 h. However, after 96 and 120 h, ME produced an inhibitory effect of about 40 % in comparison with the untreated control group ($p < 0.05$) (Fig. 4).

The inhibitory effect of ME on HA synthesis was stronger than that of MU.

Analysis of HA synthesis at the cell surface

The pericellular HA matrix was visualized using a particle exclusion assay, as described in Material and Methods. When cells were untreated with MU and ME, a pericellular halo was observed (Fig. 5 (a)). When cells were treated with 10 μM MU or ME for 96 h, the pericellular halo was barely visible (Fig. 5 (b), (c)). Cells treated with 1.0 U/ml *Streptomyces* hyaluronidase prior to the particle-exclusion assay also showed no halo (Fig. 5 (d)). These results suggested that MU and ME inhibited HA synthesis by human malignant mesothelioma cells and weakened their pericellular matrix.

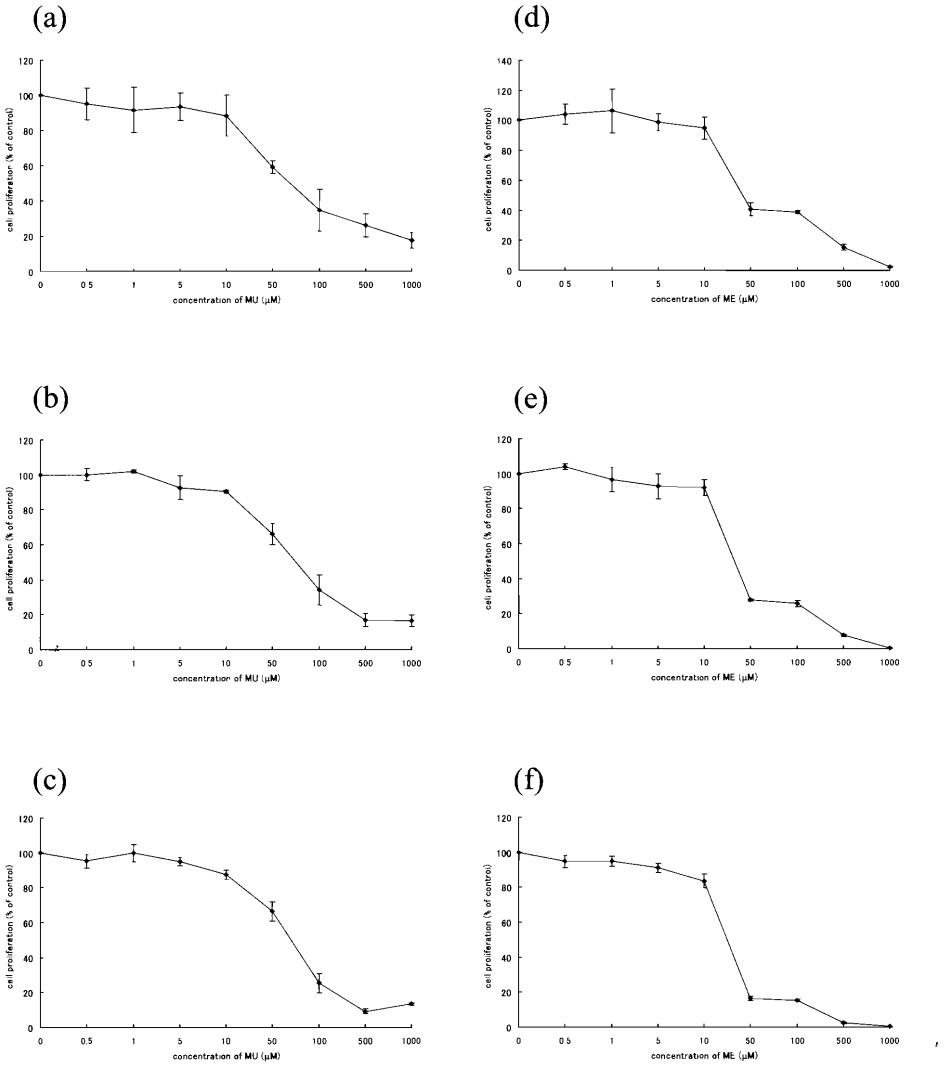


Fig. 2 Effects of MU and ME on the growth of NCI-H2052 cells. NCI-H2052 cells were incubated with 0-1000 μM MU for 72 h (a), 96 h (b) or 120 h (c), and with 0-1000 μM ME for 72 h (d), 96 h (e) or 120 h (f). At each point, live cells were evaluated using the AlamarBlue assay. Each point represents the mean of three replicate experiments.

Analysis of NCI-H2052 adhesion and locomotion

MU inhibited the adhesion of NCI-H2052 cells by about 30%, and ME by about 50% in comparison with the untreated control ($p < 0.05$) (Fig. 6 (a)). MU inhibited cell locomotion by about 30%, and ME by about 40% ($p < 0.05$) (Fig. 6 (b)). These results confirmed that MU and ME inhibited HA synthesis and would be effective as anti-cancer drugs by inhibiting the adhesion and invasion of malignant mesothelioma cells.

Our results indicated that ME would be more effective than MU as a HA synthase inhibitor against pleural malignant mesothelioma cells.

Discussion

We demonstrated that both MU and ME inhibited HA synthesis and pericellular HA matrix formation by malignant mesothelioma cells, as well as their adhesion and invasion.

Nakamura et al. have previously reported that MU could selectively inhibit the HA

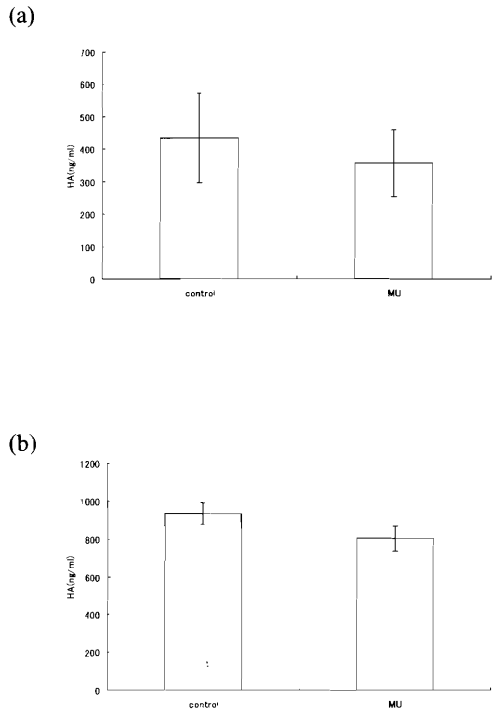


Fig. 3 Effect of MU on HA synthesis in the culture supernatant of NCI-H2052 cells. NCI-H2052 cells were incubated with 10μM MU for 96 h (a) or 120 h (b), and HA was quantified as described in Materials and Methods. Each bar represents the mean ± SEM of five replications.

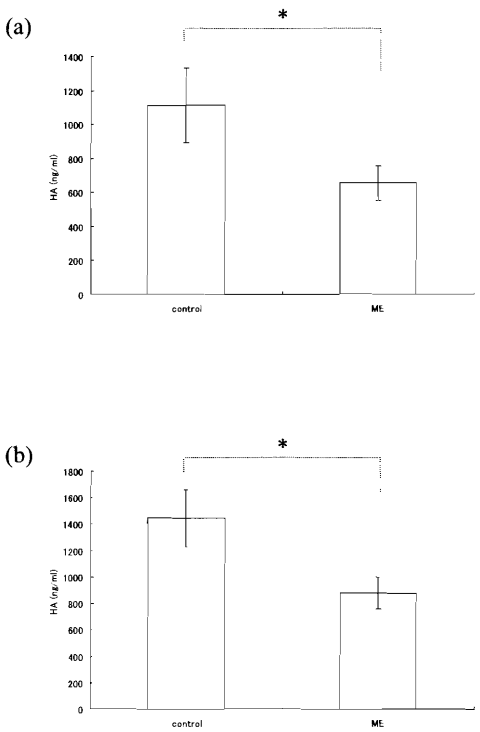


Fig. 4 Effect of ME on HA synthesis in the culture supernatant of NCI-H2052 cells. NCI-H2052 cells were incubated with 10μM ME for 96 h (a) or 120h (b), and HA was quantified as described in Materials and Methods. Each bar represents the mean ± SEM of five replications. * $p < 0.05$.

synthesis and pericellular matrix in the human skin fibroblasts, which hardly any effect on proteoglycan synthesis[12]. Furthermore, Kakizaki et al., using rat 3Y1 fibroblasts, recently demonstrated another novel mechanism of MU-mediated inhibition of HA synthesis involving the glucuronidation of MU by endogenous UDP-glucuronyltransferase (UGT), resulting in depletion of UDP-GlcUA. Because MU becomes an acceptor of UDP-GlcUA, excess glucuronidation of MU by UGT could deplete the pool of UDP-GlcUA, which is a common substrate for hyaluronan synthase (HAS) and UGT[22]. In addition, we have succeeded in demonstrating the HA-knock-down mice which had the HA-poor matrices using HA-inhibiting effect of MU. Moreover, we have examined the relationship between HA of pericellular matrix and the proliferation and metastasis by several cancer cells[12, 15]. We have also studied the structure, and effect on HA synthesis, of MU derivatives bearing hydroxy groups and methyl groups with coumarin at various positions, and found that ME had an inhibitory effect on hyaluronan, similar to that of MU[16]. We have reported that ME also inhibited the adhesion and invasion of pancreatic cancer cells, and it could prolong the survival time of mice with end-stage pancreatic cancer[17]. It is possible that the mechanism responsible for inhibition of HA synthesis by ME is the same as that for MU[16].

A close relationship has been demonstrated between HA production and malignant phenotype, and HA has been implicated either directly or indirectly in a variety of cell behaviors such as adhesion, cell motility, growth, and differentiation[23]. Many studies have shown that HA synthesis is increased in various types

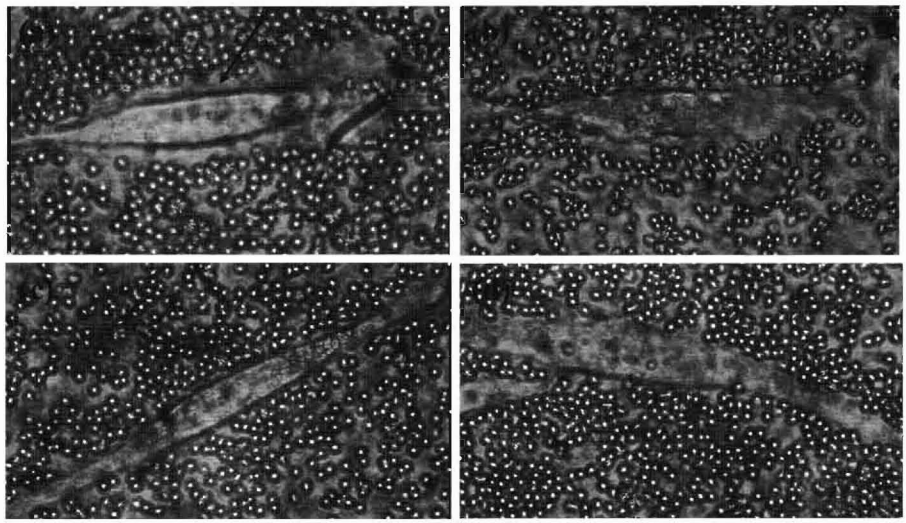


Fig. 5 Visualization of pericellular HA matrix. The pericellular HA matrix was visualized by particle-exclusion assay as described in Materials and Methods. HA matrix is indicated by arrows. NCI-H2052 cells were cultured in the absence (a) or presence of 10μM MU (b) or ME (c). In one culture, 1.0 U/ml *Streptomyces* hyaluronidase was added (d).

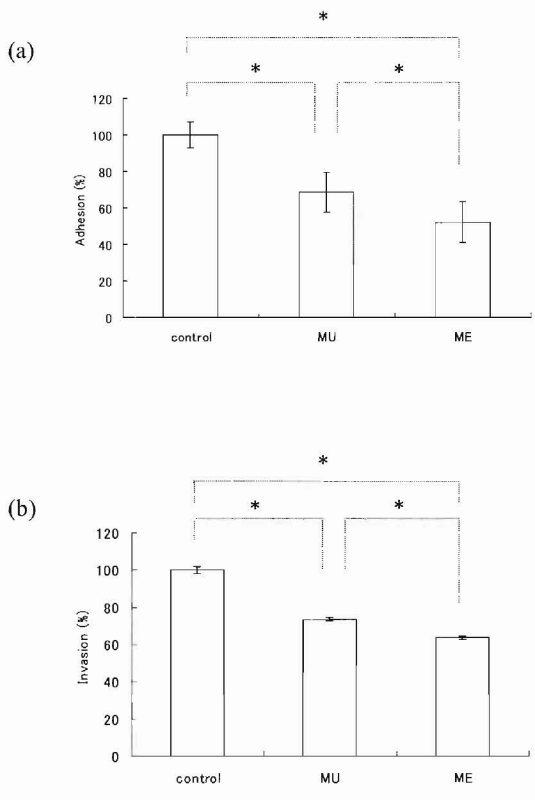


Fig. 6 Effect of MU and ME on the adhesion (a) and invasion (b) of NCI-H2052cells that had been preincubated with 10 μ M MU or ME for 96 h, as described in Materials and Methods. * $p < 0.05$.

of cancer, including colon cancer, breast cancer, and lung cancer[24-26], and that mesothelioma has a higher ability to synthesize HA than other malignancies[27-28].

In the present study, we demonstrated that both MU and ME inhibited HA synthesis and pericellular HA matrix formation by malignant mesothelioma cells, as well as their adhesion and invasion. The maximum concentration of both MU and ME that had no cytotoxicity was 10 μM, which was similar to the results obtained using pancreatic cancer cells[16]. Kudo et al. previously demonstrated that MU inhibits HA in B16F10 melanoma cells in a dose-dependent manner[14]. Therefore, with the expectation of achieving a maximum inhibitory effect on HA synthesis, we investigated whether 10 μM MU and 10 μM ME, both at the maximum non-cytotoxic concentration, would be able to inhibit HA synthesis by malignant mesothelioma cells. MU exhibited about 20% inhibition, and ME about 40%, from 96 h onwards. The inhibitory effect of MU on HA synthesis by malignant mesothelioma is weaker and occurs later than

that in pancreatic cancer[16], melanoma[14], and skin fibroblasts[13], suggesting that malignant mesothelioma has a richer pericellular HA matrix. On the other hand, we found that ME would be more effective than MU as a HA synthase inhibitor against malignant mesothelioma cells.

Mesothelioma is a malignant neoplasm arising from mesothelial cells. Since Wagner et al. first reported that mesothelioma has a relationship to asbestos exposure[1, 29], it has become apparent that it is not a rare disease. It is anticipated that malignant mesothelioma will become a major clinical issue in the near future, necessitating the development of some new form of therapy, as current treatment is ineffective.

Our present report is the first to show that MU and ME can inhibit HA synthesis by malignant mesothelioma cells, thus having potential to control their proliferation, metastasis, adhesion, and invasion. Nakazawa et al. have described that a combination of MU and an anticancer drug such as gemcitabine was effective against human pancreatic cancer cells[15]. It might be interesting to investigate new forms of chemotherapy involving combinations of anticancer drugs such as cisplatin, irinotecan, gemcitabine, and pemetrexed[30].

In conclusion, we demonstrated that both MU and ME could inhibit not only HA synthesis and pericellular HA matrix formation by malignant mesothelioma cells, but also their adhesion and invasion. Therefore, MU and ME could become a new treatment option for malignant mesothelioma.

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